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Bodh I. Jugdutt
Naranjan S. Dhalla *Editors*

Cardiac Remodeling

Molecular Mechanisms

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Cardiac Remodeling

Molecular Mechanisms

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This book is dedicated to my peers,
colleagues, teachers, students,
and friends who inspire and stimulate me
in lifelong search for knowledge that can be
applied to improve cardiovascular health
and benefit mankind;
And
to my family, who selflessly supported me in
my relentless pursuits of hidden truths:
Catherine Elizabeth (né Graham)
Bernadine Alexandra
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Sunil Keith (1973–1992)

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Preface

The concept of cardiac remodeling as a mechanism of heart disease leading to heart failure has evolved since the mid-1970s. The initial emphasis was on heart failure related to pressure and volume overload; this led to theories on adaptive and maladaptive structural and functional changes after life-threatening insults such as myocardial infarction and hypertensive heart disease. Later, the scope of cardiac remodeling expanded to pure and mixed pressure and volume overload states and a wide range of cardiomyopathies, inherited or acquired from infections or exposure to various therapeutic drugs with cardiotoxic pleiotropic effects and other cardiotoxic agents. Results of cardiovascular research at the bench and bedside levels and a host of population studies since the mid-1980s fueled the concept of adverse left ventricular remodeling during acute and subacute phases of myocardial infarction, with structural changes that have a negative impact on cardiac function. These studies have established that adverse cardiac remodeling is a major mechanism for progressive left ventricular enlargement, deterioration of ventricular function, increased suffering, and deaths from chronic heart failure. Concurrently over the last 4 decades, expanding knowledge of the basic molecular mechanisms and clinical implications of cardiac remodeling has identified several molecular pathways and potential targets, leading to drug discovery and development and improved therapies for major causes of adverse cardiac remodeling, such as myocardial infarction and hypertension. A major advance has been the appreciation that lifelong exposure to cardiovascular risk factors and cardiotoxic agents, beginning from the pediatric age through adulthood and old age, fuels the march to heart failure. This has opened up a new area of research into the biology of aging and its impact on cardiac remodeling.

Despite the advances, hearts continue to enlarge, and the heart failure burden continues to increase, especially after ST-segment-elevation myocardial infarction (STEMI). Many knowledge gaps exist. With the expanded spectrum of diseases that result in adverse cardiac remodeling, improved understanding of the underlying molecular mechanisms through research is crucial. During the last 20 years, attention focused on cellular and subcellular changes, including those at the molecular

and biochemical levels. There has been an explosion in knowledge of molecular and cellular mechanisms, importance of oxidative stress, metabolic pathways, extracellular and intracellular matrix remodeling, and the far-reaching effects of infarct and non-infarct zone fibrosis in the progression to heart failure. This has led to a profusion of original scientific and review papers dealing with several aspects of molecular mechanisms of adverse cardiac remodeling. There is therefore a need to synthesize these ideas into one book on molecular mechanisms of cardiac remodeling.

The main objective of this book has been to summarize the major research advances in molecular, biochemical, and translational aspects of cardiac remodeling over the last 2 to 3 decades under one cover and touch on future directions. The invited leaders and established investigators in the field have generously contributed 30 chapters on key topics relating to molecular mechanisms, with emphasis on selected biochemical and translational aspects of cardiac remodeling. The authors have succinctly summarized large volumes of data on these key topics and highlighted novel pathways and key molecules that need to be further explored and possibly targeted. They provide integrative reviews of the basic mechanisms and clinical correlates as well as critical assessments of publications on the key topics by the leading investigators in the field. The reference lists are fairly comprehensive and include key papers that are currently not easily accessed from Pubmed or other search engines. The book is carefully organized into two sections: Section A contains 15 chapters that focus mainly on molecular mechanisms in pressure and volume overload hypertrophy, with some overlap into brief ischemia–reperfusion injury; Section B contains 15 chapters that focus on molecular mechanisms after myocardial injury and infarction. The list of topics is by no means comprehensive but addresses some major areas needing attention. To our knowledge, there is no other book on this topic to date.

In summary, this book provides a high-profile and valuable publication resource on molecular mechanisms of cardiac remodeling for both the present and future generations of researchers, teachers, students, and trainees. It should stimulate future translational research targeted towards discovery and development for preventing, limiting, and reversing bad remodeling over the next few decades, with the ultimate goal of preventing progression to systolic and/or diastolic heart failure. The chapters suggest potential novel strategies that should receive attention for translating basic research knowledge to application in patients at the bedside. We would like to thank all the authors for their excellent contributions. We would also like to express our deepest appreciation for the preparation and editorial help provided by Catherine E. Jugdutt, Eva Little, and Dr. Vijayan Elimban in assembling this book. Cordial thanks are also due to Ms. Portia Formento and Melanie Tucker, Springer, USA, for their continuous advice and understanding during the editorial process. We hope that the book will prove useful for scientists and clinicians, students and teachers, and the industry interested in drug and discovery research.

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Part I
Molecular Mechanisms of Remodeling in Pressure and Volume Overload Hypertrophy and Heart Failure

Chapter 1
β-Adrenergic Receptor Signaling in Heart Failure

Grace Jung Ah Lee, Lin Yan, Dorothy E. Vatner, and Stephen F. Vatner

Abstract Acute activation of the sympathetic system and resultant β-adrenergic receptor (β-AR) signaling are required to maintain homeostasis, providing inotropic support in times of need, as in "fight or flight" or response to any stress, such as cardiac dysfunction and heart failure. For most of the twentieth century, it was reasoned that sympathetic stimulation of β-ARs through administration of naturally occurring catecholamines or synthetic sympathomimetic amines could provide inotropic support and should be used in heart failure therapy. However, in heart failure, sympathetic drive to the heart is excessively increased, and chronic sympathetic stimulation is deleterious, since it increases $M\dot{V}O_2$, which cannot be met by appropriate increases in coronary blood flow, thereby creating subendocardial ischemia and intensifying the cardiac dysfunction. Furthermore, continued stimulation of the β-ARs also becomes problematic because it can activate multiple cellular processes including those involved in pathological remodeling seen in the development of cardiomyopathy. However, this reasoning took a diametrically opposite turn in the latter twentieth century when the adverse effects of chronic β-AR stimulation became apparent from experimental studies in transgenic mice with cardiac-specific overexpression of $G_{s\alpha}$ and β-ARs and also from clinical studies with poor outcomes for patients on chronic sympathomimetic amine therapy. At this time it was also found that internal compensatory physiological processes countering continued β-AR stimulation in the heart were cleverer than physicians. As a protective response, β-AR desensitize, which reduces the effectiveness of β-AR stimulation and the consequent increases in myocardial oxygen demands. Taken together, these factors were fundamental to the change in course from β-AR stimulation to β-AR blockade in the treatment of heart failure.

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1.1 Introduction

β-Adrenergic receptor (β-AR) signaling is central to all aspects of the pathophysiology of heart failure. The sympathetic nervous system including the neurohormones, epinephrine, and norepinephrine is rapidly called into action by any stress, such as cardiac dysfunction and heart failure. For most of the twentieth century, it was reasoned that sympathetic stimulation of β-ARs through administration of naturally occurring catecholamines or synthetic sympathomimetic amines could provide inotropic support and should be used in heart failure therapy. However, this reasoning took a diametrically opposite turn in the latter twentieth century when it was realized that patients with β-AR blocker therapy fared significantly better. The goal of this chapter is to document the scientific and clinical basis for the changing paradigm of the role of β-AR signaling in heart failure. To do this, the chapter has the following sections: Sect. 1.2 (The Discovery of β-ARs), Sect. 1.3 (Regulation of Cardiac Contractility by β-ARs), Sect. 1.4 (Targeting β-ARs in the Treatment of Heart Failure: Use of β-AR Inotropic Agonists), Sect. 1.5 (Adverse Effects of Chronic β-AR Stimulation in the Treatment of Heart Failure), Sect. 1.6 (Advent of β-AR Blockade Therapy), Sect. 1.7 (Mechanisms Mediating Salutary Effects of β-AR Blockade Therapy in Heart Failure), Sect. 1.8 (Future Directions), and Sect. 1.9 (Conclusions).

1.2 The Discovery of β-ARs

Although the concept of β-ARs mediating the signaling from the sympathetic nervous system to regulate cardiac function is axiomatic today, this was not always the case. Throughout much of the twentieth century, it was erroneously believed that adrenergic signaling was primarily mediated by two classes of neurotransmitters, sympathin E (excitatory) and sympathin I (inhibitory), classified according to their physiological response [1, 2]. This was due, in part, to the use of natural adrenalin, which contained variable mixtures of epinephrine and norepinephrine with quite different agonistic activities, resulting in obscured conclusions that masked their distinct effects. In retrospect, the fallacy of their results is clear. Not only do epinephrine and norepinephrine have different effects, e.g., norepinephrine has α-vasoconstrictor activity as well as β-vasodilator and inotropic activity, whereas epinephrine does not have much α-activity, but both elicit reflex effects in vivo with the most prominent mediated by the arterial baroreflex, which modulates the direct actions of the catecholamines on arterial pressure, heart rate, and peripheral vascular resistance.

In 1906, Dale first introduced the concept of receptors in connection with the sympathetic nervous system [3]. In his studies, he observed the actions of ergot alkaloid antagonists on the effects of epinephrine and proposed there are two distinct receptor types. One type, in which epinephrine mediated excitatory responses, was antagonized by ergot alkaloids, whereas in the second type, ergots had no effect on the inhibitory effects of epinephrine. Then in 1948, a major step was taken by Ahlquist, who challenged this idea of sympathins by characterizing two AR types, α and β, based on the rank order of catecholamine potencies rather than the nature of their physiological response (contraction vs. relaxation) [2].

However, the idea of ARs existing as physical entities received much skepticism [4, 5]. Even Ahlquist noted in his later paper that ARs are hypothetical structures that hold momentary value until the exact mechanism of adrenergic signaling is deciphered [6]. However, his seminal studies persevered and in 1967, Lands et al. extended his classification scheme by introducing two β-AR subtypes, β_1 and β_2, based on their affinities for epinephrine and norepinephrine [7]. Whereas β_1-ARs in cardiac and adipose tissue have approximately equal affinity for epinephrine and norepinephrine, β_2-ARs relax bronchial and vascular smooth muscle and have greater affinity for epinephrine than for norepinephrine. Then in 1972, Carlsson et al. provided pharmacological evidence that both β_1- and β_2-ARs are present and functional in the feline heart and that β_1-AR is the predominant subtype in both the atria and the ventricles [8].

From these findings, Lefkowitz developed highly specific radioligand-binding assays that allowed selective labeling of β-ARs, which was responsible for the most significant progress in the field in the latter half of the twentieth century [9]. Using this method, he and his colleagues physically identified cardiac β-ARs for the first time in the canine heart in 1975 [9]. Moreover, the radioligand-binding technique made possible the quantification of the relative proportions of β_1- and β_2-ARs and in 1983, it was reported that human left ventricle (LV) consists of 86% β_1-AR and 14% β_2-AR [10], thus confirming and extending the work of Carlsson. In addition, the interactions of β-ARs with various agonists and antagonists were explored based on the concept that the radioligand competes for the binding site with an agonist. In 1980, it was discovered that binding of an agonist and antagonist was affected by GTP [11], and taking into account that adenylyl cyclase systems require GTP for activation [12], the ternary complex model, consisting of the adrenergic receptor coupling to GTP-binding G protein to activate adenylyl cyclase (AC), was proposed [13].

The advances in molecular biology techniques that shortly followed led to the successful cloning of the β_2-AR, the very first G protein-coupled receptor to be cloned [14]. Then by the 1990s, six α-AR subtypes (α_{1A}, α_{1B}, α_{1C} and α_{2A}, α_{2B}, α_{2C}) [15, 16] and three β-AR subtypes (β_1, β_2, β_3) [17–19] were firmly established. Moreover, insights on the physiological actions of various AR subtypes were made possible through generation of transgenic mice models with targeted disruption of ARs [20–23]. Today, we now understand that α-ARs have positive inotropic activity

(most prominent in rodents), but their primary role is regulating peripheral resistance [24], whereas β-ARs provide the strongest mechanism to regulate cardiac performance [25–28].

1.3 Regulation of Cardiac Contractility by β-ARs

In response to acute stress, the normal heart must be able to rapidly increase its output nearly fivefold to meet the higher metabolic demands [29]. This is primarily met by the sympathetic nervous system acting on the β-ARs to mediate positive chronotropic and inotropic responses in the heart. While all three β-ARs subtypes, namely β_1, β_2, and β_3, are expressed on the cardiomyocyte [9, 30], β_1-AR is the most abundant form (70–80% of total β-AR in the normal heart) and is one of the main regulators of cardiac performance, with β_2-AR being secondary, but also having powerful vasodilator properties in vessels [31]. The β_3-AR, however, is only minimally expressed, and its role in the heart remains controversial. Initially, the β_3-ARs were implicated to have a role in fat metabolism [32], but various studies suggest they have cardiac roles as well. For instance, in vivo stimulation of β_3-AR induces inotropic responses in rodents; however, no effect was observed in primates, suggesting its physiological response may be species specific [33]. In addition, these reports do not correspond to the findings of ex vivo studies, which showed negative inotropic effects of β_3-AR stimulation in the ventricular tissue of mice and humans potentially through the inhibitory G protein (G_i)–nitric oxide synthase (NOS) pathway [34, 35]. The apparent discrepancy between in vivo and ex vivo effects of β_3-AR signaling may depend on the type of agonist used as well as their differential effects under in vivo and ex vivo conditions. Interestingly, however, a recent study by Niu et al. demonstrated in vivo that β_3-AR has cardioprotective roles by inducing negative inotropic effects and maintaining NO and reactive oxygen species balance in the setting of catecholamine overstimulation in the failing heart [36]. Whether this effect is also observed in humans needs to be elucidated.

β_1- and β_2-ARs are the principal stimulatory G protein (G_s)-coupled receptors that drive heart rate and enhance myocardial contractility [25–28]. Upon amine binding, both β_1- and β_2-ARs activate adenylyl cyclase (AC) to increase the level of cyclic AMP (cAMP) (Fig. 1.1) [37]. The latter targets protein kinase A (PKA), which phosphorylates various proteins involved in excitation–contraction coupling. These include, but are not limited to L-type calcium channels (LTCC) [38], ryanodine receptors (RyR2) [39], phospholamban (PLB) [40, 41], and troponin I (TnI) [41], which together coordinate stronger contractions and hastened relaxation of the cardiac muscle. For instance, phosphorylation of LTCC increases the Ca^{2+} influx [42], which stimulates RyR2 to release Ca^{2+} from the sarcoplasmic reticulum (SR). The Ca^{2+}-induced Ca^{2+} release process is further enhanced by phosphorylation of RyR2 [43], thereby increasing its sensitivity to cytosolic [Ca^{2+}] and resulting in greater SR Ca^{2+} unloading necessary for stronger pumping of the myocardium [44]. The following relaxation phase is also accelerated through phosphorylation of PLB

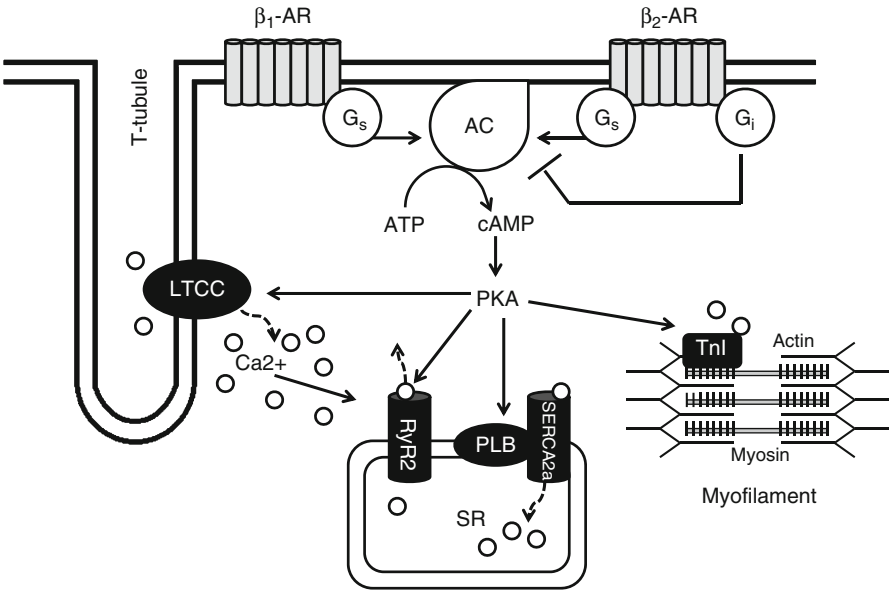

Fig. 1.1 Enhancement of cardiac contractility through β-AR signaling. Upon agonist binding, β_1- and β_2-ARs activate the stimulatory G protein (G_s)-adenylyl cyclase (AC)-protein kinase A (PKA) pathway to phosphorylate multiple calcium handling and myofilament proteins, including L-Type calcium channel (LTCC), ryanodine receptor (RyR2), phospholamban (PLB), and troponin I (TnI). Activation of these proteins by β-ARs enhances their function, resulting in stronger contractions as well as hastened relaxation. For instance, the Ca^{2+}-induced Ca^{2+} release process is further enhanced by phosphorylation of RyR2 by PKA, resulting in greater sarcoplasmic reticulum (SR) Ca^{2+} unloading during systole. The relaxation phase is also hastened by phosphorylation of PLB and TnI, resulting in faster reuptake of Ca^{2+} by the SR and faster relaxation of the myofilaments by preventing actin–myosin interaction. Interestingly, the β_2-AR also couples to inhibitory G protein (G_i), which could negate the effects of G_s–AC–PKA signaling, resulting in only modest inotropic response compared to β_1-AR

at Ser16 by PKA, which allows rapid sequestering of Ca^{2+} by relieving its inhibition of the SR Ca-ATPase (SERCA2a) pump [40, 45, 46], as well as phosphorylation of TnI, which decreases the sensitivity of myofilaments to Ca^{2+} [47–49], resulting in muscle relaxation.

Interestingly, studies from animal models reported that β_2-AR also couple to G_i, which could negate the effects of G_s–AC–PKA signaling, resulting in attenuation of enhanced contractility and hastened relaxation [50, 51]. For instance, (−)-noradrenaline hastened relaxation through β_1-ARs, but not with (−)-adrenaline through β_2-ARs in the feline ventricle [52], which was supported by the finding that β_2-ARs have modest inotropic effects compared to β_1-AR independent of receptor density in the rat heart [53, 54]. However, whether β_2-ARs couple to G_i in humans remains controversial. Kilts et al. demonstrated that β_2-ARs also couple to G_i in the human atria [55], whereas a later study by Molenaar et al. showed evidence that is inconsistent with the dual coupling feature of β_2-AR [56].

1.4 Targeting β-ARs in the Treatment of Heart Failure: Use of β-AR Inotropic Agonists

While β-AR blockers are currently widely employed in the treatment of heart failure, much of the therapeutic approaches in the twentieth century were based on the concept of increasing myocardial contractility through inotropic agents. The reasoning was simple: (1) The failing heart has poor contractility, (2) catecholamines are the most potent stimulators of myocardial contractility, and (3) catecholamines would be useful to increase contractility in patients with heart failure (quod erat demonstrandum). What was not known was either the concept of desensitization of β-AR [57–59] or the fact that there is an oxygen cost of increasing heart rate, LV wall stress, and myocardial contractility, i.e., the major determinants of $\dot{M}VO_2$, which, in turn, increase the requirement for coronary blood flow. The increased demand is easily met in normal hearts and coronary circulation, but not so in the setting of either hypertrophy or heart disease characterized by limited subendocardial coronary reserve [60, 61]. Under these conditions, the imbalance between coronary blood flow supply and myocardial oxygen demands results in myocardial ischemia, which exacerbates cardiac dysfunction.

Not recognizing these concepts, catecholamines and synthetic sympathomimetic amines were routinely administered to patients with heart failure; at first, norepinephrine or epinephrine was given [62], and then in the 1960s, isoproterenol (ISO), a nonselective β_1- and β_2-AR agonist with potent inotropic and chronotropic properties, was used in the treatment of heart failure [63]. Many patients who received this treatment developed fatal arrhythmias [63], and those with coronary artery disease had increased myocardial ischemia [64]. In an experimental correlate of the clinical situation, Hittinger et al. showed that isoproterenol infusion in conscious dogs with LV hypertrophy and failure further impaired both systolic and diastolic function [65]. The increased stress of inotropy as well as chronotropy under limited coronary flow reserve resulted in subendocardial hypoperfusion, which exacerbated the myocardial dysfunction.

In an effort to find a selective agent that could augment the inotropic state of the myocardium without affecting the chronotropic state, dopamine, a naturally occurring, nonselective α- and β-AR agonist, was used. While dopamine had less chronotropic effect compared to isoproterenol, the increase in cardiac index was still associated with elevated heart rate, resulting in ventricular tachyarrhythmias [66, 67]. Furthermore, the inotropic effect of dopamine was mediated in part by the release of endogenous noradrenaline [68–70]. In severe heart failure, where endogenous noradrenaline levels are low, the degree to which dopamine could increase cardiac output was often minimal and unpredictable [71, 72]. Moreover, infusion at high doses leads to stimulation of both α- and β-ARs with a predominant α-AR effect, resulting in excessive vasoconstriction [73, 74]. To minimize the effects on peripheral vasculature and heart rate, while retaining positive inotropic effects, dobutamine, a synthetic derivative of dopamine, was developed. Vatner et al. investigated the actions of dobutamine in healthy conscious dogs and found that it

exerted potent inotropic effects with insignificant changes in heart rate [75]. However, the lack of increase in heart rate and effects on peripheral vascular resistance was due to mixed α- and β-adrenergic stimulating properties of the drug, coupled with counteracting influences induced by activation of autonomic reflexes. Accordingly, when autonomic reflexes were blocked or when one arm of the AR system (either α or β) was blocked, effects on heart rate and peripheral vascular resistance were identified. Furthermore, considering that many inotropic agents also have vasodilating effects, administration of nitroprusside, a vasodilating agent, was also explored. In the 1970s, there were various reports showing the promising effects of nitroprusside in improving heart function in the setting of heart failure [76–78]. A later study compared the effects of chronic infusion of dobutamine with those of nitroprusside in end-stage heart failure patients and showed that patients with nitroprusside treatment had significantly higher event-free survival rate than patients with dobutamine treatment [79]. From these findings, it was proposed that peripheral vasodilator therapy with nitroprusside was superior to dobutamine. However, the combined effects of vasodilators and inotropes resulted in a higher mortality rate than vasodilator therapy alone [80]. In the early 1980s, moderate success was observed with the β_1-AR selective inotropic agent, prenalterol. Although in an acute setting, it improved hemodynamics [81, 82], chronic administration showed detrimental effects [83]. Then in the 1990s, clinical trials with β_2-AR agonist, dopexamine, initially showed some clinical benefit over the placebo [84, 85]. However, the beneficial effects were due to the vasodilatory and cardiac unloading effects of this agonist.

In an attempt to improve cardiac function, β-AR downstream targets were also explored. Milrinone, a phosphodiesterase (PDE) inhibitor, was first approved for intravenous use in the late 1980s due to its effects on increasing cAMP half-life and subsequently increasing intracellular Ca^{2+} concentration and improving cardiac contractility. In addition, because increased cAMP levels also results in arterial and venous dilation, it may lead to hypotension. In clinical trials, milrinone had no clear benefit over placebo [86]. Then in 1986, enoximone, another PDE inhibitor, was studied. A year later, it was found to be superior to both dobutamine and nitroprusside in the management of heart failure [87]. However, in 1994, it was found that enoximone administration in patients with end-stage heart failure resulted in increased mortality [88]. Then in a larger phase III trial in 2009, ESSENTIAL-I and ESSENTIAL-II, enoximone treatment did not show any benefit over the placebo, which led to the termination of enoximone development [89].

1.5 Adverse Effects of Chronic β-AR Stimulation in the Treatment of Heart Failure

Acute activation of the sympathetic system is required to maintain homeostasis, providing inotropic support in times of need, such as in “fight or flight” situations. However, in heart failure, sympathetic drive to the heart is excessively increased,

and as discussed in the last section, the chronic sympathetic stimulation is deleterious, since it increases $\dot{M}VO_2$, which cannot be met by appropriate increases in coronary blood flow. This results in subendocardial ischemia, which intensifies the cardiac dysfunction. Furthermore, continued stimulation of the β-ARs also becomes problematic because it can activate multiple cellular processes including those involved in pathological remodeling seen in the development of cardiomyopathy. Therefore, continued β-AR stimulation as occurs in heart failure induces β-AR desensitization [57–59], which is protective since it reduces the effectiveness of β-AR stimulation and the consequent increases in myocardial oxygen demands. The realization of these concepts was fundamental to the paradigm shift from β-AR stimulation to β-AR blockade in the treatment of heart failure. The background documenting this concept follows in both experimental and clinical settings.

1.5.1 Experimental Evidence

It was a considerable time before the adverse outcomes of chronic β-AR stimulation were demonstrated both in vitro and in vivo in numerous animal studies. Initially, it was thought that β-AR overexpression could be a novel therapy for heart failure. This concept was supported by studies that showed enhanced myocardial function in transgenic mice with up to 60-fold overexpression of β_2-ARs [90] without evidence of cardiac pathology [91]. Furthermore, transgene therapy with adenovirus coding the β_2-AR transgene significantly improved cardiac function in the failing rabbit hearts [92].

However, the error of this concept was realized when the chronic effects of β-AR stimulation were observed. The novel feature of the following studies was the continued monitoring of the mice as they aged. Iwase et al. reported that transgenic mice with cardiac-specific overexpression of $G_{s\alpha}$ ($G_{s\alpha}$ TG) have increased responsiveness to β-AR stimulation by ISO compared to WT mice as young adults, as evident by significantly higher left ventricular ejection fraction (LVEF) (Fig. 1.2a) [93]. However, upon aging, their function deteriorates, resulting in LV dilation, higher incidence of arrhythmias, and depression of LVEF (Fig. 1.2a) [93–95]. Furthermore, histological examination of these hearts revealed a picture of cardiomyopathy, including marked increase in hypertrophy, myocyte necrosis, and fibrosis (Fig. 1.2b) [93]. Confirming these findings, Engelhardt et al. showed that transgenic mice with cardiac-specific overexpression of β_1-AR (β_1-AR TG) have hyperfunction at a young age, but their function progressively worsens as the mice age [96], which was also further confirmed by the work of Peter et al. (Fig. 1.3a) [97]. Furthermore, premature mortality by 12 months of age was significantly higher in β_1-AR TG mice compared to WT mice (Fig. 1.3b) [98]. In support of these findings, in vitro studies in rat ventricular myocytes showed significant increases in apoptosis upon β_1-AR stimulation [100], and hypertrophy from ISO administration was abolished by a β_1-AR antagonist [101]. Furthermore, the concept of the beneficial effects of chronic β_2-AR overexpression was finally put to rest when Du et al. showed that

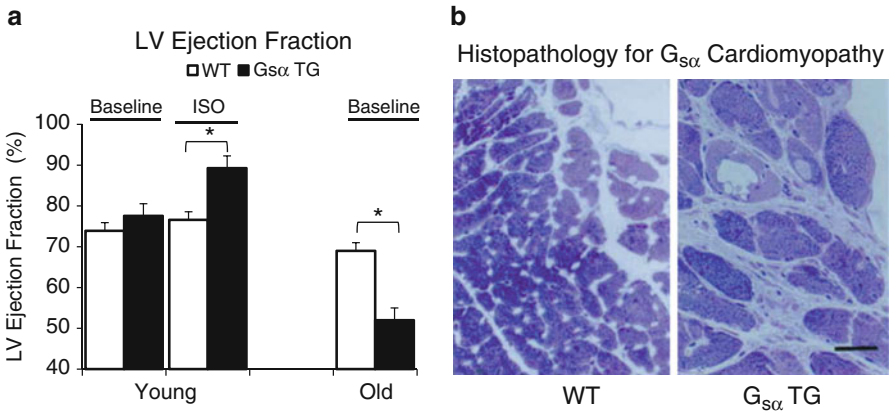

Fig. 1.2 Overexpression of $G_{s\alpha}$ in the heart induces cardiomyopathy. (**a**) $G_{s\alpha}$ TG mice have higher responsiveness to isoproterenol (ISO) compared to WT mice as young adults, but experience progressive deterioration of left ventricular ejection fraction (LVEF) with age (*$p<0.05$) [93, 94]. (**b**) LV subendocardium of 19-month-old WT mice and 15-month-old $G_{s\alpha}$ TG mice stained with toluidine blue. Old $G_{s\alpha}$ TG mice have marked cellular hypertrophy and fibrosis compared to WT mice. Bar = 25 μm [93]. Figures adapted with permission from Lippincott Williams & Wilkins and Journal of Clinical Investigation

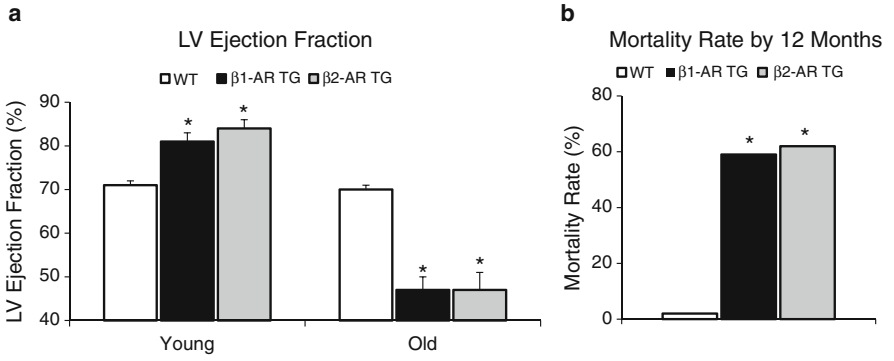

Fig. 1.3 Cardiac overexpression of β_1-AR or β_2-AR decreases heart function and increases mortality with age. (**a**) β_1- and β_2-AR TG animals have enhanced left ventricular ejection fraction (LVEF) as young adults, but their cardiac function significantly worsens as the animals age compared to the age-matched WT mice. (*$p<0.05$ vs. WT) [97]. (**b**) The mortality rate of β_1- and β_2-AR TG animals by 12 months of age is significantly higher compared to WT animals (*$p<0.05$ vs. WT) [98, 99]. Figures adapted with permission from Journal of Clinical Investigation, Lippincott Williams & Wilkins, and Oxford University Press

β_2-AR TG mice also develop progressive cardiomyopathy with increasing age, as reflected by deterioration of myocardial function [99], which was also confirmed by studies of Peter et al. (Fig. 1.3a) [97]. Similar to β_1-AR TG mice, β_2-AR TG mice also have significantly higher mortality by 12 months of age compared to the WT mice (Fig. 1.3b) [99].

Chronic stimulation of the β-AR pharmacologically with high doses of ISO also results in the rapid development of cardiomyopathy. Similar to transgenic mice models of cardiomyopathy, several studies in rodent models have reported marked increases in cellular necrosis and fibrosis [102–105] and impaired LV function [106, 107] upon administration of high doses of ISO, resulting in heart failure and increased mortality. In support of this, the effects of chronic catecholamine stress are exacerbated in mice with transgenic overexpression of components in the β-AR signaling pathway, such as AC5, one of the two major AC isoforms in the heart. It was shown that mice with cardiac-specific overexpression of AC5 (AC5 TG) have higher mortality, depressed LVEF, as well as increased fibrosis and apoptosis compared to the WT mice under chronic catecholamine stress [108].

It is now recognized that the development of cardiomyopathy from chronic β-AR stimulation is mediated by several signaling pathways (Fig. 1.4). One of these is the Ca^{2+} handling pathway. In animal models as well as heart failure patients, prolonged Ca^{2+} transients have been described [109, 110], and in humans, it was shown that this effect was due, in part, to the impairment of reuptake of Ca^{2+} into the SR during diastole [111, 112]. In fact, it was shown that in the failing heart, PLB is hypophosphorylated by PKA [113], resulting in delayed sequestering of Ca^{2+} by the SR and increased cytosolic Ca^{2+} during diastole, which is further exacerbated by enhanced activity of LTCC [114]. Engelhardt et al. showed that adverse effects of cardiac-specific overexpression of β_1-AR could be rescued by ablation of PLB [98]. The β_1-AR TG x $PLB^{-/-}$ bigenic mice had significantly lower diastolic calcium levels and upregulation of SERCA compared to β_1-AR TG mice. Moreover, the bigenic mice had significant improvement in mortality and cardiac function as well as reduction in hypertrophy and fibrosis. The cardiotoxic effects of increased intracellular Ca^{2+} could be attributed to activation of calcineurin [115] and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) pathway [116], which could induce hypertrophy. However, it may also be due to the activation of the necrosis pathway. While Ca^{2+} is not the main regulator of the necrosis pathway, previous studies have suggested that increases in intracellular Ca^{2+} induced by β-AR stimulation by ISO could induce necrosis [117, 118].

Other distal signaling pathways that have been implicated involve several kinases, which mediate hypertrophy and cardiomyopathy. For example, inhibition of proto-oncogene serine/threonine-protein kinase (Raf-1), which activates MAPK/ERK kinase (MEK) and subsequently extracellular signal-regulated kinase (ERK), may contribute to the development of cardiomyopathy. Yan et al. showed that mice with AC5 disruption (AC5KO) were protected against aging cardiomyopathy, and the mechanism behind this protection was proposed to be enhanced pro-survival Raf-1/MEK/ERK signaling [119]. This is also supported by the finding that ERK activation could prevent myocardial necrosis and apoptosis [120–122] and ERK inhibition under ischemic insult results in increased size of myocardial infarction in vivo [120] as well as increased apoptosis in vitro [123]. Therefore, it may be possible that inhibition of this pro-survival pathway may contribute to the development of cardiomyopathy. Another pathway that contributes to the development of cardiomyopathy involves activation of proapoptotic protein p38α mitogen-activated protein kinase (p38α MAPK), which was shown to be elevated in $G_{s\alpha}$ TG cardiomyopathy

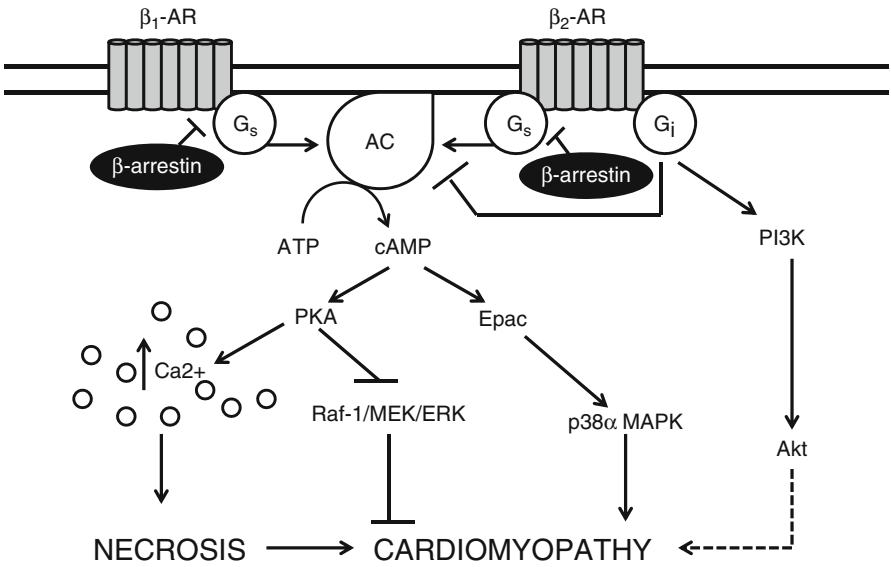

Fig. 1.4 β-AR signaling pathways involved in the development of cardiomyopathy. Chronic stimulation of β_1- and β_2-ARs drives cAMP levels via G_s-AC signaling to activate protein kinase A (PKA) and Epac. PKA induces enhanced activity of Ca^{2+} handling proteins, such as LTCC and RyR2, resulting in significant increases in cytosolic Ca^{2+} levels, which contributes to necrosis and subsequent development of cardiomyopathy. PKA could also inhibit the pro-survival proto-oncogene serine/threonine-protein kinase (Raf-1), mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK), and extracellular signal-regulated kinase (ERK) pathway, thereby relieving this protective mechanism. Similarly, Epac activates p38α mitogen-activated protein kinase (p38α MAPK) to induce cardiomyopathy. The β_2-AR also couples to G_i to activate the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) pathway. While acute stimulation of Akt may be beneficial, chronic stimulation of this protein may have adverse effects. As a protective measure, β-arrestins are recruited to decouple the receptor from downstream signaling, thereby desensitizing the β-AR

model [124]. Peter et al. reported that inhibition of p38α MAPK rescues the cardiomyopathy induced by β_2-AR [97]. While p38α MAPK disruption did not prevent β_1-AR-induced cardiomyopathy, there was upregulation of another proapoptotic protein, mammalian sterile 20-like kinase 1 (Mst-1) in the hearts of β_1-AR TG mice [97], suggesting that Mst-1 may play a role in the development of cardiomyopathy that is more associated with the β_1-AR. The last pathway, which involves phosphatidylinositol 3-kinase (PI3K)–protein kinase B (Akt) signaling, is a matter of debate. Various studies have shown that Akt mediates hypertrophy [125–127], but could also enhance cardiac contractility [128, 129], suggesting it has beneficial roles in the heart. Kim et al. showed that mice with cardiac-specific overexpression of active Akt had increased L-type Ca^{2+} density, as well as increased expression of SERCA2a protein, which resulted in enhanced myocardial contractility [128]. Furthermore, it has been well documented that Akt can protect the heart from apoptosis in vitro [130, 131]

and in vivo [132]. Okumura et al. reported that mice with AC5 disruption (AC5KO) had significantly lower apoptosis compared to WT mice after ISO administration, which was associated with marked increase in active Akt [133]. However, other studies have highlighted the deleterious effects of Akt signaling. Matsui et al. reported that chronic Akt expression in mice results in a vast array of phenotypes, including moderate cardiac hypertrophy with preserved function to significant cardiac dilation as well as sudden death [134]. In a later study, Nagoshi et al. reported that transgenic mice with cardiac-specific Akt expression had significantly larger infarcts without any restoration of function under ischemia/reperfusion injury [135]. Furthermore, it was shown that activation of Akt results in a negative feedback mechanism to inhibit PI3K, concluding that in the heart, PI3K-dependent but Akt-independent pathways are required for full cardioprotection. Given these findings, the possibility of Akt contributing to the development of cardiomyopathy cannot be fully dismissed.

One of the key series of studies instrumental in changing the concept of β-AR stimulation to β-AR blockade in heart failure therapy involved the mouse models with cardiac-specific overexpression of either $G_{s\alpha}$ or β-AR. As noted earlier, these mice responded to increased sympathetic stimulation with enhanced levels of heart rate and contractility (Fig. 1.2a) [93]. This was not a transient process, as occurs with intravenous administration of catecholamines, but was a permanent fixture of their cardiac function through constant overexpression. The $G_{s\alpha}$ mice tolerated the chronically enhanced cardiac function when they were young, but developed cardiomyopathy as they aged, as reflected by cardiac dysfunction and heart failure, myocyte necrosis and apoptosis and cardiac fibrosis, as well as premature mortality from the cardiomyopathy. When these mice were put on continuous β-AR blockade therapy, they were protected from cardiomyopathy and showed improvement in LVEF as well as improvement in survival (Fig. 1.5a) [94]. These findings were not only novel at the time, but also played a significant role in the paradigm shift from β-AR agonist to β-AR blockade in the treatment of heart failure.

1.5.2 Clinical Evidence

A major deterrent to β-AR stimulation therapy was that patients who received inotropic agonists often experienced side effects, including elevated heart rate, arrhythmias, and peripheral vasoconstriction, which overshadowed any initial inotropic benefits gained. Moreover, patients with treatment experienced worsening of their condition and significantly higher mortality rates than the placebo group. For instance, ISO induced fatal arrhythmias and intensified myocardial ischemia in patients with coronary artery disease. Patients with dopamine treatment developed ventricular tachyarrhythmias. Moreover, prenalterol significantly increased mortality. In addition, while dobutamine had beneficial effects in the acute setting, chronic administration had adverse effects. In fact, the 1999 Flolan International Randomized Survival Trial proved dobutamine to increase mortality [137]. These findings were critical in the shift from beta agonists to β-AR blockers in the treatment of heart failure.

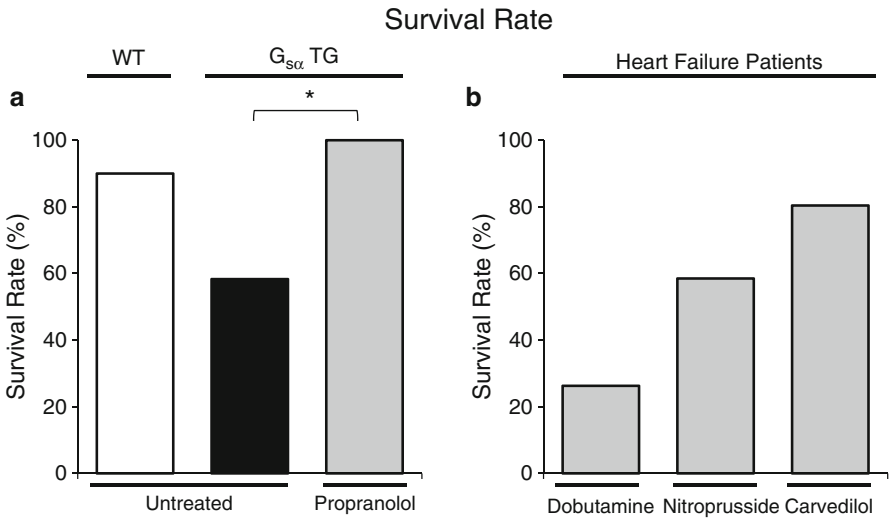

Fig. 1.5 β-AR blockade treatment increases survival rate. (**a**) Approximately 9-month-old WT and $G_{s\alpha}$ TG mice were followed for mean duration of 6.5 months with or without propranolol treatment. In the untreated group, $G_{s\alpha}$ TG mice have premature mortality, which was abolished by propranolol treatment. (*$p<0.05$ by log-rank test) [94]. (**b**) By 300 days of therapy, β-AR blocker, carvedilol, was shown to be superior in prolonging the lifespan of heart failure patients compared to both β-AR agonist (dobutamine) and vasodilator agent (nitroprusside) [79, 136] Figures moadapted with permission from Journal of Clinical Investigation and Oxford University Press

The other major clinical contribution was the concept of β-AR desensitization in patients with heart failure introduced by Bristow and his colleagues [57]. As an adaptive response to increased catecholamine levels, β-ARs desensitize through downregulation of the receptors and attenuation of downstream signaling. Bristow et al. demonstrated that isoproterenol (ISO) stimulation in failing human hearts resulted in 50–56% reduction in β-AR density, decreased AC activity, and decreased muscle contraction compared to normal hearts [57], and the half-equivalent dose of ISO was fivefold higher in the failing tissue compared to the normal tissue [138]. Dose–response study with dobutamine showed progressively lower inotropic response (dP/dt) in patients with severe heart failure compared to those with moderate heart failure [139]. The observed β-AR desensitization is mediated by two mechanisms. First, β-AR mRNA is downregulated [140, 141] potentially through degradation by A + U-rich element RNA-binding/degradation factor (AUF1) [142]. It was found that AUF1 expression is significantly elevated in individuals with heart failure, and its abundance was regulated by β-AR stimulation. In addition, AUF1 was able to interact with β-AR mRNA suggesting it may be responsible for its stability. Second, β-ARs are decoupled from downstream signaling [143–145].G protein-coupled receptor kinase 2 (GRK2), a member of the GRK family commonly known as β-AR kinase 1 (βARK1), phosphorylates agonist occupied β-AR [146] and subsequently recruits

β-arrestin, which inhibits G protein-mediated signaling (Fig. 1.4) [147]. Studies in the left ventricles of patients with dilated or ischemic cardiomyopathy showed elevated expression and activity of GRK2 [141].

Therefore, in the setting where β-ARs are already desensitized, β-AR agonist treatment will not provide much inotropic response. In fact, it will exacerbate the condition, as seen in the clinical trials of β-AR agonists. For these reasons, the concept of β-AR desensitization was important in understanding the rationale for β-AR blockers in heart failure therapy.

1.6 Advent of β-AR Blockade Therapy

β-AR blockade therapy began with the seminal studies of Sir James Black, who was awarded the Nobel Prize in 1988 [148] for developing β-AR blockers in the treatment of cardiac disease. He began his studies with the goal of reducing myocardial oxygen demands [149], and by 1962, he and Stephenson introduced a nonselective β-AR blocker, pronethalol [150]. It was reported that pronethalol had strong antiarrhythmic effects in guinea pigs and dogs [151–154] and was effective in managing angina pectoris in patients [155]. Shortly after, Black developed another nonselective β-AR blocker, propranolol, in 1965 [156]. He compared the effects of pronethalol with propranolol and showed that propranolol was superior in reducing heart rate and blocking ISO-induced hypotension without any fall in blood pressure in dogs [156].

Furthermore, unlike inotropic agonists, propranolol treatment in mice did not induce myocardial damage [157]. In fact, it was shown to be effective in reducing mortality rates and ventricular fibrillation in experimental studies of coronary artery occlusion [158, 159]. Similar effects were seen in the clinical setting, and it was reported that propranolol was effective in improving myocardial oxygenation [160] and reducing mortality in patients with myocardial infarction [161]. Furthermore, it had strong antiarrhythmic effects as well as having beneficial effects in controlling angina pectoris in heart failure patients [162–164]. The success of propranolol also led to the development of β_1-AR selective β-AR blockers including practolol, atenolol, and metoprolol. While practolol had less β-AR blocking potency than propranolol, animals studies demonstrated that practolol was effective in preventing arrhythmias and other alterations in cardiac function [165]. Furthermore, administration of atenolol or metoprolol in mice was able to prevent myocardial injury induced by epinephrine, including inflammatory cell infiltration, fibrosis, and atrophy and necrosis [166]. Then in 1975, the beneficial effects of practolol and alprenolol were illustrated clinically [167]. Six patients were treated with oral β_1-AR selective blocker practolol, and one patient received the nonselective β-AR blocker, alprenolol, for 2–12 months. After treatment, all seven patients showed improvement in ventricular function and reduction in heart size. The long-term effect of β_1-AR

selective blocker, metoprolol, was also evaluated in an international multicenter study, the metoprolol in dilated cardiomyopathy (MDC) trial [168]. Beginning from 1985, 383 patients were followed for at least 12 months. Although the beneficial effects of metoprolol were modest during the first year of treatment, the requirement for heart transplantation was dramatically decreased in the treatment group. In addition, metoprolol was well tolerated in the long term, indicated by the low withdrawal rate. In 1994, the trial on the β_1-AR selective blocker bisoprolol in the cardiac insufficiency bisoprolol study (CIBIS) was published [169] and was soon followed by the US Carvedilol (nonselective β/α_1-AR blocker) Study in 1996 [136]. Patients with carvedilol treatment had significant improvement in mortality, and the survival benefit compared to β-AR agonist, dobutamine, as well as to vasodilator agent, nitroprusside, is clear (Fig. 1.5b). The first large randomized clinical trial was the Cardiac Insufficiency Bisoprolol Study II (CIBIS-II), released in 1999, in which 2,647 heart failure patients were followed for a mean duration of 1.3 years [170–173]. The study was terminated early due to the clear benefit of bisoprolol in decreasing mortality rate (11.8% vs. 17.3% bisoprolol vs. placebo). In 2000, similar results were seen in the Metoprolol CR/XL Randomized Intervention Trial in congestive heart failure (MERIT-heart failure) [174].

1.7 Mechanisms Mediating Salutary Effects of β-AR Blockade Therapy in Heart Failure

While β-AR blockers improve cardiac function as well as hemodynamic response, the concept that β-AR blockade would be useful therapy in heart failure is counter-intuitive, with the inverse reasoning of why β-AR stimulation would be useful in heart failure, alluded to earlier. In addition, β-AR blockade has already been initiated by failing heart through desensitization and reduced β-AR density and regulation, also noted earlier. To put it simply the failing heart has an internal brain telling the physician how to design its therapy, i.e., if β-AR blockade is already initiated physiologically, then it follows that more of the same might be indicated. The counter argument is based on knowing that β-AR blockers have a profound cardiac depressant effect, which could not be tolerated in a heart already failing with marked cardiac depression. Indeed, early attempts at β-AR blockade often failed because of this. It was not until physicians recognized that by gradually and incrementally instituting β-AR blockade was it possible to achieve therapeutic levels without compounding the cardiac depression. This is combined with the fact that some of the current β-AR blockers used clinically, e.g., carvedilol, have actions other than simple β-AR blockade, such as vasodilating effects, and are as not as potent β-AR blockers as propranolol.

Empirically noting the above does not mean that all the mechanisms mediating the salutary action of β-AR blockade in heart failure are known. In fact, it remains unclear how β-AR blockers lower the risk of cardiac complications. However, it is

apparent that β-blockers offer far more than simply blocking the receptor. First of all, again with inverse reasoning to why β-AR stimulation therapy failed clinically, β-AR blockers reduce heart rate, arguably the most important regulator of $\dot{M}VO_2$. As noted above, the imbalance between myocardial oxygen supply and demand is an important mechanism resulting in myocardial dysfunction through invocation of subendocardial ischemia. Since the most important regulator of myocardial oxygen demand is heart rate followed by LV wall stress, a drug which diminishes heart rate and prevents subendocardial ischemia will then reduce LV wall stress. These hemodynamic factors alone will eventually act to prevent a further decline in cardiac function followed by gradual recovery. This also results in a "resensitizing" effect. These include upregulating β-AR density, decreasing GRK2 activity, correcting the impairment of Ca^{2+} handling proteins, and reversing downstream signaling.

1.7.1 Upregulation of β-AR Density

Reduced contractility in chronic heart failure through desensitization mechanisms is protective, as it reduces myocardial oxygen demand, and more importantly, the sudden increases that occur with stress. As noted earlier, Bristow et al. initially reported that β-AR density is reduced by 50% in failing human ventricles, and later studies have confirmed this finding. Therefore, it is conceivable that increases in cardiac performance with β-blockade result, in part, from quantitative restoration of β-AR. In the normal rat heart, chronic infusion of nonselective β-AR blocker, propranolol, was associated with significant increases in both β_1- and β_2-AR density [175]. In addition, heart failure patients with β_1-AR blocker treatment, metoprolol, also showed upregulations of cardiac β-ARs [176, 177]. The mechanism behind this action is uncertain. However, the synthesis and degradation of β-ARs seem to be regulated on the RNA level. As mentioned earlier, AUF1 is significantly increased in heart failure and was shown to degrade β-ARs and thereby reducing its density [142]. In addition, the increase of β-ARs following of β-AR blockade was seen on the protein level as well as on the mRNA level [140]. Interestingly, however, carvedilol and bucindolol, nonselective β-AR blockers with vasodilating properties through α_1-AR inhibition, did not exert β-AR upregulating effects in heart failure patients, despite being as effective in improving cardiac function [176, 178]. It may be possible that these classes of β-AR blockers have other mechanisms mediating their beneficial effects.

1.7.2 Decreasing GRK2 Activity

The increase in inotropy seen in patients with β-AR blockade may indicate that there is an enhancement of β-AR signaling from its depressed state. GRK2 is a G protein-coupled receptor kinase responsible for phosphorylating the β-ARs and

decoupling the receptor from downstream signaling via recruitment of β-arrestin. Patients with heart failure have elevated GRK2, consistent with the β-AR desensitization concept. In addition, studies in transgenic mice have shown that increase in GRK2 has negative inotropic response, which could be ameliorated by GRK2 inhibition [179]. Given this finding, it may be possible that improvement in contractile response seen in patients with β-AR blockade may be partly due to decreased GRK2 activity. In fact, studies in mice [180] and pigs [181] have shown that treatment with bisoprolol, atenolol, and carvedilol downregulate GRK. This effect was also seen in heart failure patients. Treatment with metoprolol or bisoprolol decreased GRK2 activity in the right atrium compared to patients who did not receive β-AR blockers [182, 183]. Therefore, downregulation of GRK2 may be an important mechanism by which β-AR blockers confer beneficial effects.

1.7.3 Correcting the Impairment of Ca^{2+} Handling Proteins

The decreased β-AR signaling also leads to subnormal phosphorylation of Ca^{2+} handling proteins. In fact, PLB and TnI have been shown to be hypophosphorylated in the failing hearts [113, 184, 185]. Decreased phosphorylation of PLB results in delayed SR Ca^{2+} uptake, which in part explains the relaxation deficit in heart failure patients, as well as decreased SR unloading, resulting in weaker contractions. Interestingly, however, RyR2 have been reported to be hyperphosphorylated in heart failure [39]. The result is a "leaky RyR2" that leads to higher diastolic Ca^{2+} concentrations and a decreased Ca^{2+} loading of the SR, which leads to higher propensity for arrhythmias. Given that β-AR signaling is attenuated in heart failure, it may be possible that β-AR blockade could correct these impairments through resensitizing the system, thereby restoring the phosphorylation of these proteins to normal levels. In fact, patients with carvedilol, metoprolol, or atenolol treatment had restoration of normal phosphorylation of RyR2 associated with improved cardiac muscle function [186].

1.7.4 Reversing Adverse Effects of Distal Signaling

It is also important to note that β-AR blockers could reverse the maladaptive signaling in distal mechanisms. For instance, the increase in levels of proteins involved in cellular growth and death, such as p38α MAPK, Jun N-terminal kinase (JNK), and Akt, seen in $G_{s\alpha}$ cardiomyopathy model was downregulated upon propranolol treatment [124]. In parallel to this evidence, patients who responded to either metoprolol or carvedilol had restoration of adult α-myosin heavy chain isoform and reduction of the fetal β-isoform levels [187, 188]. This effect could be due to decreased circulating catecholamine levels from β-AR blockade because re-induction of fetal genes and shift in myosin heavy chain isoform occur in parallel to the elevated levels of

catecholamines seen in heart failure patients [189]. From these findings, it is clear that β-AR blockade does not simply antagonize the receptor, but also affects multiple levels of the signaling pathway to salvage the heart.

1.8 Future Directions

The treatment of heart failure and the reduction in mortality and morbidity have all improved markedly over the past several decades; however, this disease remains a leading cause of mortality and morbidity. Accordingly, there is considerable room for improvement in therapy. Advances will occur in finding new components of the β-AR signaling pathway to inhibit that are distal to the β-AR and have less adverse depressant effects on LV function. This could be at the level of inhibiting adenylyl cyclase [190] or even more distal signaling mechanisms, e.g., Raf/MEK/ERK, p38 MAPK, and other kinases yet to be defined. It is interesting that inotropic therapy is not dead, and new agents are being devised to stimulate the failing heart, without the adverse consequences of increasing $\dot{M}VO_2$. One example is the development of cardiac myosin activators, which improve the performance of the failing heart in chronically instrumented conscious dogs and patients with heart failure [191–193]. In contrast to sympathomimetic amines, such as dobutamine or dopamine, the cardiac myosin activators do not increase $\dot{M}VO_2$ (Fig. 1.6a), most likely because they reduce heart rate and do not increase LV wall stress. The most unique aspect of their action is to increase stroke volume and cardiac output by increasing the duration of cardiac contraction, and not by increasing the rate of LV pressure development (LV dP/dt) (Fig. 1.6b, c). These drugs along with those affecting Ca^{2+} and particularly at the level of the ryanodine receptor [39] could be responsible for the next advances in heart failure therapy.

1.9 Conclusions

This chapter summarized the development of β-AR stimulating agents for the treatment of heart failure from the discovery of β-ARs to how they regulate cardiac contractility and their use for most of the twentieth century in cardiac therapy. There are very few therapeutic approaches that demonstrated a diametrically opposite turn as did the transition from β-AR agonists to antagonists in the treatment of heart failure. The reasoning for treating heart failure with β-AR agonists was simple: (1) the failing heart has poor contractility, (2) catecholamines were the most potent stimulators of myocardial contractility, and (3) catecholamines would be useful to increase contractility in patients with heart failure (quod erat demonstrandum). What was not known was either the concept of desensitization of β-AR or the fact that there is an oxygen cost of increasing heart rate, LV wall stress, and myocardial contractility, i.e., the

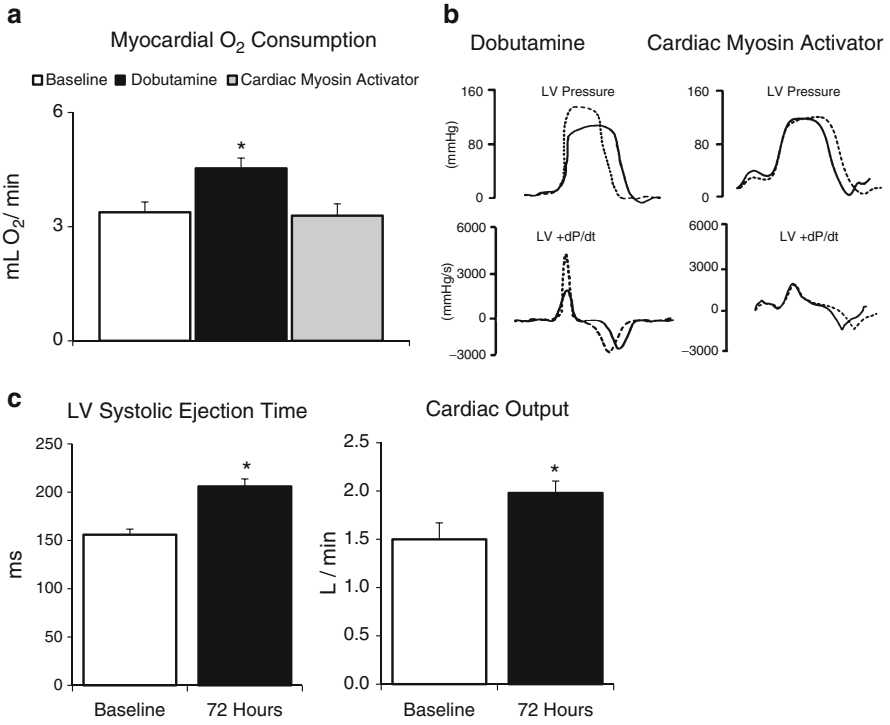

Fig. 1.6 Cardiac myosin activators improve cardiac function in hearts with systolic dysfunction. (**a**) Unlike dobutamine, cardiac myosin activators do not increase myocardial O_2 consumption (*$p<0.05$ vs. baseline) [191]. (**b**) Compared to dobutamine, cardiac myosin activators do not increase LV pressure development (solid line: before treatment, dashed line: after treatment) [191]. (**c**) 72-h infusion of cardiac myosin activator significantly increases LV systolic ejection time and cardiac output from the baseline (*$p<0.05$ vs. baseline) [191]. Figures adapted with permission from Lippincott Williams & Wilkins

major determinants of $M\dot{V}O_2$, which, in turn, increase the requirement for coronary blood flow. The increased myocardial metabolic demand is easily met in normal hearts and coronary circulations, but not so in the setting of either hypertrophy or heart disease characterized by limited subendocardial coronary reserve. Under these conditions, the imbalance between coronary blood flow supply and myocardial oxygen demands results in myocardial ischemia, which exacerbates cardiac dysfunction. All of these factors were fundamental to the change in course from β-AR stimulation to β-AR blockade in the treatment of heart failure, reinforced by poor clinical outcomes of patients on β-AR stimulation therapy. This chapter elucidates the experimental and clinical evidence delineating the mechanisms mediating increased cardiac function with β-AR agonists and conversely the deleterious effects of chronic stimulation, which can lead to heart failure, resulting in a transition for heart failure therapy from β-AR stimulation to blockade.

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Chapter 2
Remodeling of Potassium Channels in Cardiac Hypertrophy

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Abstract The potassium channel is a major target of remodeling in cardiac hypertrophy. To maintain physiological cardiac function in the face of increased workloads, hypertrophied cardiac myocytes undergo downregulation of K^+ channels that results in a prolongation of action potential duration (APD) and upregulation of Ca^{2+} entry channels. Increased intracellular calcium in cardiac hypertrophy activates calcineurin/nuclear factor of activated T cell pathway to permit remodeling of the K^+ channels, resulting in a positive feedback between the K^+ channel remodeling and alteration of Ca^{2+} handling. Although the I_{to} channel is the major target of the K^+ channel remodeling in hypertrophied cardiomyocytes, alteration of other K^+ channels and/or K^+ channel regulators plays an important role in the remodeling and arrhythmogenicity. In this chapter, we list types of K^+ channels and their mRNA that undergo remodeling in cardiac hypertrophy and discuss molecular mechanisms of the remodeling.

Keywords Cardiac remodeling • Hypertrophy • Potassium channels • I_{to} channels • Gene expression • Ca^{2+} handling • Action potential prolongation • Arrhythmias

2.1 Introduction

Hypertrophic growth of the heart is an adaptive process in response to increased workloads. Hypertrophy has been shown to be associated with arrhythmias which can be caused by an alteration in cellular electrophysiology and cardiac remodeling,

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including a prolongation of the action potential duration (APD). Although changes in several ionic currents have been reported in hypertrophied cardiomyocytes, accumulated experimental data have highlighted an importance of K^+ channel remodeling in the APD prolongation in models of hypertrophy [1]. Although a reduction in the transient outward potassium current (I_{to}) is the most consistent ionic current change in cardiac hypertrophy, the target type of K^+ channels varies between species and experimental conditions for hypertrophy. Furthermore, it is believed that heterogeneous reduction, either transmurally or regionally, in I_{to} density associates with QT dispersion that accounts for a mechanism to develop arrhythmias.

Subsequent studies employing molecular biological approaches revealed that modulations of Ca^{2+}-responsive signaling pathways underlie the K^+ channel remodeling [1–3]. Recently, involvement of other pathways has been reported. These molecular mechanisms may provide possible new therapeutic targets against lethal arrhythmias and sudden death in cardiac hypertrophy.

2.2 Electrical Remodeling in Cardiac Hypertrophy

Electrical remodeling in cardiac hypertrophy is considered as a compensatory response to increased workloads. Although the activated signal mechanisms differ in experimental model, it has been reported that renin–angiotensin–aldosterone system, sympathetic nervous system, and other neurohumoral mechanism are activated in cardiac hypertrophy. Mechanical stretch on myocyte also activated signal cascades in heart tissue and cardiomyocyte. These activated signal transductions induce changes in gene expression and function of ion channels, initially to maintain cardiac function. Once these compensatory mechanisms are disrupted, the maladapted responses cause several pathological features including cardiac arrhythmias.

2.2.1 Changes in Action Potential Duration

The most striking feature of excitable cell is its action potential profile. The cardiac myocyte is characterized as having long APD with plateau of maintained depolarization before repolarization. Changes in APD result from alterations in the expression and function of depolarizing and repolarizing currents. Electrical remodeling in cardiac hypertrophy is primarily characterized by prolongation of APD. The prolongation of APD is a consistent finding in hypertrophy by several experimental models including pressure and/or volume overload by aortic constriction [4–13] and hypertension [14–18]. Since APD is primarily responsible for the time course of repolarization, this APD prolongation represents delays in cardiac repolarization. The prolongation of APD is not a unique phenotype in cardiac hypertrophy. Several experimental studies utilizing failing heart induced by myocardial infarction [19–21],

pacing tachycardia [22–24], and genetically modified animal model [25–28] exhibit the APD prolongation as observed in hypertrophy. In addition, myocardial infarction model has higher arrhythmogenicity [29, 30]. Thus, some of findings in failing heart are also useful to comprehend the mechanisms of remodeling in hypertrophy. Since the heart tissue consists of myocyte layers with different action potential profiles, APD varies across the myocardial wall and the region of the heart [31–34]. The regional heterogeneity in APD is enhanced by hypertrophy [35]. In addition, cardiac hypertrophy also enhanced temporal dispersion of APD. The enhanced spatial and temporal dispersions contribute to the arrhythmogenicity in hypertrophied animal models. This finding is consistent with increased dispersion of monophasic action potential duration and electrocardiographic QT intervals and increased prevalence of ventricular arrhythmia in human [36–38].

2.2.2 Experimental Models of Cardiac Hypertrophy

2.2.2.1 Experimental Models to Induce Hypertrophy

Cardiac hypertrophy has been induced by various experimental techniques. Although the prolongation of APD is a common electrophysiological change, signals and ion currents contributing to this APD prolongation differ in each model (Table 2.1).

The pressure overload model by constriction of aorta is widely used to induce left ventricular hypertrophy (LVH). The extent of hypertrophy depends on the site and degree of aortic constriction, resulting in a variety of changes in electrical remodeling. Hypertension model is also considered as pressure and/or volume overload and is another tool to investigate electrical remodeling in hypertrophy. The hypertension model has slower progression of LVH than aortic constriction, which contributes different response of remodeling. Monocrotaline-induced pulmonary hypertension is used as a right ventricular hypertrophy (RVH) model, mostly in rats [8, 39]. Constriction of the pulmonary artery is also utilized for inducing RVH in large mammals [10].

In most of models, hypertrophy is accompanied with a variable amount of heart failure. The balance of heart failure and hypertrophy may explain the difference among several models including complete atrioventricular block [40–43], catecholamine treatment [44], and pacing-induced tachycardia model [22–24] in addition to pressure overload.

2.2.2.2 Animal Species and Expression of Channels

The species of animal is an important factor to understand electrical remodeling in cardiac hypertrophy. APD prolongation is observed regardless of animal species, and it is caused by alterations in K^+ channels in most studies. However, the mechanism of APD prolongation is different between small and large mammals due to the different expression of subtype of K^+ channel (Table 2.1).

Table 2.1 K^+ channel remodeling in cardiac hypertrophy

Model			Currents				
Methods	Species	APD	I_{to} ($I_{to\text{-}f}$, $I_{to\text{-}s}$)	I_{K1}	I_K (I_{Kr}, I_{Ks}, I_{Kur})	Comment	References
Pressure/volume overload							
Ao (abdominal)	Feline	↑		↔	↓	I_K slowed activation, faster deactivation	[4]
PAC (RVH)	Feline			↑	↓	I_K slowed activation, faster deactivation	[7]
Ao	Dog	↑	↑				[9]
Ao	Rabbit	↑	↑	↓	↔		[5]
Ao	Guinea pig	↑		↔	↔	$I_{Ca,L}$↑	[11]
PAC (RVH)	Ferret		↓			Slowed TTP, decay and recovery	[10]
Ao (ascending)	Rat	↑ epi	$I_{to\text{-}f}$ ↓	↔	↔		[13]
Ao (abdominal)	Rat	↑	↓apex, free wall				[6]
Ao (abdominal)	Rat	↑	↓				[12]
DOCA/salt	Rat	↑	↓			Small negative shift gating	[15]
DOCA/salt	Rat	↑	$I_{to\text{-}f}$ ↓		I_{Kur} ↔		[17]
SHR	Rat	↑	↓	↔			[14]
RVHTN	Rat	↑	↑			Slowed I_{to} decay	[16]
RVHTN	Rat					↓Kv4.2/4.3 mRNA, ↔Kv1.2, 1.4, 1.5, 2.1, KCNQ1	[18]
Monocrotaline	Rat	↑ RV	↓ RV				[8]
Monocrotaline	Rat	↑ RV > LV	↓			↓KChIP2 mRNA	[39]
AV block							
	Canine	↑			I_{Ks} ↓, I_{Kr} in RV↓	↓ KCNQ1/KCNE1 mRNA	[41, 43]
	Rabbit	↑QT	$I_{to\text{-}f}$ ↔	↑	I_{Ks} ↓, I_{Kr} ↓		[42]
	Mouse	↑QT	$I_{to\text{-}f}$ ↓			↓Kv4.2/KChIP2 mRNA	[40]
Pacing tachycardia							
	Dog	↑	↓	↓			[23]
	Rabbit	↑	↓	↔			[24]
Catecholamine treatment							
Iso	Rat	↑	↓				[44]
Iso	Rat	↑	↓ Subepi > Subendo			Indirect assessment by 4AP	[35]

(continued)

Table 2.1 (continued)

Model			Currents				
Methods	Species	APD	I_{to} ($I_{to\text{-}f}$, $I_{to\text{-}s}$)	I_{K1}	I_K (I_{Kr}, I_{Ks}, I_{Kur})	Comment	References
Genetic							
Growth hormone-secreting tumor	Rat	↑	↓				[28]
Calsequestrin	Mouse	↑	↓	↓		I_{NCX}↑, I_{Na}↓, $I_{Ca,L}$↓	[25]
H-Ras-v12	Mouse	↑	↓			I_{NCX}↑	[27]
Clinical							
Severe HF	Human	↑	↓	↓	↔		[100]
Failing	Human	↑	↓ Subendo				[69]
Failing LV	Human		$I_{to\text{-}f}$↓ Subepi			$I_{to\text{-}f}$ slowed recovery in subendo	[70]
HF	Human					↓Kv4.3 mRNA, ↔Kv1.4, Kvβ1, Kir2.1, herg	[46]
HF	Human	↑	$I_{to\text{-}f}$↓	↓	I_{Ks} ↓		[47]

↑ increase, ↓ decrease,↔ unchanged, *RVH* right ventricular hypertrophy, *AoC* aortic constriction, *PAC* pulmonary artery constriction, *TTP* time to peak, *SHR* spontaneously hypertensive rat, *DOCA* deoxycorticosterone acetate, *RVHTN* renovascular hypertension, *GH* growth hormone, *RAS* renin–angiotensin system, *Iso* isoproterenol, *HF* heart failure

Rodents (mice and rats) have shorter cardiac APD than large mammals to adapt to the extremely high heart rate (~600 bpm in mice). I_{to} is a major repolarizing current throughout this short action potential, but the contribution of delayed rectifier potassium current (I_K) and inward rectifier potassium current (I_{K1}) remains subtle. The hypertrophy model in rodents revealed reduction of I_{to} in most of the studies. I_{to} consists of two components: fast ($I_{to\text{-}f}$) and slow ($I_{to\text{-}s}$) components according to the fast and slow rates of inactivation, respectively. In rodents, $I_{to\text{-}f}$ is considered as the major target channel rather than $I_{to\text{-}s}$ [45]. Reduction of I_{to} is achieved by change in gating kinetics and/or the change in expression level of channels.

In large mammals, I_{to} is responsible for the initial rapid phase of action potential repolarization, representing a notch preceding plateau phase. The major contributor for repolarization is I_K (I_{Kr} and I_{Ks}). The hypertrophy model in large mammals showed reduction of I_K, but the change in I_{to} varies among experimental models.

Although data on K+ channel remodeling in human cardiac hypertrophy are lacking, reports using human failing heart showed downregulation of I_{to} in left and right ventricle [46] and downregulated I_{Ks} in right ventricle [47].

2.3 Remodeling of Potassium Channels

In models of hypertrophied myocardium, previous investigations have highlighted the importance of remodeling of potassium channels in the APD prolongation. The APD of cardiomyocytes is determined by a plateau phase that is controlled by a fine balance between small inward (calcium) and outward (potassium) ionic currents (Fig. 2.1). Functional downregulation of K^+ currents has consistently been observed especially in severe hypertrophy and failure regardless of species, and has been implicated as a major contributor to the APD prolongation. Distinct molecular complexes of potassium channels involve in hypertrophy depending on experimental conditions and species as described in Sect. 2.2. In this section, previous and recent publications regarding remodeling of potassium channels in channel transcript levels are summarized.

2.3.1 Transient Outward Potassium Current (I_{to})

Downregulation of I_{to} is arguably the most consistent alteration of potassium currents in hypertrophied myocardium. Because I_{to} plays an important role in the early repolarization phase of action potential in most species, it is believed that suppression of I_{to} provides an explanation of QT prolongation and QT dispersion leading to fatal ventricular arrhythmias. Exceptions are studies of some compensatory pressure overload hypertrophy models, in which there was no change or increase in I_{to} densities [16, 48, 49]. I_{to} has been classified into $I_{to\text{-}f}$ and $I_{to\text{-}s}$ as described in Sect. 2.2. These two components are encoded with distinct channel genes and are modulated differently and regionally in response to hypertrophic stresses.

2.3.1.1 The Fast Transient Outward Potassium Current ($I_{to\text{-}f}$)

In mammalian hearts, the fast transient outward potassium current ($I_{to\text{-}f}$) is conducted by channels comprised of voltage-gated α-pore-forming subunits (Kv4.3 in human/canine and Kv4.2 and Kv4.3 in rodents/ferrets) and an auxiliary β-subunit (Kv channel interacting protein 2; KChIP2). Expression of Kv4 channels correlates with regional heterogeneities in $I_{to\text{-}f}$, which is reflected by a predominant expression in the mid-myocardium and epicardium. In many hypertrophy models, reduction of $I_{to\text{-}f}$ density generally correlates with decreased Kv4 channel transcript levels. It is notable that hypertrophic stress induced by non-myocyte cell-conditioned growth medium or phenylephrine ablates regulated expression of I_{to} and Kv4 mRNA, which is seen during normal heart development [50].

KChIP2 has at least two functions, which are to assist transport of Kv4 channels to the plasma membrane and to regulate gating kinetics of Kv4 channels [51]. The former function is in line with no $I_{to\text{-}f}$ functional expression in KChIP2 knockout

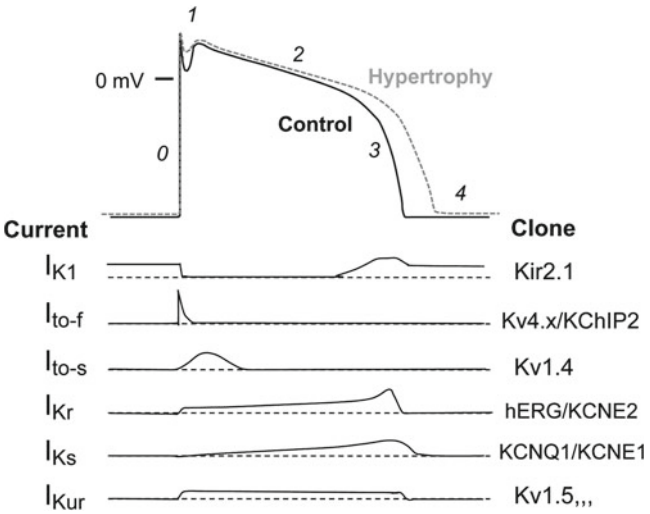

Fig. 2.1 Schematic diagram illustrating the major potassium currents that underlie the action potential in the mammalian ventricle. Top panel shows a control (*solid line*) and hypertrophied (*gray dotted line*) action potential profile. The phases of the action potential are labeled. *Lower panels* show schematic time courses of major K^+ currents as well as clones that encode the currents

mice [52], and KChIP2 accelerates recovery from inactivation in the latter case [51]. Hypertrophy stimulations decrease gene expression of KChIP2 as well as Kv4 α-subunit, which results in reductions in $I_{to\text{-}f}$ [10, 53]. Actually, recent data in hypertrophied rat neonatal cardiomyocytes suggest that decrease in KChIP2 expression controls α1AR-induced reduction in $I_{to\text{-}f}$ through regulation of NF-κB [54].

2.3.1.2 The Slow Transient Outward Potassium Current ($I_{to\text{-}s}$)

Kv1.4 is the predominant gene which encodes the voltage-gated α-pore-forming subunit of the endocardial $I_{to\text{-}s}$ channel. In contrast to $I_{to\text{-}f}$, responses of $I_{to\text{-}s}$ to hypertrophic stimulations are controversial. In LVH/RVH rats, there was no change in Kv1.4 mRNA, although Kv4.2 and Kv4.3 mRNA are the target of remodeling [8, 18, 55]. In a transgenic mouse model of hypertrophy with overexpressed L-type Ca^{2+} channels, Kv1.4 mRNA is increased, whereas Kv4.2 and Kv4.3 mRNA are reduced [56]. Interestingly, Kv1.4 mRNA levels are high at birth, increase during postnatal days (P0 to P10), and subsequently decrease to very low levels in adult rat ventricles, while Kv4.2 increase between birth and adult, becoming the predominant K^+ channel protein [57]. Such K^+ channel isoform switch during postnatal development is diminished by incubation with non-myocyte cell-conditioned growth medium or phenylephrine that induces cardiomyocyte hypertrophy, resulting in reversion of fetal phenotype of transient K^+ channel currents [50].

2.3.2 *Delayed Rectifier Potassium Current (I_K)*

I_K plays an important role in cardiac repolarization process. Reduction of I_K currents in hypertrophied cardiomyocytes has been reported in different models, which may contribute to delayed repolarization in heart failure. In feline ventricular myocytes, hypertrophic stimulation has reduced I_K current density with slowed activation and accelerated deactivation, which may result in a greater predisposition to developing early after depolarization [4, 7]. In hypertrophy models in rabbits, both two components of the I_K currents, I_{Kr} and I_{Ks}, are downregulated [42]. Actually, most of the studies consistently show reduction of I_{Ks} [42, 47, 58], while I_{Kr} (or HERG) is reduced or unchanged [46, 57]. The different results of I_{Kr} changes are model specific. Reduction of I_{Ks} may be associated with alteration of transmural APD heterogeneity. In ventricles of canine failing hearts, the percentage of I_{Ks} reduction was greater in epicardial and endocardial cells than that in midmyocardial cells, which may result in elimination of the transmural I_{Ks} gradient [58].

2.3.3 *Other Potassium Current*

Kv1.5 reduction in hypertrophy is also observed [59–61]. Kv1.5 encodes the ultrarapid delayed rectifier K^+ currents (I_{Kur}) which is dominantly found in atrium not in ventricles. Kv1.5 remodeling in post-MI LVH appears to be related to thyroid hormone (T3) level [61]. Because the Kv1.5 gene has a T3 responsive element in the promoter region, T3 enhances Kv1.5 expression in rat heart [62]. Downregulation of KChIP2 also contributes to the Kv1.5 remodeling, because KChIP2 interacts with Kv1.5 to assist cell surface expression [63]. Antisense oligodeoxynucleotides directed against Kv1.5 mRNA revealed atrial-specific Kv1.5 remodeling [64]. Thus, role of Kv1.5 remodeling may be more significant in atrial fibrillation rather than in ventricular hypertrophy [65]. There are several studies to show downregulation of I_{K1} or alteration of kinetics in heart failure [23], but several studies reported no change of Kir2.1 mRNA in failing heart compared with normal heart [46, 66].

2.4 Regional Heterogeneity of Electrical Remodeling

Regional heterogeneity of cardiac repolarization times results in the T wave in the electrocardiogram. Transmural and/or interregional heterogeneities of potassium channel expression are major contributors to the regional heterogeneity of repolarization times. The densities of $I_{to\text{-}f}$ vary transmurally in the heart, that is, more prominent in epicardium than in endocardium [32, 67, 68]. Hypertrophic stimulation alters the transmural heterogeneity [69, 70]. In rats with LVH induced by isoprenaline [71] and aortic constriction [6], APD is predominantly prolonged in epicardial

cells resulting from greater reduction in $I_{to\text{-}f}$ amplitude compared to endocardial cells. In rat ventricular myocytes that are remote from infarct zone, hypertrophy in epicardium reduced current densities of $I_{to\text{-}f}$ and protein expression of Kv4.2 and Kv4.3 greater than in endocardium [72].

There is interregional heterogeneity of left ventricular repolarization [6, 73]. APD and $I_{to\text{-}f}$ density vary gradually from apex to septum. APD is shortest in left ventricular apex, longest in septum, and intermediate in left ventricular free wall, reflecting from differential $I_{to\text{-}f}$ amplitude [6]. Hypertrophied rat cardiomyocytes induced by abdominal aorta constriction ablate the interregional heterogeneity by greater $I_{to\text{-}f}$ reduction in apex and free wall [6]. Myocardial infarction also eliminates the interregional heterogeneity of APD, I_{to}, and Kv4.2 expression between RV free wall and intraventricular septum [74].

2.5 Possible Mechanisms for Remodeling of Potassium Channels

The involvement of the calcineurin/NFAT pathway in reducing $I_{to\text{-}f}$ has been reported [75–79] (Fig. 2.2). Calcineurin is a Ca^{2+}/calmodulin-dependent protein phosphatase, and its activity is increased in hypertrophied hearts [80–82]. A calcineurin inhibitor cyclosporine A abolishes reduction of $I_{to\text{-}f}$ and Kv4.2/Kv4.3 mRNA expression in hypertrophied rat ventricle [83]. In fetal rodents, calcineurin/NFAT increased $I_{to\text{-}f}$ by transcriptional upregulation of Kv4.2 [84].

On the other hand, APD prolongation by I_{to} reduction in mice and rats leads to calcineurin activation via increase in $[Ca^{2+}]_{in}$ [76, 85–87]. In case of human or canine, reduction in I_{to}, I_{K1}, and I_{Ks} contributes to APD prolongation [47, 58], leading to increase in $[Ca^{2+}]_{in}$. But contribution of the regulation of channels to activation of calcineurin in human or canine is unclear.

The ubiquitous transcriptional factor NFAT has four reported isoforms, which are designated as NFAT1, NFAT2, NFAT3, and NFAT4. Unlike NFAT1, −2, or −4 in immune tissues, NFAT3 is the prominent isoform in the heart. Ca^{2+}-dependent activation of calcineurin induces NFAT dephosphorylation and NFAT translocation to the nucleus where it can interact with GATA4 to activate transcription of hypertrophic responsive genes [88]. Cardiac K^+ channel genes (Kv1.5, Kv2.1, Kv4.2, Kv4.3, and KChIP2) which have putative NFAT binding sites in the promoter regions are downregulated through NFAT3 pathways after myocardial infarction [89]. Among these K^+ channel genes, downregulation of Kv4.2 is the most sensitive to increase in NFAT3 activity compared with other genes (Kv1.5, Kv2.1, Kv4.3, and KChIP2) which require robust increase in NFAT3 activity [78]. Magnitudes of NFAT3 activation vary among hypertrophic models, which can partly explain why some studies do not show downregulation of Kv1.5 and Kv2.1. In a murine hypertrophic model, activation of NFAT3 by calcineurin transduces variations in $[Ca^{2+}]_i$ into differences in I_{to} density in endocardial and epicardial myocytes [89]. $[Ca^{2+}]_i$ and calcineurin/NFAT3

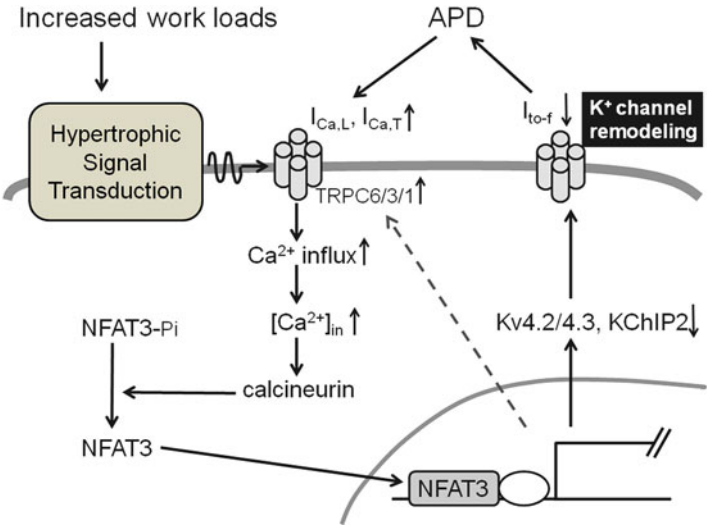

Fig. 2.2 Potential positive feedback between increased Ca^{2+} influx and K^+ channel remodeling

activity is higher in endocardium than in epicardial myocytes, resulting in lower Kv4.2 expression and I_{to-f} density in endocardial cells, at least in mice [90, 91]. This hypothesis is supported by the finding that suppression of Kv4 expression and I_{to} density in post-myocardial infarction hypertrophy is abolished by calcineurin inhibitor cyclosporine A in a rat model [83] and NFAT knockout mice [89].

Involvement of signaling pathways other than the calcineurin/NFAT pathway in K^+ channel remodeling has been also reported. Oxidative stress through an Rac-dependent NADPH oxidase activation and superoxide production is involved in decreases in Kv4.3 mRNA [92, 93]. Kv4.2 and Kv1.4 mRNA expression is also regulated by endogenous oxidoreductase systems [72, 94, 95]. Involvement of NF-κB signaling in downregulation of I_{to-f} through control of KChIP2 and Kv4.2 expression has been reported [54]. Downregulation of I_{to-f} by either α1 adrenergic receptor or TNF-α stimulation may require NF-κB signaling-dependent decreases KChIP2 expression [54]. Mitogen-activated protein kinase (MAPK)/MEK and CaMK II are also involved in reduction of KChIP2 mRNA expression [53, 96]. One can speculate that TRPC6 may affect K^+ channel remodeling by increase in pathological calcineurin/NFAT signaling [97].

2.6 Conclusions

Interruption of the Ca^{2+}-dependent positive feedback loop is a potential new therapeutic target against arrhythmias in cardiac hypertrophy [1, 98]. Considerable progress has been made in understanding the molecular mechanisms of cardiac

remodeling of K^+ channels. We now realize that these mechanisms are highly complex reflecting the crosstalk between a variety of regulatory proteins, which can be altered in diseased hearts [99]. Clearly, future research is still needed to understand the clinical relevance of these regulatory proteins.

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Chapter 3
Role of Gender in Ca^{2+} Cycling and Cardiac Remodeling Due to Heart Failure

Naranjan S. Dhalla, Amrit Malik, Shelly Zieroth, and Paramjit S. Tappia

Abstract Heart failure (HF) is invariably preceded by cardiac hypertrophy. Contractile dysfunction has been linked to ventricular remodeling and progressive defects in cardiomyocyte Ca^{2+} handling as well as changes in the sensitivity of myofibrils to Ca^{2+}. Since the renin–angiotensin system and the sympathetic nervous system are activated in HF, it is likely that subcellular alterations, at the level of the sarcoplasmic reticulum and sarcolemma as well as molecular changes that affect Ca^{2+} homeostasis in the heart, may be due to prolonged exposure to angiotensin II and excess production of catecholamines. In addition, activation of different neurohormonal systems is also linked to cardiac remodeling and subsequent insufficiency in contractile performance. Female sex hormones such as estrogen and progesterone are considered to play a cardioprotective role; however, women lose this protection against various risk factors of heart disease at postmenopausal stage. Accordingly, this chapter highlights some of the mechanisms that cause Ca^{2+}-handling abnormalities in the pathogenesis of HF and the role of gender and menopausal status in influencing the nature and extent of these alterations. Furthermore, the impact of different pharmacological agents including angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, β-adrenoceptor blockers, and Ca^{2+} antagonists on the changes in Ca^{2+}-cycling

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proteins in HF is discussed. Finally, areas where there is a deficiency in knowledge with respect to the mechanisms of subcellular remodeling in HF that need to be addressed are also identified.

Keywords Cardiac hypertrophy • Heart failure • Subcellular remodeling • Ca^{2+}-cycling proteins • Sarcolemma • Sarcoplasmic reticulum • Myofibrils • Sex differences • Estrogen

3.1 Introduction

Heart failure (HF) is a complex problem which is invariably preceded by cardiac hypertrophy as an adaptive mechanism mainly due to the activation of renin–angiotensin system (RAS), sympathetic nervous system (SNS), and several other neurohormonal systems [1–11] (Fig. 3.1). Although the mechanisms for the transition of cardiac hypertrophy to HF are poorly understood, ventricular remodeling due to marked changes in extracellular matrix has been suggested to be closely associated with the development of contractile dysfunction [12, 13]. On the other hand, in view of the ability of sarcoplasmic reticulum (SR) and sarcolemma (SL) to transport Ca^{2+} and regulate the contractile apparatus [14–16], it has been suggested that cardiac dysfunction in HF is also due to progressive defects in the abilities of SR and SL to handle Ca^{2+} as well as changes in the sensitivity of myofibrils (MF) to Ca^{2+} in cardiomyocytes [14–19]. In fact, Ca^{2+}-handling abnormalities have been demonstrated to occur in cardiomyocytes isolated from failing hearts [19–25]. Furthermore, depression in the sensitivity of MF to Ca^{2+} and MF Ca^{2+}-stimulated ATPase activity in the failing heart has been shown to be due to changes in myosin isozyme composition, myosin light chain kinase (MLCK), troponin phosphorylation, and troponin Ca^{2+}-binding activities [14–16, 18, 26]. Such subcellular alterations are considered to be due to occurrence of intracellular Ca^{2+} overload, elevated levels of tumor necrosis factor-alpha (TNF-α), development of oxidative stress, and activation of proteases [2, 14–16, 27, 28] (Fig. 3.2). Furthermore, varying degrees of changes in SR Ca^{2+}-pump ATPase, Ca^{2+}-release channels, and phospholamban (PLB) as well as mRNA levels for these SR proteins are found to occur in failing hearts [14–16, 20, 21]. Alterations in SL enzyme and their gene expression in different types of failing hearts are related to Na^+–K^+ ATPase, Ca^{2+}-channels, and Na^+–Ca^{2+} exchange [22–26, 29, 30]. Likewise, changes in MF proteins and their gene expression for α-myosin heavy chain, β-myosin heavy chain, and regulatory proteins (MLCK, troponin I, and troponin T) are observed in HF [14, 31–34]. While the activation of protein kinase C (PKC) has been shown to depress the SR Ca^{2+}-pump ATPase expression [35–39], PKC and other kinases such as protein kinase A, Ca^{2+}-calmodulin-dependent kinase (CaMK), and mitogen-activated protein kinase [40–43] are involved in the pathway of signal transduction for subcellular remodeling in failing hearts (Fig. 3.3). Since RAS and SNS are activated in HF [44–48] and

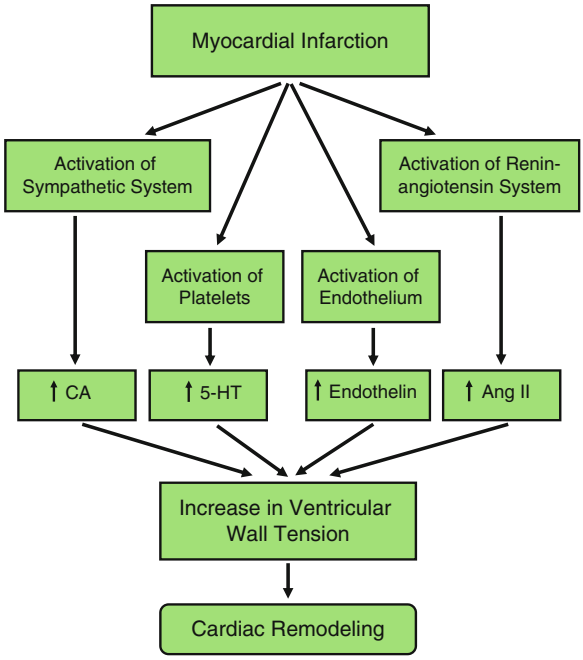

Fig. 3.1 Different neurohormones involved in cardiac remodeling in response to myocardial infarction. *CA* catecholamines, *5-HT* 5-hydroxytryptamine, *Ang II* angiotensin II

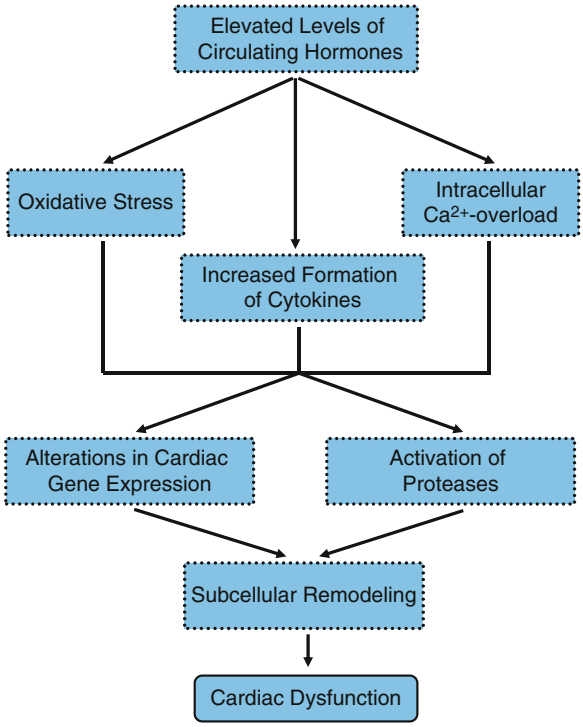

Fig. 3.2 Role of elevated circulating hormones in subcellular remodeling and subsequent cardiac dysfunction

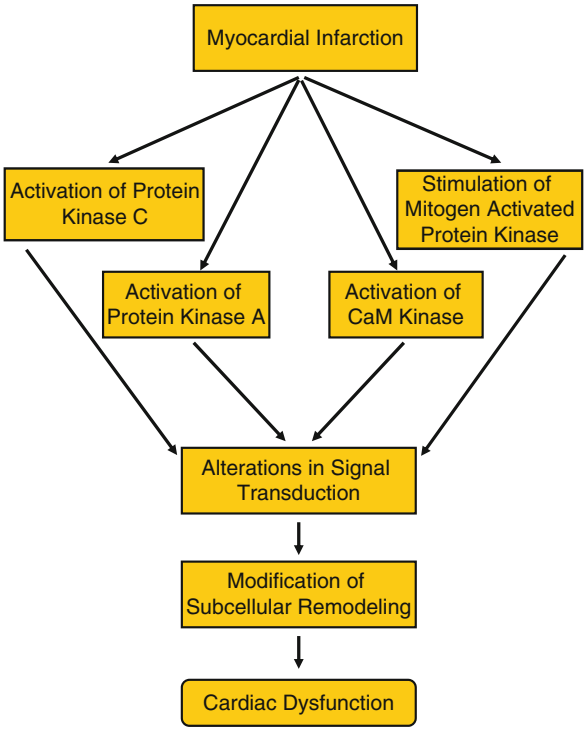

Fig. 3.3 Activation of different kinase signal transduction systems due to myocardial infarction. *CaM kinase* Ca^{2+}/calmodulin-dependent kinase

prolonged exposure of the heart to angiotensin II (Ang II) as well as catecholamines causes changes in cardiac gene expression [49–52], it is likely that subcellular alterations in the hearts may be due to the activation of both RAS and SNS. It is noteworthy that most of the information on the mechanisms of HF is obtained from males whereas little is known about the cellular and molecular mechanisms of cardiac dysfunction during the development of HF in females. Figure 3.4 summarizes the subcellular compartments that undergo alterations during cardiac remodeling in the development of HF.

3.2 Gender Difference and Pathophysiology of HF

Several epidemiological studies have established that gender is a major determinant of cardiovascular risk factors and heart disease [53–57]. Premenopausal females have a lower risk of developing coronary artery disease compared to men; however, this female advantage is lost after menopause [58–60]. Furthermore, women show more diastolic HF with normal ejection fraction, whereas men have more systolic

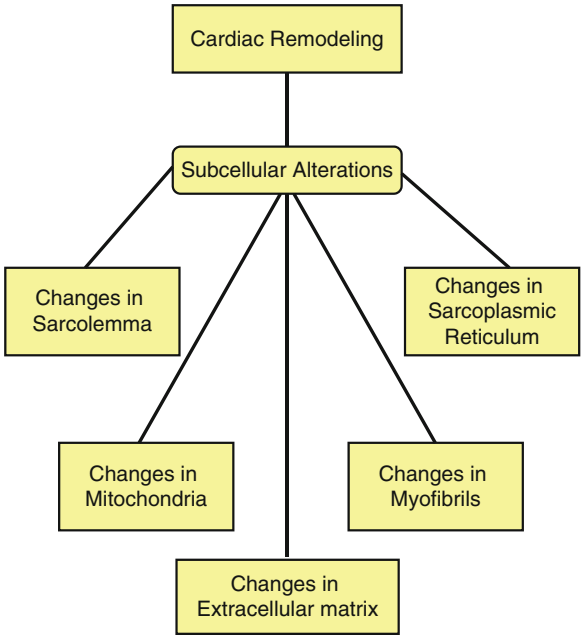

Fig. 3.4 Cardiac remodeling due to alterations in the different subcellular compartments

HF with depressed ejection fraction [61–63]. Although HF in men, unlike women, is normally preceded by eccentric cardiac hypertrophy [62], gender difference in several types of HF is associated with differences in cardiac remodeling and cardiac dysfunction. HF in male rats due to volume overload was accompanied by a greater degree of cardiac dysfunction, cardiac remodeling, and apoptosis in comparison to females [64–68]. Gender differences in aortic regurgitation-induced cardiac function and cardiac remodeling have also been observed in patients [69, 70]. Volume overload in boys with HF was found to produce a greater decrease in mRNA levels for SR Ca^{2+}-pump protein than that seen in girls [71]. Gender and sex-related differences were observed for cardiac function in patients with HF due to aging [72] as well as for apoptosis in HF patients with ischemic and dilated cardiomyopathy [73, 74]. HF due to pressure overload in males was also observed to produce greater changes with respect to cardiac remodeling and cardiac dysfunction [75, 76] as well as a greater reduction in gene expression in SR Ca^{2+}-pump protein and Na^{+}–Ca^{2+} exchanger [77] in comparison to those seen in females. Gender differences in the expression of Na^{+}–Ca^{2+} exchanger current and β-AR responsiveness were also seen in HF due to tachycardia in pigs [78]. Induction of myocardial infarction (MI) for 4–6 weeks in female rats produced a lesser increase in ventricular wall thickness and fewer abnormalities in diastolic filling compared to male rats without any difference in infarct size, left ventricle (LV) cavity enlargement, or systolic dysfunction [79–81]. Sex-related differences in changes in cardiac function as well as

cardiac remodeling, without any difference in infarct size, were also seen in mice upon inducing MI for 2–12 weeks [82–84]. It is pointed out that while gender differences with respect to both cardiac dysfunction and cardiac remodeling have been shown to occur in different types of HF, very little information regarding molecular and cellular mechanisms for the gender-related difference in HF due to MI is available in the literature.

It is generally held that after menopause, females lose advantage for protection against various risk factors of heart disease and the female sex hormones such as estrogen and progesterone are considered to play an important role in cardioprotection. Conversely, greater susceptibility of males to heart disease may be due to the markedly high levels of circulating testosterone compared to females. Sex-related differential changes in gene expression and DNA synthesis in normal male and female hearts were considered to be due to the regulatory effects of sex hormones [85]. The regulatory influence of sex hormones in normal male and female animals was shown in studies on the effects of ovariectomy with or without estrogen and progesterone treatment as well as castration with or without testosterone treatment. Ovariectomy in normal animals depressed cardiac function and induced changes in SR Ca^{2+}-pump protein, Ca^{2+}-release channels, and SL Na^{+}–Ca^{2+} exchange activities; these effects were abolished by estrogen replacement [86, 87]. Both ovariectomy in female and castration in male normal rats were also found to decrease cardiac function and produce a shift in myosin enzymes; these effects were attenuated by treatment with estrogen and testosterone, respectively [88]. Furthermore, castration in normal male rats also decreased mRNA levels for SL Na^{+}–Ca^{2+} exchanger, L-type Ca^{2+} channels, and β-ARs; varying degrees of alterations in these effects in castrated animals were observed upon treatment with testosterone [89–91]. On the other hand, ovariectomy attenuated the volume overload-induced cardiac dysfunction and cardiac remodeling, and these effects were prevented by estrogen treatment [65–68, 92, 93]. Alterations in β-AR signal transduction and apoptosis due to volume overload in ovariectomized animals were also attenuated by estrogen [66–68]. The cardioprotective effect of estrogen was seen against volume overload-induced structural and functional changes in males [94]. Ovariectomy augmented pressure-induced hypertrophy and changes in Akt–eNOS signaling pathway in females [95] as well as chronic β-AR stimulation-induced calpain activation and impairment in Akt–eNOS pathway [96]. Chronic administration of estrogen normalized MI-induced cardiac remodeling and the increase in endothelin-1 receptor expression [97]. These observations support the view that estrogen is cardioprotective in attenuating cardiac dysfunction and cardiac remodeling in HF; however, the role of other sex hormones in this regard is poorly understood. Furthermore, it should be noted that MI is the major cause of HF when compared with other risk factors such as pressure overload, volume overload, valvular defects, and different types of cardiomyopathies [28, 98, 99], and thus, it is of critical importance that molecular and cellular mechanisms of MI-induced HF in both males and females be carefully examined to gain further information on gender difference with respect to HF.

3.3 Pathogenesis of Cardiac Dysfunction in HF Due to MI

Several animals such as rabbits, cats, and dogs exhibit varying degrees of MI upon occlusion of the coronary artery [100–107]; however, due to high mortality and relatively small infarct size, these animals do not form good experimental models of HF. Some investigators used mice as a model for inducing MI [82–84], but the viable LV from these animals is not sufficient for biochemical analysis of subcellular organelles during the development of HF. On the other hand, rat was found to be an excellent model of HF for studying changes in the viable myocardium following occlusion of the left coronary artery; a wide spectrum (15–60% of the LV) of scar occurred and healed completely [108–115]. The degree of depression in the LV function was observed to depend upon myocardial infarct size as well as duration of MI [116, 117]. Ventricular remodeling due to an increase in collagenous material in the extracellular matrix was found to occur following MI [118–123], and this was suggested to explain cardiac dysfunction in HF. In addition, both plasma and cardiac RAS were activated in the infarcted animal and promoted the formation of Ang II [124]. We have examined time-course changes in heart function and observed that mild, moderate, and severe stages of HF occurred at 4, 8, and 16 weeks after inducing MI in rats, respectively [125]. Furthermore, the number of Ca^{2+} channels, which permit Ca^{2+} entry into the cardiac cell, as well as K^+ channels, was depressed at the moderate (8 weeks) and severe (16 weeks) stages of HF following MI in rats [112, 125]. SR Ca^{2+} uptake activity was depressed at 4, 8, and 16 weeks after inducing MI [126, 127]; this change was associated with a depression in SR Ca^{2+}-pump ATPase as well as gene expression [128–130]. Likewise, the density of beta-adrenergic receptors (β-ARs) decreased without any changes in the affinity ($1/K_d$) at 4 and 8 weeks whereas the number of alpha-adrenergic receptors (α-ARs) increased at 8 and 16 weeks after inducing MI [131]. Further experiments revealed depressions in β_1-AR and adenylyl cyclase activities in failing LV of the infarcted animals [132]. The activities of SL Na^+–K^+ ATPase [133, 134] and SL Na^+–Ca^{2+} exchange [135] were depressed without any change in the SL Ca^{2+}-pump activity at 8 and 16 weeks of MI. Alterations in membrane potentials as well as Ca^{2+} currents, Na^+–Ca^{2+} exchange currents, and expression of K^+ channels were also observed in the infarcted myocardium [136–140]. Furthermore, decrease in cardiac contractility of the infarcted heart was shown to be partly due to changes in myosin isozymes as well as actomyosin ATPase activity [114, 141]. Alterations in myosin light chain have been found [142, 143], and a decrease in the sensitivity of the viable myocardium to Ca^{2+} has been reported [144, 145] in HF due to MI. We also reported marked changes in MF ATPase as well as myosin protein and gene expression in hearts failing due to MI [146, 147]. These studies suggest that heart dysfunction in rats with HF due to MI is not only associated with changes in extracellular matrix, but alterations in SL and SR as well as MF in cardiomyocytes are also involved intimately.

Treatment of infarcted animals with HF by various angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor (AT_1R) antagonists prevented

ventricular remodeling, improved heart function, changed hormone profile, and reduced mortality [148–160]. Likewise, the treatment of infarcted rats with captopril attenuated the loss of cardiac β-AR responses and enhanced intracellular Ca^{2+} handling in the viable LV [161, 162]. Blockade of RAS by agents such as imidapril, enalapril, and losartan also ameliorated changes in the β-AR signal transduction system in HF due to MI [163, 164]. Different β-AR blockers, propranolol and atenolol, also partially prevented MI-induced changes in cardiac performance as well as MF and SR activities [165, 166]. Favorable effects in reducing LV remodeling and improving heart function in rats with MI were obtained upon exercise training as well as upon treatment with growth hormone, thyroid hormones, and antioxidants [167–170]. Treatment of animals with antiplatelet agents, sarpogrelate (a $5\text{-}HT_{2A}$ receptor antagonist) and cilostazol (a phosphodiesterase inhibitor), revealed attenuation of LV remodeling, cardiac dysfunction, and remodeling of both SR and MF in HF due to MI [171–173]. Since Ca^{2+} antagonists, metabolic therapy, and treatment with Na^{+}–H^{+} exchange inhibitors have also been reported to produce beneficial effects in the infarcted animals [174–179], it is suggested that various molecular targets in cardiomyocytes are involved for improving cardiac function in HF. Extensive studies in our laboratory on treatment of MI-induced HF with different agents [180–192] have shown that improvement of cardiac performance in the failing heart is associated with the prevention of alterations in cardiac gene expression specific for SL, SR, and MF proteins. Furthermore, our work on MI-induced HF has indicated that changes in SR Ca^{2+}-cycling proteins are more dramatic in comparison to alterations in the function of both SL and MF.

3.4 Conclusions

From the foregoing review of literature, it is clear that most of the data regarding the molecular and cellular mechanisms of cardiac dysfunction in HF due to MI, which represents the major form of HF, are obtained by employing male hearts. It is also evident that gender differences with respect to development of cardiac remodeling and cardiac dysfunction in different types of HF have been identified; however, the mechanisms for such differences are poorly understood. It is therefore emphasized that a comprehensive study on the mechanisms of gender difference in HF should be undertaken. Since abnormalities in the function of SR, by virtue of its ability to release and accumulate Ca^{2+} for the occurrence of cardiac contraction and relaxation, are considered to be the major determinants of cardiac dysfunction in HF [2, 14, 16, 26], a careful examination of SR Ca^{2+}-release channels and Ca^{2+}-pump activities, protein content, and gene expression in the MI-induced HF in both males and females should be carried out. In view of the early, moderate, and severe stages of HF in male rats at 4, 8, and 16 weeks of inducing MI, respectively [125, 126, 131–133], both male and female hearts will be used for the assessment of changes in Ca^{2+}-cycling proteins at different times of inducing MI to establish delayed transition of cardiac hypertrophy to HF in females compared to males. Since the activation of CaMK is known to

increase SR Ca^{2+}-release channel and Ca^{2+}-pump activities [40, 182], CaMK activity and protein content should be determined to establish if gender difference in MI-induced HF is due to difference in the regulation of Ca^{2+}-cycling proteins by CaMK. In view of the role of prolonged activation of RAS in the development of HF, various components of peripheral and cardiac RAS should be monitored in MI-induced HF to investigate if gender difference in HF is determined by difference in the activation of RAS. Since sex hormones are known to impart gender difference [85–88], the beneficial effects of estrogen and progesterone in modifying MI-induced HF and changes in Ca^{2+}-cycling proteins should be examined in females with or without ovariectomy whereas the adverse effects of testosterone need to be studied in males with or without castration. The mechanisms of the beneficial effects of estrogen in females with or without ovariectomy should be investigated by monitoring alterations in signal transduction in HF due to MI whereas the role of estrogen receptors should be examined by measuring gene expression for Ca^{2+}-cycling proteins in cardiomyocytes.

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Chapter 4
The Failing Heart: Is It an Inefficient Engine or an Engine Out of Fuel?

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Abstract To meet its high-energy demand, the heart is very flexible in its choice of energy substrates. It can use a variety of energy substrates which include fatty acids, glucose, lactate, pyruvate, ketones, and amino acids. In the failing heart, significant changes in cardiac energy substrate metabolism occur, although there is no consensus as to exactly what these changes are. Energy starvation in heart failure has been extensively discussed, where reduced oxygen and energy substrate delivery to the heart, reduced cardiac energy substrate uptake, reduced mitochondrial oxidative phosphorylation, and decreased metabolic flexibility have been implicated as contributing factors to the declining mechanical function in heart failure. In addition to energy starvation, there is also the possibility of inefficient energy utilization in the failing heart. This inefficiency can occur at the level of ATP production where the preferential dependence on fatty acids consumes more oxygen per unit ATP and/or the overexpression of uncoupling proteins can increase energy loss as heat rather than ATP production. Increased ATP utilization for non-contractile purposes, such as ionic homeostasis and futile cycling of fatty acids, can also contribute to

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inefficiency in the failing heart. Impaired phosphocreatine/creatine kinase shuttle activity may also contribute to inefficient transport of ATP from the mitochondria to the contractile myofibrils. The degree and type of energy inefficiency in the failing heart are likely dependent on the pathogenesis and severity of heart failure. In this chapter, we review the various contributors to energy inefficiency in heart failure and discuss the potential to optimize cardiac energy metabolism as a potential treatment for heart failure.

Keywords Heart failure • Energy starvation • Cardiac inefficiency • Fatty acid oxidation • Glycolysis • Glucose oxidation

4.1 Introduction

The heart is a continuously contracting organ with a great need for energy. This is illustrated by the high turnover rates of adenosine triphosphate (ATP) in the heart. Although cardiac ATP content is very low (5 μmol/g wet weight), the heart has high ATP hydrolysis rates (~30 μmol/g wet weight/min). Thus, the heart has virtually no energy reserves, and the ATP pool of the heart is essentially turned over every ten beats [1–4]. To secure the energy needed to perform its tasks (such as mechanical work, ionic homeostasis, cardiac signaling, and synthetic function), the heart is flexible in the choice of energy substrates it utilizes. Many different energy substrates can be used, which include fatty acids, glucose, lactate, pyruvate, ketone bodies, and amino acids. The relative contribution of each of these energy substrates to overall cardiac ATP production depends on substrate availability, the workload of the heart, circulating hormones (such as insulin and catecholamines), age, oxygen availability, and pathophysiologic changes that accompany various diseases including heart failure [5, 6], hypertrophy [7], cardiomyopathy [8], and ischemia–reperfusion injuries [9]. However, under physiologic conditions, the adult heart normally derives most its ATP from fatty acid oxidation, with the remainder primarily being derived from the oxidation of carbohydrates (glucose, lactate, and pyruvate) [10–14].

Heart failure is defined as failure of the heart to pump an adequate amount of blood to meet the peripheral needs of the body. It develops following various insults to the heart including, but not limited to, ischemic insults, hypertrophy following long-standing untreated hypertension, genetic or acquired cardiomyopathies, storage diseases, or hyperdynamic circulation such as severe anemia or hyperthyroidism [15]. During the progression to heart failure, adaptive changes occur in the heart, including structural and metabolic remodeling. Systemic neurohumoral compensatory mechanisms develop to maintain adequate cardiac output. This includes activation of renin–angiotensin–aldosterone system (RAAS) and sympathetic system [16]. However, as heart failure progresses, these initially adaptive mechanisms become maladaptive leading to increased workloads and energy demand of the heart as well as increased energy cost of work ending in decompensation [17].

The study of mechanisms contributing to heart failure is complicated by the many causes of heart failure. However, numerous animal models of heart failure have been studied, with each mimicking a subtype such as pressure overload (e.g., transverse aortic constriction and abdominal aortic constriction), volume overload (e.g., aortocaval shunt), stress overload (e.g., rapid ventricular pacing), genetic alterations (transgenic animals), or post-infarction (coronary artery ligation) (see [18] for excellent review). As will be discussed, each of these experimental models, as well as complementary human studies, has provided valuable information as to how alterations in energy metabolism contribute to the severity of heart failure.

4.2 Energy Generation and Utilization in the Heart

The heart is a highly dynamic organ. The human heart pumps about 10 t of blood on a daily basis and beats about 100,000 times a day [19]. To accomplish this task, the heart uses multiple energy substrates to generate the needed energy. In this regard, the heart is the highest energy substrate consumer in the body on a gram weight basis [19]. The process of energy generation and utilization in the heart involves several processes, namely, energy substrate delivery, glycolysis, carbohydrate oxidation, β-oxidation, tricarboxylic acid cycle (TCA cycle) activity, mitochondrial oxidative phosphorylation, and the transfer of the high-energy phosphates to the contracting myofilaments.

4.2.1 Energy Substrate Delivery

This process entails the uptake of various energy substrates by the cardiomyocytes. These substrates are then metabolized, either via glycolysis or via mitochondrial oxidative metabolism to form acetyl CoA that feeds into the TCA cycle. Normally, TCA cycle activity is coupled to oxidative phosphorylation for ATP production [19, 20].

Fatty acids are primarily delivered to the heart either as free fatty acids bound to albumin or as triacylglycerols (TGs) present in chylomicrons and very low density lipoproteins [1, 20]. Fatty acid supply to the heart is a key determinant of fatty acid use by the heart [1]. In early heart failure, levels of fatty acids can be elevated compared with non-heart failure patients [16]. In contrast, circulating levels of glucose, lactate, ketones, pyruvate, and amino acids are not that different in heart failure patients (see [20] for review). As a result, while alterations in fatty acid supply may alter energy metabolism in heart failure, potential differences in energy substrate use in the failing heart are more likely due to alterations in energy substrate uptake and utilization, as opposed to differences in energy substrate supply to the heart.

4.2.2 Glycolysis

Glucose is transported into the cardiomyocyte primarily by either GLUT1 or insulin-dependent GLUT4 transporters. Once inside the cardiomyocytes, glucose is phosphorylated to glucose-6-P, with the majority of this glucose-6-P then entering glycolysis to eventually produce pyruvate [20]. The advantage of glycolysis is that it produces ATP without the expense of oxygen. However, the amount of ATP generated is small in comparison to the amount produced by mitochondrial oxidative phosphorylation, and as a result, glycolysis normally produces less than 5% of the total ATP requirements of the heart [1]. However, as will be discussed, this proportion can change in the failing heart.

4.2.3 Fatty Acid β-Oxidation, Glucose Oxidation, and the TCA Cycle Activity

Fatty acids, delivered to the heart as free fatty acids bound to albumin or liberated from TGs by the activity of lipoprotein lipase, are taken up by the heart, esterified to CoA, and the acyl group transported into the mitochondria via carnitine palmitoyl transferase-1 (CPT1) to undergo fatty acid β-oxidation. Each cycle of fatty acid β-oxidation shortens the fatty acid moiety by two carbons and produces one acetyl CoA molecule. Acetyl CoA then feeds into the TCA cycle that is, in turn, coupled with electron transfer chain for ATP generation. Oxidation of fatty acids produces more ATP per molecule than any other energy substrate. However, this process consumes more oxygen; hence, fatty acids are an inefficient source of energy. The contribution of fatty acid β-oxidation to cardiac energy generation varies, depending on fatty acid availability, energy demand, cardiac pathologic status, and competition with the other energy substrates (see [20] for review).

Glucose oxidation is involved in the decarboxylation of glycolytically derived pyruvate by the pyruvate dehydrogenase complex (PDC) to form acetyl CoA. Two acetyl CoA molecules are produced from each glucose molecule. Pyruvate can also be produced from lactate via the activity of lactate dehydrogenase. Acetyl CoA from the PDC then feeds into the TCA cycle. A major product of the TCA cycle is nicotinamide adenine dinucleotide (NADH), which then feeds into the electron transport chain. Glucose oxidation produces less ATP per molecule than fatty acid oxidation. However, glucose oxidation consumes less oxygen; hence, it is considered a more efficient energy substrate (see [2–4, 11, 21] for review).

4.2.4 Oxidative Phosphorylation for ATP Production

Under aerobic conditions about 95% of the hearts, ATP requirements are met through the mitochondrial oxidative phosphorylation of ADP. The reducing equivalents are

transferred to the mitochondria via flavine adenine dinucleotide (FADH2) and NADH, which are generated via dehydrogenase reactions in fatty acid β-oxidation, the TCA cycle, the glycolytic pathway, and from the oxidation of pyruvate derived from glucose or lactate. The relative contribution of the various energy substrates to ATP production is affected by many factors including the developmental stage of the heart, cardiac workload (pre- and afterload), heart rate and force of contraction, and the presence of various cardiac pathologies (e.g., hypertrophy, failure, or ischemia–reperfusion injury) [19, 20, 22, 23].

4.2.5 ATP Transfer and Phosphocreatine/Creatine Kinase Shuttle

Transfer of the high-energy phosphate bond in ATP that is generated inside the mitochondria (via oxidative phosphorylation) to the site of ATPases (i.e., the myofibrils, sarcoplasmic reticulum, and sarcolemma) is facilitated by the creatine kinase shuttle system. Creatine kinase (CK) is a reversible kinase that initially phosphorylates creatine to phosphocreatine (PCr) using the high-energy phosphate bond in ATP [24]. The CK shuttle is facilitated by mitochondrial and cytoplasmic CKs, which eventually results in the rephosphorylation of ADP to ATP in the cytoplasm.

In cardiomyocytes, about two-thirds of creatine are phosphorylated and act as a reservoir for ATP. Thus, when energy production declines, PCr levels decline while ATP levels are initially maintained. This is accompanied by an increase in ADP levels [25, 26]. Because of this, total creatine levels and PCr/ATP ratio can be used as indices of cardiac energetic status. In general, both indices decline in early phases of heart failure before an actual decline in total ATP levels occurs [24–26].

Since creatine is not synthesized de novo in cardiac muscle [27], the activity of creatine transporters plays a regulatory role in the intracellular availability of creatine in cardiomyocytes. Reduction of creatine transporter expression and/or activity can be one of the contributing factors to deterioration of PCr/CK shuttle observed in many cardiac diseases, including heart failure [24–26].

The association of various isoforms of CK with subcellular structures ensures adequate transfer of ATP-derived energy for adequate coupling of energy generation and utilization. For example, the tight association of myofibrillar CK (MM-CK) with smooth endoplasmic reticulum ATPase (SERCA) is thought to enable efficient energy transfer for Ca^{2+} uptake [28]. Similarly, the mitochondrial CK (miCK) being located in the inner mitochondrial membrane near the adenine nucleotide translocase is thought to help export the high-energy phosphate of ATP through the formation of ADP and PCr, thus maintaining mitochondrial respiration by prevention of intramitochondrial ATP accumulation [29] (for further review, see [30]).

4.3 Nature of the Metabolic Changes in Heart Failure

Heart failure is associated with a number of changes in energy metabolism [19, 20, 23, 30, 31]. Whether these changes contribute to the contractile failure of these hearts, hence considered as maladaptive and should be discouraged [32, 33], or whether they represent an adaptive response that should be encouraged is not yet firmly established [34]. Moreover, there is no agreement regarding the actual nature of the energy metabolic changes that occur in heart failure, and two main concepts have emerged (Fig. 4.1). The first concept is that the failing heart is energetically starved, similar to an engine out of fuel [19, 35]. This implies lower rates of energy metabolism and lower rates of production of the energy currency, ATP. The second concept is that the failing heart may not necessarily be energy starved, but rather is inefficient in its use of energy [36].

4.3.1 Heart Failure and Cardiac Inefficiency

Cardiac energy is expended for mechanical work generation, ionic homeostasis, and other vital cellular functions, such as synthesis and degradation of various intracellular molecules and their trafficking to various cellular compartments or to the extracellular compartment [37–39]. In 1949 Bing et al. [40] defined cardiac mechanical efficiency as the "cardiac work generated per energy consumed." Since most of cardiac energy generated (about 95% under aerobic conditions) is derived from fatty acid and carbohydrate metabolism [21], and the fact that metabolic rates in the cardiac muscle are tightly coupled with energy demand [41, 42], oxygen consumption (MVO_2) by the cardiac muscle can be used as a measure of energy production. If energy production increases without a parallel increase in cardiac work, then a decrease in cardiac efficiency would occur. A decrease in the efficiency in utilizing energy for mechanical function has the potential to be a major contributor to cardiac dysfunction in heart failure [21, 43–49]. This cardiac inefficiency can develop primarily at three different levels: (1) increased oxygen cost of acetyl CoA and ATP production, (2) increased ATP consumption for homeostatic activities, and (3) impaired energy transfer to myofibrils.

4.3.1.1 Increased Oxygen Cost of ATP Production Due to an Increased Dependence on Fatty Acids

As mentioned, under aerobic conditions, the heart derives most of its energy requirements from fatty acids [21, 43–49]. Moreover, the activated neurohumoral mechanisms during early stages of heart failure favor lipolysis, leading to increased circulating fatty acids and more exposure of the cardiomyocytes to fatty acids [16]. When one molecule of palmitate is fully metabolized, it yields 105 ATP molecules, compared to the metabolism of glucose where only 31 ATP molecules are produced.

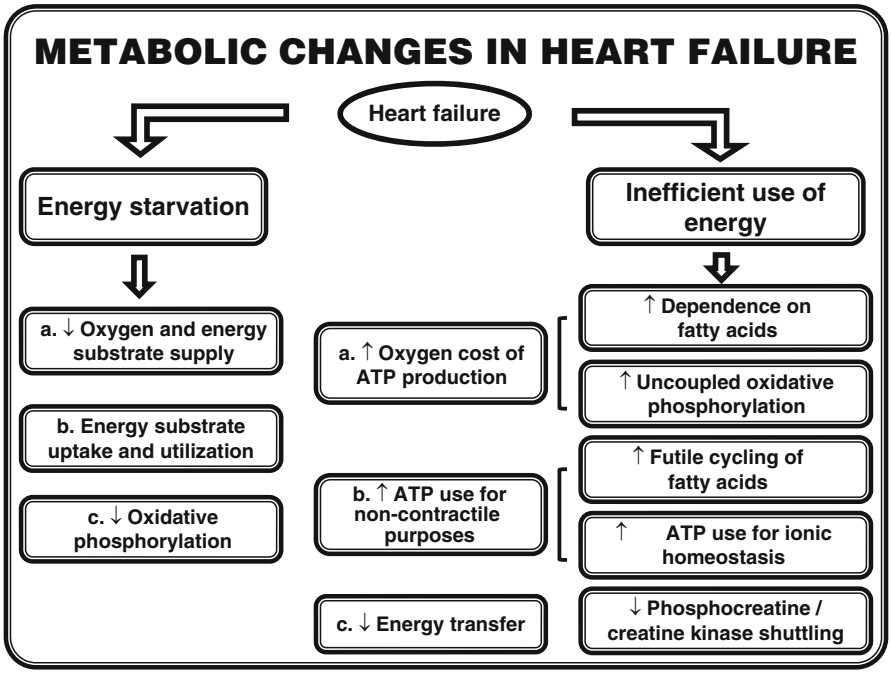

Fig. 4.1 Energy metabolic changes in heart failure. The energy starvation theory describes the failing heart as an engine out of fuel with (**a**) reduction of oxygen and substrate delivery to the heart, (**b**) reduction in energy substrate uptake and utilization, and (**c**) reduced mitochondrial oxidative phosphorylation. The cardiac inefficiency theory considers the inefficient energy utilization for mechanical work due to (**a**) increased oxygen cost of ATP production which in turn can be due to the preferential dependence on fatty acids or uncoupling of oxidation phosphorylation, (**b**) increased use of ATP for homeostatic activities that include futile cycling of fatty acids and ionic homeostasis secondary to increased Na^+ and Ca^{2+} accumulation induced by mismatched glucose metabolism, and (**c**) reduced energy transfer to myofibrils due to impaired phosphocreatine/creatine kinase shuttle

However, despite this higher ATP yield of palmitate, a higher MVO_2 is also needed [20]. This is because while the initial cytoplasmic metabolism of glucose (i.e., glycolysis) produces two ATP, the initial cytoplasmic metabolism of fatty acids (i.e., the formation of long chain acyl CoA from fatty acids and CoA) actually consumes two high-energy phosphates (i.e., ATP to AMP). Also, the fact that palmitate oxidation yields both $FADH_2$ and NADH, as compared to only NADH produced during glucose metabolism, contributes to palmitate being an inefficient source of energy. Because $FADH_2$ bypasses complex I of the electron transfer chain, it pumps less protons and generates less ATP than NADH (reviewed in [20]). This means that a preferential dependence on fatty acids for energy generation in early stages of heart failure could lead to more MVO_2 for the same energy yield, hence causing inefficiency in ATP generation. This effect was shown many years ago when increasing fatty acid supply

to the heart secondary to adrenergic stimulation-induced lipolysis was found to increase cardiac MVO_2 without changing the external work [46, 49]. In theory, a complete dependence on fatty acids for energy generation leads to 10–13% reduction in the calculated efficiency. However, the observed differences in efficiency are even higher, indicative of involvement of other mechanisms [20, 23].

In heart failure, there is not a uniform consensus as to what happens to cardiac fatty acid oxidation rates. Both human and experimental studies have shown an increase in fatty acid β-oxidation rates, a decrease in fatty acid β-oxidation rates, or no change in fatty acid β-oxidation rates (see references [11, 20], and [23] for reviews). As a result, it cannot be conclusively stated as to whether the heart is more inefficient due to an increased reliance on fatty acid as an energy source. What is evident, however, is that inhibiting fatty acid β-oxidation in the failing heart does appear to improve cardiac efficiency and function [11, 19, 20, 23].

4.3.1.2 Uncoupled Mitochondrial Oxidative Phosphorylation

The mitochondrial electron transfer chain (also known as the mitochondrial respiratory chain) utilizes reducing equivalents in the mitochondrial matrix to generate ATP. The electrochemical gradient of protons across the inner mitochondrial membrane is essential for this process. This gradient is generated by the movement of protons from the mitochondrial matrix to the intermembrane space by complexes I, III, and IV. The protons then move down the electrochemical gradient to the mitochondrial matrix to provide the energy needed for ATP synthase activity, with the subsequent phosphorylation of ADP to ATP [20, 50, 51].

The uncoupling proteins (UCP) provide a potential alternative route for transport of protons across the inner mitochondrial membrane without the involvement in ATP synthesis [51]. These UCPs were originally discovered in brown adipose tissue where they help oxidatively generated energy to dissipate in the form of heat secondary to failure of coupling with phosphorylation of ADP for ATP generation [52, 53]. In the heart, UCP2 and UCP3 are preferentially expressed [54]. UCP3 has been suggested to mediate fatty acid-induced uncoupling of oxidation phosphorylation [55]. Moreover, high circulating fatty acid levels can increase UCP3 expression in the heart [56]. The aforementioned observation provides a potential explanation why the reported difference in efficiency due to dependence on fatty acid oxidation is higher than the theoretically calculated difference. Several reports from heart failure animal models describe overexpression of UCPs (see [57] for review). In an abdominal aorta constriction model of heart failure in the rat, decreases in ATP, ADP, AMP, and PCr contents in the failing heart were associated with a significant increase in UCP2 expression [58]. Adenovirus-mediated overexpression of UCP2 in neonatal cardiomyocytes was also found to increase basal oxygen consumption without affecting total ATP content [59], indicative of an increase in cardiac inefficiency. Moreover, UCP2 overexpression disrupted mitochondrial membrane potential secondary to failure to control calcium-induced calcium release from sarco/endoplasmic reticulum calcium ATPase (SERCA) [59]. In cardiac mitochondria

isolated 10 weeks following coronary artery ligation in rats, UPC3 was also shown to be upregulated, mitochondria were less coupled (lower ADP/oxygen ratio), and a significant reduction of efficiency was observed [60]. Furthermore, a positive correlation between UPC3 levels and circulating non-fasting fatty acid levels was observed, an observation which supports the regulatory role of circulating fatty acids on cardiac UPC3 expression and thus increased inefficiency [60].

4.3.1.3 Futile Cycling in the Heart

Normally, the heart consumes generated ATP for mechanical work, ionic homeostasis, and synthesis of various cellular molecules [22, 37, 39]. However, utilization of ATP can be preferentially directed to additional homeostatic activities leading to further deterioration of inefficiency in heart failure.

As fatty acid supply to the myocardium increases early in the disease progression secondary to increased sympathetic activity, excess fatty acyl CoA has the potential to accumulate in the mitochondrial matrix. A mitochondrial thioesterase has been identified, which may cleave this unneeded fatty acyl CoA into fatty acid anion and free CoA [61, 62]. This process maintains the CoA pool in the mitochondria for further activation of fatty acids for β-oxidation. However, not only is the high-energy CoA bond lost due to the thioesterase activity, the resulting fatty acid anions have been proposed to be exported to the cytosolic compartment via the activity of UCP3 [63, 64]. While this process prevents intramitochondrial accumulation of potentially harmful fatty acid anions, it increases the futile utilization of ATP for the subsequent esterification of these fatty anions prior to subsequent fatty acid β-oxidation [20, 23].

Another source of futile ATP utilization is the glycerolipid/TG cycling which is another fate for fatty acids taken up by the heart. In this case, fatty acids cycle between the intracellular TG pool and the free fatty acid state. Excess ATP is thus utilized at the esterification step prior to incorporation of fatty acids into the TG pool or prior to fatty acid β-oxidation [65].

Whether these two pathways of futile fatty acid cycling are involved in the cardiac inefficiency in heart failure is not yet clear. However, as indicated above, the increased sympathetic activity in heart failure is expected to mobilize fatty acids from adipose tissue, increasing the circulating levels of fatty acids and cardiac exposure to fatty acids [17].

4.3.1.4 Reversion to Fetal Glycolytic Metabolism Favors Mismatched Glucose Metabolism and the Subsequent Higher Energy Cost of Ionic Homeostasis

It is known that the fetal heart is largely glycolytic [66]. Studies with newborn rabbit hearts have shown that rapidly after birth, the expression of fatty acid oxidation regulating enzymes such as peroxisome proliferator-activated receptor α (PPARα)

increases, while those regulating glycolysis decline. These changes mark the conversion to fatty acid oxidation as the main source of energy [20, 67–69].

In experimental models of heart failure in which an initial pathologic hypertrophy develops (such as salt-sensitive hypertensive rats [7], pressure overload models such as transverse aortic constriction [70], and volume overload models such as aortocaval fistula [66, 71, 72]), reversion to a fetal glycolytic metabolism occurs. Proteomic analysis of mitochondrial proteins also confirmed this shift to glycolytic metabolism in rats 8 weeks after coronary artery ligation [73].

The reversion to glycolytic metabolism that is associated with a decline in mitochondrial oxidative capacity is thought to help produce energy from a substrate that utilizes less oxygen per ATP; hence, efficiency is improved. On the other hand, this increase in glycolysis without a parallel increase in glucose oxidation can favor mismatched metabolism, whereby hydrolysis of glycolytically derived ATP can accelerate proton production with the development of intracellular acidosis [74]. This can subsequently lead to an increase in Na^+ and Ca^{2+} accumulation [74], resulting in an impaired contractility (Fig. 4.2). Correction of intracellular accumulation of Na^+ and Ca^{2+} increases ATP utilization for ionic homeostasis, rather than for contractile activity.

Reversion to fetal glycolytic metabolism is associated with changes in the expression of gene and protein levels and activity of enzymes known to regulate both glycolysis and fatty acid β-oxidation. Members of the peroxisome proliferator-activated receptor (PPAR) family are specifically involved. PPARα, which is predominantly expressed in the heart and skeletal muscles, is known to regulate fatty acid oxidation, and thus, its inhibition can be responsible for the reversion to fetal glycolytic metabolism [75]. In this regard, genetic deletion of PPARα has been shown to result in hypertrophy and inhibition of fatty acid β-oxidation in mice exposed to transverse aortic constriction [76]. This is associated with decreased expression of fatty acid oxidation genes [77] and a decline of fatty acid oxidation rates accompanied by a stimulation of glucose oxidation [78]. Previous findings led to the notion that stimulating PPARα in failing hearts could improve function through prevention of the development of fetal glycolytic metabolic profile, via prevention of the downregulation of fatty acid β-oxidation [79]. As a result, pharmacologic approaches to activate PPARα (i.e., with the use of fenofibrate) have been used in an attempt to decrease heart failure severity. This was found to produce a uniform upregulation of PPARα-regulated genes, although results have been variable regarding the improvement of heart function [80, 81]. A worsening of ex vivo LV function was observed in hypertrophied hearts derived from rats exposed to transverse aortic constriction [80]. In post-infarct rat hearts [81] and in dogs with heart failure due to excessive tachycardia [82], there was no improvement of LV function or reduction of chamber volume following chronic fenofibrate treatment. On the other hand, the use of fenofibrate in a porcine tachycardia model of heart failure resulted in lessened deterioration of LV dysfunction [83]. The variability in the reported functional consequences of PPARα activation hints at the lower likelihood of PPARα involvement in progression to heart failure. However, these findings do not appear to be a concern in heart failure patients who are already using

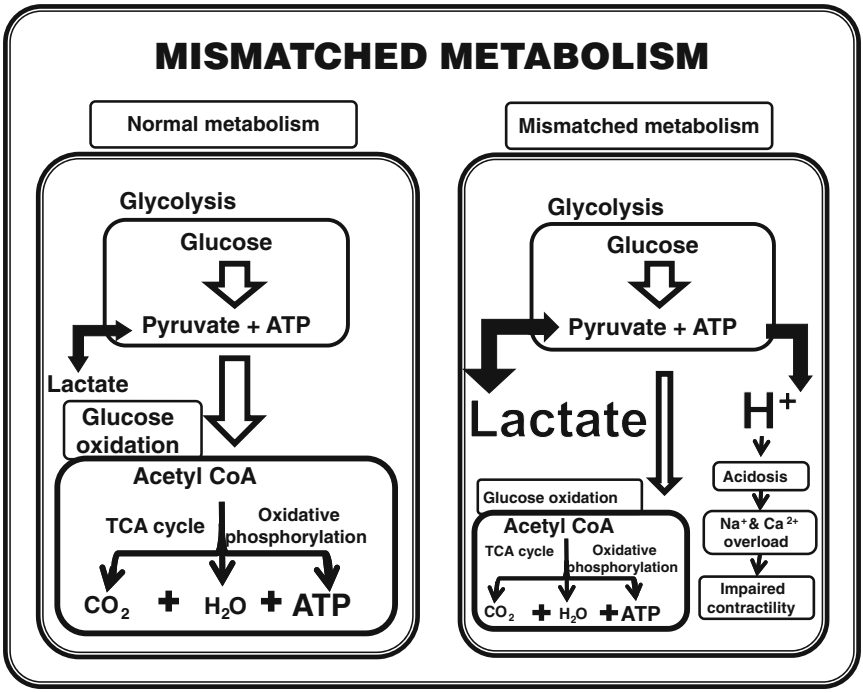

Fig. 4.2 Normal versus mismatched glucose metabolism. Intracellular proton production arises when the rate of glycolysis exceeds the rate of glucose oxidation. If glycolysis increases and/or glucose oxidation decreases (mismatched rates of glucose metabolism), proton production accelerates and causes intracellular acidosis. Efflux of proton leads to Na^+ overload and ultimately Ca^{2+} overload that causes cardiac mechanical dysfunction and inefficiency

fenofibrate to reduce cholesterol. In fact, fenofibrate may possess cardioprotective effects mediated by non-metabolic pathways. For example, it was reported that fenofibrate lessens cardiac fibrosis and diastolic dysfunction in salt-sensitive hypertensive rats, probably via non-metabolic effects involving the suppression of inflammation via inhibiting NF-*k*B [84].

PPARβ/δ is another member of the PPAR family which may be involved in the reversion of the heart to a fetal glycolytic metabolism. Although not specific for cardiac muscle, it is expressed in high amounts in the heart [85]. The role of PPARβ/δ in cardiac metabolism has been studied by cardiac-specific deletion of PPARβ/δ in mice. These mice develop severe cardiomyopathy ending in heart failure and premature death associated with decreased cardiac expression of genes responsible for fatty acid oxidation, decreased cardiac fatty acid oxidation rates, and increased cardiac lipid deposition [86]. In contrast, cardiac-specific PPARβ/δ overexpression increases expression of genes involved in fatty acid β-oxidation. However, fatty acid oxidation rates in these mice were normal, indicative of possible post-translational modifications. Neither genes involved in TG synthesis nor those involved in fatty

acid uptake were increased. Surprisingly, cardiac-specific overexpression of PPARβ/δ was associated with increased expression of genes involved in glucose uptake (such as GLUT4 and the key regulatory enzyme phosphofructokinase), which was associated with an increase in cardiac glucose uptake and oxidation [75, 87–89]. Despite the fact that both PPARα and PPARβ/δ share overlapping pathways for control of fatty acid oxidation [75], cardiomyocyte PPARβ/δ restriction in PPARα null mice resulted in no further inhibition of fatty acid β-oxidation, but rather a pronounced inhibition of mitochondrial biogenesis [90]. Whether the reversion to fetal glycolytic phenotype is associated with increased PPARβ/δ expression in the failing hearts is yet to be studied.

4.3.1.5 Impaired Energy Transfer to Myofibrils: Consequences of Compromised Phosphocreatine/Creatine Kinase Shuttle

Impaired PCr/CK shuttling may also be a contributing factor to the development of inefficiency in heart failure. PCr acts as a reservoir to replenish ATP content via the activity of CK, a reaction that is capable of producing ATP ten times faster than the rate of ATP synthesis from oxidative phosphorylation [91]. This fact is confirmed in various models of heart failure. For example, Hearse et al. [92] observed a rapid decline in myocardial PCr levels and contractility in isolated working rat hearts during post-ischemic reperfusion. Similar results were reported by Whitman et al. [93] using isolated perfused rabbit hearts. The same concept was further confirmed in a porcine model of transverse aortic constriction-induced cardiac hypertrophy and failure [94].

PCr/ATP ratio is usually used as an index of the energy status of the heart [95, 96], which can also predict possible protection against supervening ischemic insults and the subsequent development of heart failure. Changes in PCr levels have been reported in many heart failure patient studies. Winter et al. [97] reported an earlier decline in PCr levels in non-ischemic heart failure patients using magnetic resonance spectroscopy, where a significant reduction in cardiac creatine levels was shown by a significant reduction of water/creatine index. This decrease in PCr was also reported early in patients with dilated cardiomyopathy [98]. These findings conform to the studies of Neubauer et al. [99], who used ^{31}P-NMR spectroscopy and found a 70% reduction in PCr levels in heart failure patients with dilated cardiomyopathy while ATP levels were unaltered. The PCr/ATP ratio was thus markedly reduced in these patients. The authors found that the decline of PCr/ATP ratio correlated with the severity of heart failure and that the ratio improved by recompensation. The authors also found that PCr/ATP ratio is a valid predictor of mortality in these patients. They found that patients with normal PCr/ATP (more than 1.6) had an all-cause mortality of 10% following a 2.5-year surveillance. On the other hand, patients with low PCr/ATP ratio had 40% all-cause mortality [99].

Not only do PCr levels decline in heart failure but the ability of the failing heart to maintain adequate ATP levels in response to exercise challenge is also

compromised to various degrees depending on heart failure etiology. Using the handgrip exercise, Weiss et al. [100] observed that PCr/ATP ratio in the left ventricular wall is maintained in non-ischemic heart failure patients, but is greatly reduced in coronary artery disease heart failure patients. Similar changes were also observed by Yabe et al. [101] at the level of subendocardium.

4.3.2 Heart Failure and Energy Starvation

The energy starvation theory was initially proposed by Hermann in 1939 [102], who reviewed the chemical nature of heart failure and addressed the reduction of myocardial creatine in the heart. From this perspective, the failing heart can be described as an engine out of fuel [19]. This implies an ability to improve or at least prevent further deterioration of cardiac function by stimulating the various stages of high-energy generation [35]. Because this theory gained much interest and is described in detail elsewhere, we will only briefly highlight this concept (refer to [19] for review).

As heart failure progresses to decompensation, the reduced cardiac output reduces peripheral tissue perfusion including the heart tissue itself. The associated pulmonary congestion reduces oxygen saturation leading to further deterioration of oxygen supply. In general, reduced intracellular oxygen availability does not limit peak oxygen utilization. However, as heart failure progresses, the workload increases, and cardiac MVO_2 can reach a maximum and becomes limited leading to subsequent deficiency of energy generation and utilization [103]. This is accompanied by reduced mitochondrial oxidative phosphorylation [104]. In addition, flexibility in substrate utilization is lost [105]. Moreover, membrane expression of the insulin-sensitive glucose transporter GLUT4 is reduced in advanced heart failure [106]. On the other hand, expression of fatty acid uptake protein (CD36) and intracellular fatty acid transporter (fatty acid binding protein) are increased in both ischemic and dilated cardiomyopathy patients [107].

4.4 Proof of Principle for Cardiac Inefficiency and Energy Starvation in Heart Failure

Energy starvation can be confirmed by the generalized decline in metabolic rates seen in advanced cases of heart failure, whether in patients or experimental models [5, 108]. Lending support to this theory is the observation that mechanical function in advanced heart failure can be improved by using metabolic modulators. In this regard, stimulation of glucose oxidation (e.g., by dichloroacetate) in patients with congestive heart failure was found to improve mechanical efficiency and hemodynamic parameters [109]. Also, intracoronary infusion of pyruvate in heart failure

patients can induce a short-term functional improvement in heart function [110]. Furthermore, cardiac-specific overexpression of GLUT1 can prevent the development of left ventricular dysfunction after transverse aortic banding [111], and induction of mitochondrial biogenesis can prevent cardiomyopathy in mice [35]. Treatments that limit the energy demand of the failing heart by reducing mechanical work such as β-adrenoceptor antagonists are also known to be useful adjuncts to the traditional heart failure therapy [112–115].

Support for cardiac inefficiency in heart failure arises from the beneficial effect of fatty acid β-oxidation inhibitors to treat heart failure. Inhibition of fatty acid oxidation improves mechanical function in heart failure, evidence which clearly stands against the energy starvation theory (see [11, 19, 20], and [22] for reviews). A number of approaches to inhibit fatty acid β-oxidation have been used, which include (1) reduction of circulating fatty acid levels with β-adrenoceptor antagonists such as nebivolol that inhibit lipolysis [113, 114], (2) the use of PPARγ agonists such as thiazolidinediones that favor sequestration of fatty acids in adipose tissue [116–118], (3) inhibition of mitochondrial fatty acid uptake by etomoxir [119] or perhexiline [120–122], and (4) inhibition of mitochondrial β-oxidation with agents such as trimetazidine [123–128]. The resulting indirect stimulation of glucose oxidation as a consequence of lower rates of fatty acid β-oxidation improves the coupling of glycolysis to glucose oxidation, leading to reduced protons production by hydrolysis of glycolytically derived ATP (Fig. 4.2). Also, shifting substrate preference from fatty acid β-oxidation is expected to increase efficiency of energy production without changes in MVO_2. Indeed, the observed improvement of mechanical function in patient with congestive heart failure with the use of glucose oxidation stimulators such as dichloroacetate [109] can be explained on basis of improved matching between glycolytic flux and glucose oxidation. In this regard, the increased glycolytic flux observed in severe heart failure was associated with downregulation of glucose oxidation [5], indicative of an even greater mismatch in the pathways of glucose metabolism.

4.5 Conclusion

Both cardiac energy starvation and cardiac inefficiency may contribute to the severity of heart failure. These two contributing mechanisms are also not mutually exclusive, and may coexist. However, it is likely that energy starvation occurs later in the course of heart failure, while inefficiency may be present throughout the course of heart failure, including the very early stages where metabolic interventions are expected to yield maximum benefit. It would thus be desirable to consider cardiac inefficiency when designing metabolic modulators to treat heart failure, since stimulation of substrate utilization may not always be beneficial. This is supported by reduced efficiency observed with acceleration of fatty acid β-oxidation or of glycolysis.

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Chapter 5
Regulation of Cardiac Hypertrophic Remodeling by the USP15/SLIM1 Pathway

Hiroto Nakajima

Abstract Many lines of evidence support the important role of the ubiquitin (Ub)–proteasome system (UPS) in the development of heart diseases. Cardiomyopathy or cardiac responses to pressure or volume overload are also accompanied by alterations in the UPS. At the beginning of this review, we provide an overview of recent studies regarding the features and roles of the UPS and its components in cardiac hypertrophic remodeling. Then, we focus on our animal model of cardiac remodeling, which was induced by cardiac-targeted overexpression of Ub-specific protease 15 (USP15) in mice. Skeletal muscle LIM protein 1 (SLIM1), a direct interactor and substrate of USP15, was increased in the remodeling heart of this model. We discuss possible mechanisms of the hypertrophic responses in the model on the basis of several novel findings on USP15 and SLIM1.

Keywords Deubiquitination • DUB • FHL1 • NFATC1 • TGFβ • BMP • SMAD • Cardiac remodeling • Cardiac hypertrophy • Cardiomyopathy

5.1 Introduction

The ubiquitin (Ub)–proteasome system (UPS) is an ATP-dependent proteolytic system that requires the polyubiquitination of a protein intended for degradation by the 26S proteasome (reviewed in [1]).

Protein ubiquitination consists of the covalent linkage of Ub to one or more lysine residues of a target protein or the preceding Ub. In general, polyubiquitination of a target protein through lysine 48 results in degradation, while polyubiquitination

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through lysine 63 or monoubiquitination of lysine residues can be used for other purposes such as DNA repair, re-localization, endocytosis, cellular signaling, or regulation of protein function [1]. The process of ubiquitination involves the concerted action of Ub enzymes composed of E1 (Ub-activating enzyme), E2 (Ub-conjugating enzymes), and E3 (Ub ligases). The E3 Ub ligases confer substrate specificity to the whole system, and around a thousand E3 enzymes have been identified in human genome based on specific, commonly shared structural motifs [1].

On the other hand, removal of covalently attached Ub from proteins is called deubiquitination and is catalyzed by deubiquitinating enzymes (DUBs). Functions of DUBs are generally categorized as follows in the Ub pathway (reviewed in [2]):

1. Processing of Ub precursors
2. Editing or rescue of Ub conjugates, which are generally adducts to other proteins in the cell but can also be ligated to abundant small nucleophiles such as glutathione
3. Recycling of Ub or Ub oligomers from Ub–protein conjugates targeted for degradation
4. Disassembly of unanchored Ub oligomers

The human genome encodes approximately 90 DUBs, and they fall into at least five distinct subfamilies based on their sequence similarities and likely mechanisms of action (reviewed in [3, 4]). The largest one of these subfamilies consists of the Ub-specific proteases (USPs), cysteine proteases containing a conserved catalytic triad within a defined catalytic domain [4].

Recently, much attention has been made on the UPS in the development of cardiac diseases. In the following sections, we provide an overview of the recent studies regarding the features and roles of the UPS and its components in cardiac hypertrophic remodeling, and then focus on our animal model of cardiac remodeling, which was induced by cardiac-targeted overexpression of USP15.

5.2 Profile of the Ub Pathways in Cardiac Hypertrophic Remodeling

A number of studies have shown that the accumulation of ubiquitinated proteins and lower levels of transcription of the proteasome components in human heart failure progressed from compensated hypertrophy, compared with non-failing hearts [5, 6], suggesting impaired removal of abnormal proteins by UPS. Additionally, in an experimental mouse model of cardiac hypertrophy and heart failure induced by transverse aortic constriction (TAC), both high levels of ubiquitinated proteins and depression of proteasome activities have been shown [7].

However, Depre and colleagues have reported the upregulation of the levels of UPS components and proteasome activities during the development of cardiac hypertrophy caused by either pressure overload or cardiac-specific expression of Hsp22 in mice [8, 9]. The authors have demonstrated that pharmacological inhibition of

proteasome reverses cardiac remodeling in overloaded hearts and stabilizes the contractile function by repressing collagen accumulation via reduced NF-kB activity [10]. Proteasome inhibition also results in beneficial effects on cardiac remodeling induced by hypertension in rat hypertension models [11, 12].

Several E3 Ub ligases have been described as restrictedly expressed in cardiac and skeletal muscles (reviewed in [13, 14]), and transcripts of some of these genes are upregulated, although to small extents, in pressure-overloaded rat hearts [15]. These E3 ligases protect against the development of physiological or pathological cardiac hypertrophy. Overexpression of muscle atrophy F-box protein (MaFBx, also called atrogin-1), muscle ring finger protein-1 (MuRF1), or minute double minute 2 (Mdm2) inhibits phenylephrine- or endothelin-1-induced hypertrophy in cardiomyocytes [16–18]. Overexpression of atrogin-1 in hearts of transgenic (TG) mice reduces the levels of substrate protein calcineurin and blunts cardiac hypertrophy induced by TAC [16]. Conversely, whereas atrogin-1-deficient mice exhibit no cardiac phenotype under basal conditions, they develop marked physiological cardiac hypertrophy after voluntary running exercises [19]. Similar to atrogin-1-deficient mice, MuRF1-deficient mice show no signs of cardiac hypertrophy under basal conditions, but develop exaggerated hypertrophy after TAC [20].

Regarding DUB, upregulation of the expression of USP18 and UCHL1 has been reported in a mouse in vivo model of cardiac hypertrophy induced by TAC ([21], cited in [22]) as well as an in vitro model of cardiac myocyte hypertrophy induced by the stimulation with fibronectin [22]. In the latter model, transcripts of several USPs, including USP15, are upregulated besides USP18 and UCHL1 [22].

The expression of USP7 [also called herpes virus-associated ubiquitin-specific protease (HAUSP)] and USP20 (also called VDU2) was investigated in human dilated cardiomyopathy, and both were shown to be upregulated [23, 24]. Birks and colleagues investigated the activity of multiple DUBs in myocardial samples from patients with dilated cardiomyopathy and non-failing donors for transplantation by using a thiol-reactive ubiquitin-derived probe. They detected the activity of USP15 and several other DUBs in both samples [23].

5.3 USP15-Mediated Cardiac Remodeling

5.3.1 Interaction of USP15 and SLIM1

Expression of the transcripts of USP15 has been shown in skeletal muscle, heart, and a few other tissues [25, 26], and its enzymatic activity has been confirmed in human hearts as described above [23].

The biological roles of USP15 have been unknown until recently. By analysis of the COP9 signalosome complex, USP15 has been revealed to associate with the complex and stabilize the E3 Ub ligase, ring-box 1 (RBX1, also called ROC1) [27–29]. Several substrates of USP15 have been successively reported such as NF-κB inhibitor

IκBα [30], the tumor suppressor protein adenomatous polyposis coli (APC) [31], and procaspase-3 [32]. The entire scope of the biological roles of USP15, however, remains unclear, as a large number of previously unknown protein interactions with USP15 have been proteomically discovered [33], and their functions are not yet fully investigated.

We have identified a novel interaction of USP15 with skeletal muscle LIM protein 1 (SLIM1, also called FHL1, FHL1A, KyoT, or KyoT1) in an effort to find novel protein–protein interactions by computational prediction and its biochemical confirmation [34]. We have shown that the interaction is direct because USP15 and SLIM1 directly bound under cell-free conditions and co-immunoprecipitated from the lysates of the cells, where they were co-expressed. SLIM1 was detected in both polyubiquitinated and non-ubiquitinated forms when SLIM1 and Ub were co-expressed in mammalian cells in the presence of a proteasome inhibitor. Polyubiquitinated forms of SLIM1 were markedly reduced with the co-expression of USP15, but not the catalytically inactive mutant USP15. Furthermore, co-expression of USP15 increased the protein levels of SLIM1 depending on its deubiquitinating activity, suggesting an escape from the proteasomal degradation.

Thus, the protein levels of SLIM1 are regulated by the UPS, and USP15 interacts physically and functionally with SLIM1. SLIM1 is a member of the four-and-a-half LIM protein (FHL) family [35, 36], which is comprised of an N-terminal half LIM or single zinc finger followed by four LIM domains, and is highly expressed in skeletal muscle with an intermediate expression in the heart [36–38]. Accumulating evidence indicates a key role of SLIM1 in the pathogenesis of myopathies and cardiomyopathies. Gene expression analyses have shown the downregulation of SLIM1 in dilated cardiomyopathy [39] and upregulation in hypertrophic cardiomyopathy [40–42]. Additionally, in mouse models of hypertrophic and dilated cardiomyopathy, upregulation of cardiac ventricular expression of SLIM1, but not the related proteins in FHL family, SLIM2 or SLIM3, has been reported [38]. More recently, mutations in the SLIM1 gene have been identified in various types of human X-linked myopathies associated with or without cardiomyopathy (reviewed in [43, 44]). Furthermore, a critical role of SLIM1 as a mediator of hypertrophic biomechanical stress responses has been demonstrated by using SLIM1-deficient mice [45], and the involvement of SLIM1 in skeletal muscle mass control has been shown by using TG mice which overexpress SLIM1 in skeletal muscle [46].

5.3.2 Hypertrophic Responses of the Heart in USP15-TG Mice

The above results and information suggest that USP15 may have the potential to induce cardiac or skeletal muscle remodeling. To test this hypothesis, we generated TG mice with cardiac-specific overexpression of human USP15, which is more than 97% identical and almost 100% similar to murine USP15, under the control of α-myosin heavy chain (MHC) promoter (USP15-TG mice) [34].

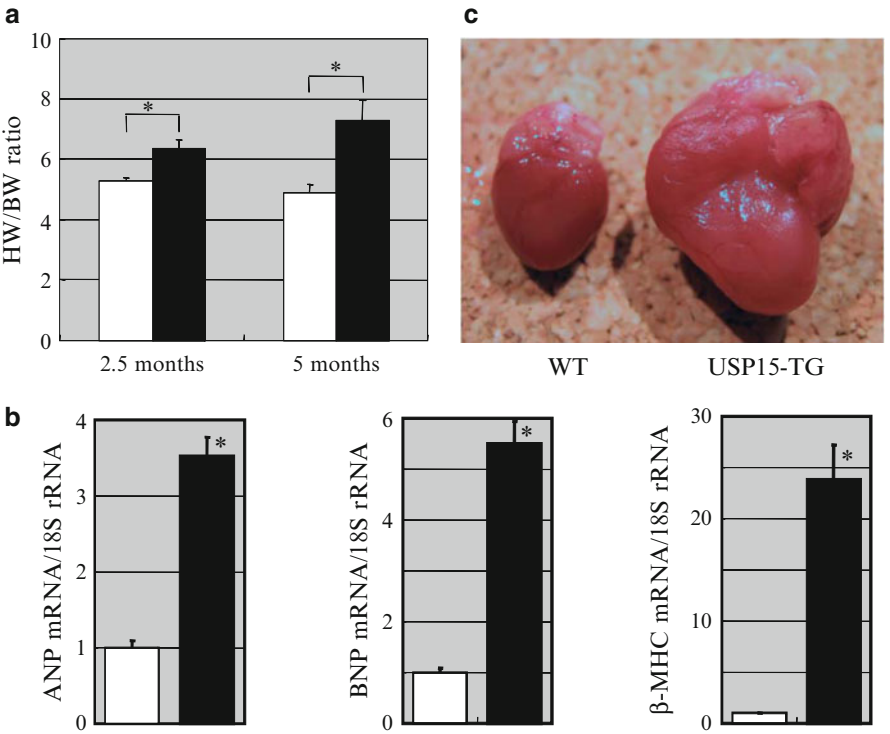

Fig. 5.1 Hypertrophic responses in the heart of USP15-TG mice (reproduced from [34] with permission). (**a**) HW/BW ratios (mg/g) in WT (*open bars*) and USP15-TG (*filled bars*) mice were determined at the indicated months after birth. Means ± SE are shown ($n = 6$–11). * Significantly different between WT and USP15-TG mice ($p < 0.05$). (**b**) Total RNA was isolated from the hearts of WT (*open bars*) and USP15-TG (*filled bars*) mice, and mRNA levels for ANP (*left*), BNP (*center*), and β-MHC (*right*) normalized to 18S rRNA were determined by real-time quantitative RT-PCR. mRNA levels in hearts of WT mice were arbitrarily assigned to 1, and fold differences were calculated. Means ± SE are shown ($n = 5$). * Significantly different between WT and USP15-TG mice ($p < 0.05$). (**c**) Hearts were removed from WT and USP15-TG mice at the age of 8 months and photographed

As a result, cardiac-specific overexpression of USP15 in TG mice induced cardiac remodeling with massive enlargement in heart size. As shown in Fig. 5.1a, b, heart weight to body weight (HW/BW) ratios and mRNA levels of fetal gene markers, such as atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and β-MHC, in the heart turned out to be significantly higher in USP15-TG mice compared with wild-type (WT) mice. These characteristics are features of cardiac hypertrophy, although we need histological examination to say clearly. The hypertrophic responses in the heart of USP15-TG mice depend on the deubiquitinating activity of USP15 because another line of TG mice, in which transfected human USP15 was spontaneously multi-mutated to lose deubiquitinating activity, showed neither an increase of HW/BW ratio nor an increase of these fetal gene markers.

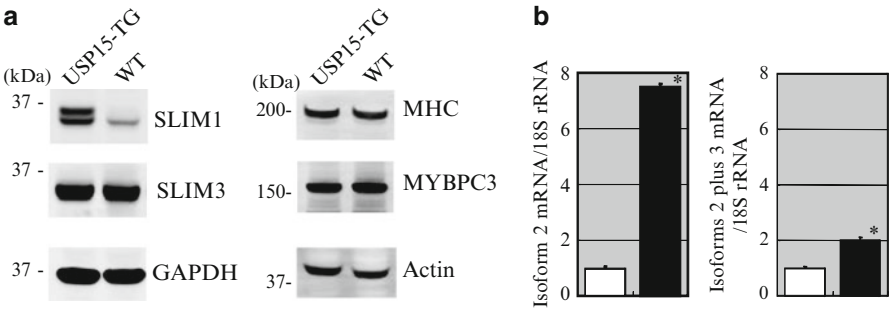

Fig. 5.2 SLIM1 protein and mRNA levels are high in the heat of USP15-TG mice (reproduced from [34] with permission). (**a**) Total proteins were extracted from the hearts of WT and USP15-TG mice, separated by SDS-PAGE, and analyzed by Western blotting with anti-SLIM1, anti-SLIM3, anti-GAPDH, anti-MHC, anti-MYBPC3, and anti-actin primary antibodies. (**b**) Total RNA was isolated from the hearts of WT (*open bars*) and USP15-TG (*filled bars*) mice and mRNA levels for SLIM1 isoform 2 (*left*) and SLIM1 isoforms 2 plus 3 (*right*) normalized to 18S rRNA were determined by real-time quantitative RT-PCR. mRNA levels in hearts of WT mice were arbitrarily assigned to 1, and fold differences were calculated. Means ± SE are shown ($n = 5$). (*Astritch*) denotes significantly different between WT and USP15-TG mice ($p < 0.05$)

USP15-TG mice showed no morphologic abnormalities in appearance; however, they started to die after the age of approximately 8 months. Figure 5.1c shows the morphology of the heart from a moribund USP15-TG mouse at the age of 8 months, exhibiting gross enlargement, which is more in atrium than in ventricle. Although the expression of human USP15 transgene, driven by α-MHC promoter, is supposed to be uniformly distributed over the heart according to the literature [47], the effect may not be homogenous throughout the heart (see below).

5.3.3 Increase of SLIM1 in the Heart of USP15-TG Mice

The cardiac remodeling in USP15-TG mice is accompanied with a marked increase of endogenous SLIM1, as shown in Fig. 5.2a [34]. However, the levels of SLIM3 (another member of the FHL family), MHC (mostly composed of α-MHC), myosin-binding protein C3 (MYBPC3, SLIM1-interacting sarcomeric protein [48]) are almost the same as those of normal hearts in WT mice. Immunoblot with anti-SLIM1 antibody in Fig. 5.2a also shows that one isoform of SLIM1 (resident type) is present in the heart extract of WT mice, while two isoforms of SLIM1 are in that of USP15-TG mice: the resident type and an extra one. On the basis of the molecular weight of these isoforms and the epitope of the anti-SLIM1 antibody, we determined the resident SLIM1 as isoform 3 in Entrez Gene and the extra one as SLIM1 isoform 2, which has extra 16 amino acids fused with the N terminus of isoform 3.

As we have shown that USP15 interacts physically and functionally with SLIM1 and stabilizes SLIM1 by deubiquitination in the transient expression system in

mammalian cells, we suppose this mechanism works to increase endogenous SLIM1 in the heart in USP15-TG mice, although this is yet to be proven. From the results of real-time quantitative RT-PCR, however, the increase of isoform 2 is attributable, at least partly, to the transcriptional activation [34]. Figure 5.2b shows that the levels of mRNA encoding specifically the isoform 2 in the heart of USP15-TG mice are seven times those in the heart of WT mice, but only twice as much difference for the levels of mRNA encoding either of the two isoforms (isoforms 2 plus 3). It is of note that USP15 associates with some of the components of U5/U6-snRNP such as PRPF4 and SART3 and suggests the involvement of USP15 in mRNA splicing [33]. Overexpression of USP15 may influence the stoichiometric balance of these components or accompanying regulatory factors in the splicing machinery, resulting in the increased expression of the splicing variant isoform 2.

SLIM1 has been shown to play a crucial role in cardiac hypertrophy by sensing biomechanical stress responses as a component within the cardiomyocyte sarcomere complex and by initiating changes in the titin- and mitogen-activated protein kinase (MAPK)-mediated responses [45]. Moreover, skeletal muscle-specific overexpression of SLIM1 induced skeletal muscle hypertrophy in TG mice showed an increase in fiber size and oxidative slow fiber-type expression, which led to increased muscle strength and endurance [46]. The authors also showed that SLIM1 directly complexes with nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (NFATC1) and enhances its transcriptional activity and that SLIM1 overexpression in a myoblastic cell line results in hypertrophic myotube formation, which is sensitive to cyclosporine A, the inhibitor of the upstream regulator calcineurin [46]. NFATC1 and four other members of this family are all present in the heart [49]. NFATC4, another member of this family, induces cardiac hypertrophy in TG mice, whereby constitutively nuclear mutant of NFATC4 is overexpressed [50]. Meanwhile, NFATC3, but not NFATC4, has been claimed to mediate calcineurin-mediated cardiac hypertrophic responses [49]. Any or multiple factors of this family are most likely involved in the hypertrophic responses in the remodeling heart of USP15-TG mice; however, further work is needed for confirmation.

Expression of SLIM1 is spatially controlled in adult normal hearts: higher in the atrium than in the ventricle in mice [51] and only detectable in the atrium but not the ventricle in rabbits [37]. Nevertheless, SLIM1 expression is upregulated in the TAC-induced hypertrophied ventricles in mice [38]. Viewed together, it may be speculated that the increased levels of SLIM1 are still skewed for the atrium over the ventricle in the heart of USP15-TG mice, which may influence the morphology change of the remodeling heart (see above).

5.3.4 Possible Signals to Cardiac Remodeling in USP15-TG Mice

Several substrates of USP15 have been reported: the NF-κB inhibitor IκBα [30] and the tumor suppressor protein APC [31] and procaspase-3 [32]. Deubiquitination and stabilization of IκBα result in the downregulation of NF-κB, which has ambivalent

effects on cellular survival [13], and those of the latter two gene products are generally considered to lead to a cytostatic or apoptotic effect.

Quite recently, studies have shown that USP15 is involved in the signaling pathways of transforming growth factor (TGF) β superfamily at two different phases [52, 53]. Eichhorn et al. have reported using glioblastoma cells that USP15 forms a complex with small mother against decapentaplegic (SMAD) 7 and SMAD-specific E3 ubiquitin protein ligase 2 (SMURF2) and is recruited to the TGFβ receptor complex, where it deubiquitinates and stabilizes type I TGFβ receptor (TβRI), leading to an enhanced TGFβ signal [52]. Both TGFβ and its receptors are present in the heart and expressed in cardiac myocytes and nonmyocytes (reviewed in [54]). TGFβ has clearly been implicated in cardiomyocyte hypertrophy and may participate in the pathogenesis of human hypertrophic cardiomyopathy (reviewed in [55]). Noncanonical pathways via the activation of TGFβ-activated kinase TAK1 are implicated in TGFβ-mediated hypertrophic response, although more proof is required [55].

On the other hand, Inui and colleagues have shown that USP15 deubiquitinates a mono- or di-ubiquitinated form of SMAD1, 2, and 3 [53]. These SMADs, classified as receptor-regulated SMAD (R-SMAD), act as signal transducers and transcriptional modulators in the downstream of the TGFβ superfamily members such as TGFβ and bone morphogenetic proteins (BMP) (reviewed in [56]). Upon stimulation with TGFβ or BMP, R-SMAD is phosphorylated by the receptors and oligomerized with SMAD4, common partner SMAD (co-SMAD), and the complex translocates into the nucleus, acting as a transcriptional modulator (canonical pathways) [56]. Inui et al. have shown that monoubiquitination of R-SMADs targets the DNA-binding domains of R-SMADs and prevents promoter recognition and that R-SMAD deubiquitination by USP15 is required for full responsiveness to TGFβ or BMP [53]. These isoforms of SMADs are all present in the cardiovascular system (reviewed in [57]). Activation of SMAD2 and 3, downstream of TGFβ stimulation, is considered to be related to deleterious development of cardiac fibrosis and failure [57], and activated SMAD3 complexed with SMAD4 has been shown to induce apoptosis in cardiomyocytes [58]. Meanwhile, BMP-2 and BMP receptors are expressed in freshly cultured neonatal rat cardiac myocytes [59]. In the downstream of BMP stimuli, activated SMAD1 complexes with SMAD4 and promotes survival of cardiac myocytes [59].

Although unproven, it is conceivable that these SMAD-mediated signals as well as noncanonical signals are intensified by overexpressed USP15 and strongly affect the cardiac remodeling in USP15-TG mice. In the heart, expression of BMP-10 is restricted only in the right atrium in adult normal mice [60] although upregulated in the hypertrophied ventricle of hypertensive rats [61], and BMP-2, BMP-4, and BMP-5 are shown to be present in human aortic valves [62]. These features of spatially differentiated expression of BMP genes may also influence the morphology of the remodeling heart in USP15-TG mice (see above).

In addition, SLIM1 has been shown recently to interact with SMAD2, 3, and 4 [63]. Ding et al. have shown that upregulation of SLIM1 induces the phosphorylation of SMAD2 and 3, depending on casein kinase (CK) 1δ but not on TGFβ receptors, and

increases the formation and nuclear accumulation of the oligomeric complex with SMAD4, resulting in transcriptional modulation of their target genes, e.g., activation of the cyclin-dependent kinase inhibitor *p21* and suppression of the oncogene c-myc [63]. Thus, USP15 may activate SMAD signaling not only directly but also indirectly through the increase of SLIM1 by closely linked interactions.

5.4 Conclusion

Cardiac-specific overexpression of USP15 increases SLIM1 in the heart and induces cardiac hypertrophic remodeling. SLIM1 plays an essential role in hypertrophic responses of cardiac myocytes probably through activation of NFATs. Moreover, USP15 interacts with TβRI and SMADs and reinforces their activities, which may possibly contribute to the cardiac hypertrophic responses in USP15-TG mice. In addition, the interaction of SLIM1 with SMADs may also possibly modify the cardiac remodeling in USP15-TG mice. Importantly, catalytic activity of USP15 has been detected in human hearts, and its transcription is upregulated in an in vitro model of cardiac myocyte hypertrophy. Although more research is needed, these lines of evidence implicate USP15 in the processes of cardiac remodeling and may provide a novel target for therapeutics in heart failure.

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Chapter 6
Role of Galectin-3 Pathways in the Pathogenesis of Cardiac Remodeling and Heart Failure

Lili Yu and Rudolf A. de Boer

Abstract Myocardial injuries stemming from pressure overload or myocardial infarction lead to cardiac remodeling and represent major health problems worldwide. An ever accumulating body of experimental and clinical research appoints galectin-3, a β-galactoside-binding lectin, as a key player in this maladaptive response to myocardial injury. Herein, a specific role for galectin-3 in inflammation and fibrogenesis has been elucidated in experimental and clinical studies. Galectin-3 was first associated with pathological conditions leading to cardiac remodeling, such as inflammation and fibrosis. Then, as the carbohydrate recognition domain of galectin-3 reacts with glycosylated proteins such as laminin, fibronectin, and tenascin, a multifunctional role of galectin-3 in the extracellular matrix was postulated. Notably, experimental animal studies clearly showed that galectin-3 is a mediator of crucial steps in fibrogenesis and further induces cardiac inflammation, hypertrophy, and dysfunction. Possible mechanisms pertaining to galectin-3 inflammatory and fibrotic properties have been suggested to involve macrophage activation, galectin-3-induced chemotaxis, and activation of the TGF-β–Smad3 signaling pathways. Additionally, the link between plasma galectin-3 and fibrosis was also established in clinical biomarker studies. Galectin-3 and its pathways may be explored further in order to develop more efficient strategies to target cardiac remodeling in heart failure leading to fibrosis.

Keywords Galectin-3 • Heart failure • Remodeling • Biomarker

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6.1 Structure, Expression, and Function of Galectin-3

Galectin-3 is a 29–35 kDa chimera-type galectin. Galectins form a large family of galactosidase-binding lectins, but galectin-3 is the only member of the galectin family with an extended N-terminal domain (110–130 amino acids). This N-terminal domain is composed of tandem repeat sequences comprising nine amino acid residues and is connected to a C-terminal carbohydrate recognition domain (CRD) of about 130 amino acids [1, 2]. The CRD interacts with various glycosylated proteins and modulates cell–cell adhesion and cell signaling in the extracellular compartment [1]. Further, the galectin-3 CRD and collagen-like domains communicate with a variety of extracellular matrix proteins (ECM), carbohydrates (e.g., *N*-acetyllactosamine), and also unglycosylated proteins such as cell surface receptors (macrophage antigens CD11b/CD18) and extracellular receptors (collagen IV) [3–6], and they furthermore modulate cell–cell adhesion and cell signaling in the extracellular compartment.

Galectins are expressed in various cells and tissues and are important for diverse physiological and pathological processes, such as immune and inflammatory responses, tumor development and progression, neural degeneration, atherosclerosis, diabetes, as well as wound repair. Galectin-3 has been detected in many proliferative cells including tumor cells, eosinophils, neutrophils, and activated macrophages [7, 8]. Notably, many of these cell types are operative in the inflammatory response and the formation of fibrosis (fibrogenesis).

Galectin-3 is predominantly located in the cytoplasm. While intracellular galectin-3 plays a pivotal role in diverse cell growth, anti-apoptosis signaling, and mRNA splicing, extracellular (or cell surface bound) galectin-3, on the other hand, participates in cell–cell and cell–matrix adhesion, by binding to glycosylated ECM components, including laminin, fibronectin, tenascin, and Mac-2-binding protein [3, 4, 9–12]. Thus, extracellular galectin-3 appears in tight communication with factors involved in fibrogenesis.

Differential expression of galectin-3 has been reported for different murine organs. Herein, lower expression was found in cerebrum, heart, and pancreas, while moderate expression was found in liver, ileum, kidney, and adrenal gland and high expression in lung, spleen, stomach, colon, uterus, and ovary [13]. Figure 6.1 displays an overview of galectin-3 distribution in various healthy tissues of different species—our data are in concert with results published by Kim and colleagues [13]. Notably, a growing body of evidence reveals that high expression levels of galectin-3 are closely associated with pathological conditions, specifically conditions pertaining to inflammation and fibrosis which are also key conditions in cardiac remodeling.

6.1.1 Localization of Galectin-3 in Damaged Tissue

Inflammation and fibrosis are intertwined pathological states, and galectin-3 has consistently been observed to be involved in these damaged states.

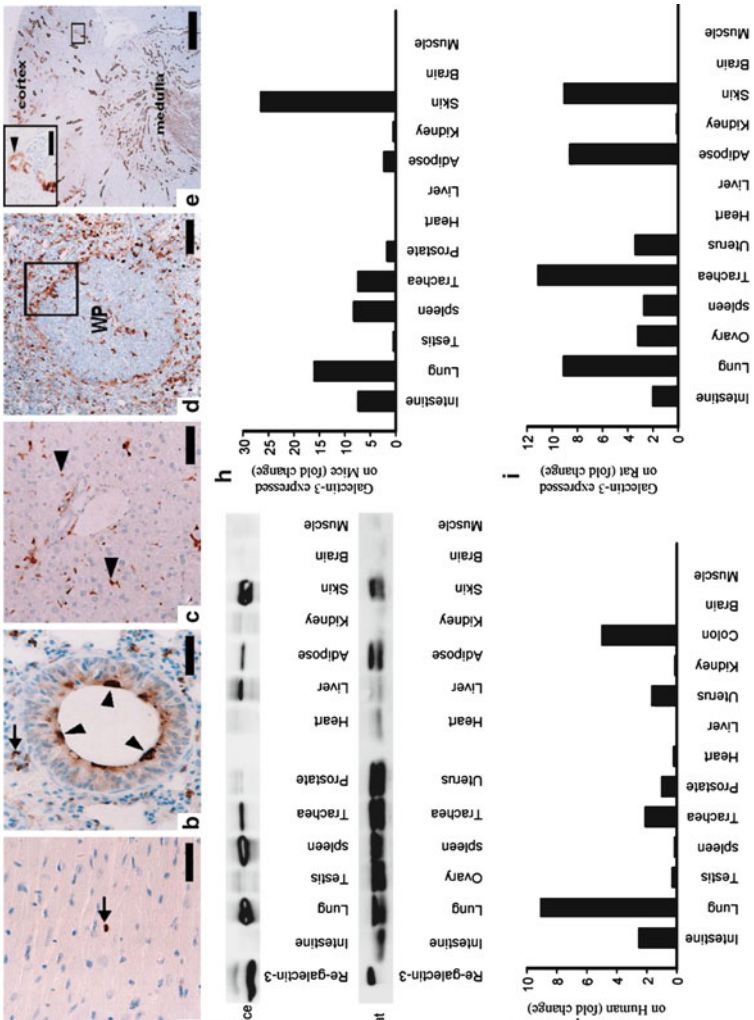

Fig. 6.1 Immunohistochemical localization and protein and mRNA expression of galectin-3 in various tissues of different species. Galectin-3 immunoreactivity has been observed in heart (**a**), lung (**b**), liver (**c**), spleen (**d**), and kidney (**e**). (**f–i**): Galectin-3 protein expression (assessed by Western blot) and mRNA expression (assessed by qPCR) have been predominantly observed in (mouse, rat, human) lung, testis/ovary, spleen, prostate, adipose tissue, and skin. Picture (**a–e**) reprinted from [13] with permission from the publisher; figure (**f–i**): unpublished data (Yu et al.). Scale bars: 50 μm (**a** and **b**); 60 μm (**c**); 90 mm (**d**); 400 μm (**e**)

Galectin-3 is not only secreted by activated macrophages, but in situ hybridization and immunohistochemistry analysis showed that galectin-3 is highly distributed in the fibrotic area of myocardium and co-localizes to macrophages [14]. Moreover, an ever growing body of experimental evidence found that galectin-3 manifests in various damaged tissue, in particular in tissues with increased collagen deposition and localization in the fibrotic area [14–23]. Further, galectin-3 was expressed in proliferating fibroblast and was also found in nucleus when cells were exposed to apoptotic stimuli in vitro [24]. Moreover, galectin-3 expression has been detected in isolated cardiac fibroblast to localize galectin-3-binding sites. Interestingly, contrary to cardiac fibroblast, galectin-3-binding sites were absent from cardiomyocytes [7, 8, 14, 25–27].

6.2 Galectin-3 in Experimental Fibrosis

6.2.1 Early Experimental Support in Liver, Kidney, and Lung Fibrosis

A prominent role for galectin-3 in fibrogenesis has been elucidated in recent years. Herein, work has specifically described fibrotic states in the liver, kidneys, pancreas, and lungs. For example, galectin-3 showed to be temporarily and spatially associated with fibrosis with a minimal expression in healthy rat liver, while highest expression was found at peak fibrosis, and virtually no galectin-3 expression was present after recovery from fibrosis [19]. Then, galectin-3 deficiency in bile duct-ligated (BDL) rats was recently shown to significantly diminish BDL-induced hepatic TGF-β1 and procollagen expression and associated hepatic fibrosis [28]. Additionally, in a murine model of cirrhosis, bone marrow cell transplantation significantly decreased liver fibrosis which was associated with decreased hepatic galectin-3 expression [29]. A recent report by Mackinnon et al. [23] extended the involvement of galectin-3 to fibrosis in the lung. The authors reported a significantly reduced TGF-β1- and bleomycin-induced lung fibrosis in galectin-3-deficient mice.

Likewise, galectin-3 expression and secretion by macrophages has been identified as a major contributor to renal fibrosis. Mice with galectin-3 deficiency did not show macrophage recruitment upon interferon gamma/LPS stimulation [19]. Henderson et al. also observed that galectin-3 mediated TGF-β-induced myofibroblast activation, a crucial step in the fibrogenesis cascade [17]. Lastly, the therapeutic potential of targeting galectin-3 to relieve fibrotic burden has recently been investigated by Kolatsi-Joannou and colleagues [30] who demonstrated that folic acid induced kidney fibrosis and associated galectin-3 expression was significantly reduced by 1% treatment with modified citrus pectin (MCP). MCP is a pectin derivative binding to the galectin-3 CRD, thereby inhibiting galectin-3 aforementioned effects.

Altogether, a growing body of literature consistently supports a role of galectin-3 in fibrogenesis. This appears a generalized effect, not confined to one organ.

Fibrosis is also central to the maladaptive response to myocardial injury, such as pressure overload and myocardial infarction leading to cardiac remodeling [14, 21, 22]. Supporting the notion that galectin-3 constitutes a global player in fibrogenesis, it is not surprising that galectin-3 has also been shown to be involved in myocardial fibrogenesis.

6.2.2 Galectin-3 in Cardiac Remodeling Pathway: Recent Experimental Support

The first evidence showing an involvement of galectin-3 in heart failure stems from a landmark study of Sharma and colleagues [14]. In a comprehensive microarray study, galectin-3 was identified as the most robustly overexpressed gene in heart failure-prone hypertrophied hearts compared to functionally compensated hearts in homozygous transgenic TGFmRen2-27(Ren-2) rats. Ren-2 rats overexpress the mouse Ren-2d renin gene and spontaneously develop heart failure after 13–15 weeks. Further, continuous low-dose infusion of recombinant galectin-3 into the pericardial sac caused left ventricular dysfunction in healthy Sprague–Dawley rats, associated with collagen deposition and other signs of cardiac remodeling [14]. These initial observations warranted galectin-3 to be considered as a new target for intervention in heart failure.

Then, more recently, Sharma et al. [21] found a significant and high expression of galectin-3 in cardiac tissue of Ang-II-induced hypertension in mice. In this model, galectin-3 was released by infiltrating macrophages in the myocardium and led to interstitial collagen deposition. Treatment with *N*-Acetyl-Ser-Asp-Lys-Pro (Ac-SDKP), an endogenous tetrapeptide specifically degraded by angiotensin-converting enzyme (ACE), reduced macrophage activation and galectin-3 expression herein and prevented galectin-3-induced cardiac inflammation, fibrosis, and remodeling.

Additionally, Liu et al. showed that in galectin-3-induced cardiac remodeling, galectin-3 increased the number of macrophages and mast cell infiltration and activated the TGF-β/Smad3 pathway. Ac-SDKP partially or near completely prevented galectin-3-induced cardiac inflammation, fibrosis, hypertrophy, and dysfunction, possibly by inhibition of the TGF-β/Smad3 signaling pathway [22].

Recent studies by Thandavarayan et al. [31], by Kamal et al. [32], and by Psarras et al. [33] further reveal a specific association of galectin-3 with left ventricular dysfunction and fibrosis. Specifically, Thandavarayan et al. [31] found a significant increase in myocardial hypertrophy and fibrosis, as well as apoptosis, all associated with upregulated galectin-3 in 14-3-3η protein mutant (DN-14-3-3η) mice after induction of diabetes. 14-3-3η protein has a regulatory role in apoptosis, adhesion, cellular proliferation, differentiation, survival, and signal transduction pathways [34]. As the authors stated, upregulated galectin-3 appears to be a general phenomenon in

LV dysfunction [31]. Moreover, Kamal et al. [32] demonstrated that cardiac hypertrophy, progressive fibrotic cardiac remodeling, and increased collagen deposition were accompanied with significantly increased galectin-3 expression in cardiomyopathic hearts in a rat model of myosin-induced experimental autoimmune myocarditis (EAM). Notably, herein galectin-3 overexpression was dramatically reduced by treatment with T-3999, a novel phenylpyridazinone, indicating an inhibitory function of T-3999 on galectin-3.

Then, Psarras et al. [33] found that desmin-deficient (des−/−) mice exhibit marked myocardial degeneration and fibrosis, which were associated with osteopontin (OPN) and galectin-3 overexpression (226× for OPN and 26× for galectin-3). OPN, like galectin-3, has chemotactic properties and is thus recruited to inflammatory sites [35, 36]. Psarras et al. [33] further compared des−/− OPN −/− mice with des−/− OPN +/+ mice and found that des−/− OPN −/− mice not only displayed remarkable improvements in ventricular function (53%) but also in myocardial fibrosis (44%) while also significantly reducing galectin-3 gene expression (by ≈80%) compared to des−/− OPN +/+ mice, indicating that the observed diminished inflammatory and fibrotic response in OPN-deficient des−/− mice could be partly explained by the significant reduced myocardial galectin-3 level [33].

So, accumulating experimental evidence implicates a role of galectin-3 in the development of organ fibrosis. Whether galectin-3 is a potential therapeutic target in left ventricular (LV) remodeling and heart failure is unknown. We have conducted experimental studies, and our results suggest that galectin-3 may be a target for therapy. Genetic disruption and pharmacological inhibition of galectin-3 attenuated adverse LV remodeling, fibrosis, and subsequent HF development. We perturbed mice with angiotensin II (Ang-II) and transverse aortic constriction (TAC), causing LV hypertrophy, decreased LV contractility, and increased LV end-diastolic pressure, associated with increased fibrosis in wild-type (WT) mice. However, galectin-3 knockout (Gal3-KO) mice did not develop LV dysfunction and fibrosis. Additionally, in homozygous TGR(mREN)27 rats, pharmacological inhibition of galectin-3 with an oligosaccharide almost completely prevented LV dysfunction and fibrosis [54, 55].

This indeed suggests that drugs binding to galectin-3 may be potential therapeutic candidates for the prevention of heart failure with extensive fibrosis. It remains unclear what mechanisms underpin these effects.

6.3 Role of Galectin-3 in Modulation of Fibrosis

6.3.1 Potential Mechanism of Galectin-3 in Extracellular Matrix

Fibroblasts, myofibroblasts, and macrophages have been identified as important cells in the initiation and progression of fibrogenesis, scar formation, and tissue remodeling [37–39]. Extracellular matrix remodeling (ECM) is a crucial aspect in fibrogenesis,

and galectin-3 seems to play a multifunctional role in the ECM environment, as its CRD reacts with glycosylated proteins in the ECM, such as laminin, fibronectin, tenascin [3, 4], as well as membrane proteins, such as αM/β2 (CD11b/18) [40].

6.3.2 *Potential Mechanisms of Galectin-3 in Myofibroblast Differentiation*

Fibroblast to myofibroblast differentiation and activation by inflammatory cytokines, like TGF-β, preceded by influx of cells such as macrophages are some of the initial steps in the process of fibrogenesis. A large body of research supports a role for galectin-3 in this process. Herein, it appears that macrophages and TGF-β induce myofibroblast activation via galectin-3, but that macrophage recruitment and TGF-β expression are independent of galectin-3.

First, galectin-3 was visualized in the fibrotic area co-localizing with fibroblasts and macrophages [14]. Second, it was shown that infusion of recombinant galectin-3 into pericardial sac leads to inflammatory cell infiltration, cardiac fibroblast proliferation, collagen synthesis, and deposition, essentially contributing to interstitial and perivascular fibrosis [14, 21, 22]. Then, Dvorankova et al. demonstrated myofibroblast activation in vitro upon treatment with a moderately high-dose recombinant galectin-3 [41]. Further, galectin-3 deficiency significantly reduced myofibroblast activation in carbon tetrachloride (CCL4)-induced hepatic fibrosis and renal fibrosis (in a model of unilateral ureter obstruction, UUO) [17, 19].

Then, macrophages regulate fibroblast and myofibroblast activation in ECM, and macrophage-derived galectin-3 presents in various fibrotic pathologies. Injured tissue displays a marked increase in galectin-3 expression by activated macrophages and also an increased TGF-β expression; all these factors promote fibroblast proliferation and myofibroblast activation [17]. Macrophage depletion, then, significantly inhibits myofibroblast activation and decreases fibrosis [19]. For example, Henderson and colleagues showed that galectin-3-deficient macrophage recruitment could not drive myofibroblast accumulation and activation. Utilizing a crossover experiment with wild-type or galectin-3-deficient macrophage supernatant and galectin-3-deficient renal fibroblast, these authors further observed that proliferation of galectin-3-deficient renal fibroblasts was activated by wild-type macrophages and attenuated by a galectin-3 inhibitor bis-(3-deoxy-3-{3-methoxybenzamido}-β-D-galactopyranosyl-sulfane), while galectin-3-deficient macrophages did not induce proliferation in galectin-3-deficient renal fibroblasts.

On the other hand, galectin-3 deficiency markedly reduces activated myofibroblast but does not affect macrophage recruitment or pro-inflammatory cytokine profiles in injured tissue, such as IL-6 and TNF-α. Additionally, while galectin-3 deficiency led to reduced collagen deposition and reduced myofibroblast activation, TGF-β expression, or Smad2/3 phosphorylation was not influenced [19].

Furthermore, our unpublished galectin-3 chemotaxis assay results show that recombinant galectin-3 significantly induces monocyte migration, which could be markedly attenuated after treatment with galectin-3 inhibitors including modified citrus pectin (MCP) and lactose. These inhibitors act as a ligand, binding to galectin-3's CRD.

Altogether, these events clearly indicate galectin-3 to be a key player in the signal axis of fibrosis generation, specifically inducing macrophage and TGF-β-induced myofibroblast activation [17, 19, 23, 42].

6.4 Clinical Utility of Galectin-3

Clinical trials have consistently indicated a potential clinical utility of galectin-3 as a biomarker for prognosticating heart failure. Herein, van Kimmenade et al. were the first to evaluate the prognostic and predictive value of galectin-3 as a biomarker in acute heart failure [43], in the pro-BNP investigation of dyspnea in the emergency department (PRIDE). While N-terminal pro-brain natriuretic peptide (NT-pro-BNP) was a superior predictor for diagnosis of acute heart failure compared to galectin-3 and apelin (herein galectin-3 was a better predictor than apelin) (Fig. 6.2a), galectin-3 was the superior predictor compared to NT-pro-BNP and apelin for prognosis in acute heart failure. Multivariate logistic regression analysis revealed that elevated plasma levels of galectin-3 were indeed the most powerful predictor for death or the combination of death and recurrent heart failure within 60 days.

Plasma galectin-3 levels were related to detailed echocardiographic examinations in a substudy ($N = 115$) of the PRIDE [44]. Galectin-3 levels were not strongly related to markers of LV structure or systolic function, but related to measures of RV function and diastolic dysfunction, and highest galectin-3 concentrations were strongly associated with a higher risk of 4-year mortality, which is independent from LV dimensions, function, and RV pressure, supporting the role galectin-3 may play in fibrosis and progressive cardiac failure.

Further and specifically relating galectin-3 to fibrosis, plasma galectin-3 levels were significantly correlated with several serum markers of cardiac ECM turnover, such as PIIINP, MMP-2, and TIMP-1, in 106 patients with chronic heart failure (New York Heart Association class II–III; mean LV ejection fraction [LVEF], 35 + 9%) [45]. Subsequently, Milting et al. described the kinetics of galectin-3 in 55 end-stage heart failure patients with the need for mechanical circulatory support (MCS). Notably, this study found that fibrosis-related biomarkers, such as tissue inhibitor of metalloproteinase (TIMP), tenascin C (TNC), OPN, BNP, and galectin-3, were all increased in patients with terminal heart failure. Interestingly, MCS only reduced the loading-related biomarker BNP, but none of the other fibrosis-related biomarkers. Additionally, patients who did not survive on MCS had higher baseline galectin-3 levels when compared with patients who lived until transplantation [45].

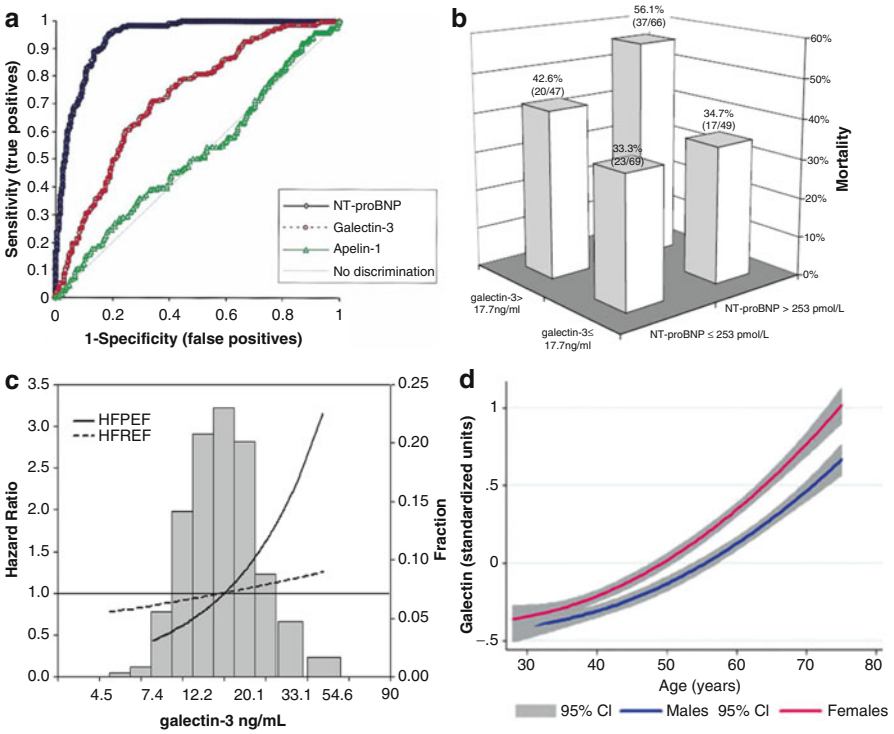

Fig. 6.2 Galectin-3 as a biomarker; data from several clinical trials. (**a**) Combined receiver-operating characteristic (ROC) curves for amino-terminal pro-brain natriuretic peptide (NT-pro-BNP), galectin-3, and apelin for the diagnosis of heart failure in dyspneic patients. The ROC analysis for NT-pro-BNP showed an area *under* the *curve* (AUC) for NT-pro-BNP of 0.94 ($p=0.0001$). The ROC analysis for galectin-3 showed an AUC of 0.72 ($p=0.0001$). The AUC for apelin for diagnosis of acute heart failure was 0.52 ($p=0.23$). This figure was reprinted from van Kimmenade et al. [43], with permission. (**b**) Mortality as a function of baseline galectin-3 and NT-pro-BNP categories. The median value of NT-pro-BNP (253 pmol/L) was used to define two levels of NT-pro-BNP concentration. Of the 232 subjects, 231 had both galectin-3 and NT-pro-BNP measurements. The number of patients in each category is as follows: high galectin-3 and high NT-pro-BNP ($n=66$), low galectin-3 and low NT-pro-BNP ($n=69$), low galectin-3 and high NT-pro-BNP ($n=49$), and high galectin-3 and low NT-pro-BNP ($n=47$). Reprinted from [46] by Lok et al., with permission. (**c**) Graphical depiction of the risk estimates for experiencing the primary outcome in patients with HFPEF and HFREF with increasing levels of plasma galectin-3. The distribution of (log-transformed) galectin-3 is depicted in the background in *brown bars*. A similar increase in galectin-3 causes a much more pronounced increase in risk in patients with HFPEF compared to patients with HFREF. Figure reprinted from [47] by de Boer et al. with permission. (**d**) Graph showing galectin-3 levels in male (*blue line*) and female subjects (*red line*) from the general population. *Gray-shaded areas* indicate 95% confidence intervals. Galectin-3 levels increase with increasing age, particularly in female subjects. This figure is reprinted from [49] by de Boer et al. with permission

Then, a larger study by Lok et al. comprising 232 patients with *chronic* heart failure (New York Heart Association function class III or IV) demonstrated that patients with high baseline levels of both galectin-3 and NT-pro-BNP had around 1.5- to 2-fold higher mortality rate (Fig. 6.2b) [46]. This study demonstrated incremental value of galectin-3 over NT-pro-BNP alone.

Additionally, a large study of 592 patients with heart failure (Coordinating Study Evaluating Outcomes of Advising and Counseling in Heart Failure, COACH trial, [47]), with mean follow-up of 18 months supported prognostic value of galectin-3 to predict re-hospitalization and death after correction for age, gender, BNP, eGFR, and diabetes, but not after correction for LVEF. A subanalysis revealed that increased plasma galectin-3 levels represents a stronger incremental risk in patients with preserved LVEF (HFPEF) compared to the patients with reduced LVEF (HFREF) ($P < 0.001$) even when absolute galectin-3 levels did not differ between patients with HFPEF and HFREF [47] (Fig. 6.2c).

Furthermore, in the HF-ACTION study where plasma galectin-3 levels were assessed in 895 subjects with heart failure from a randomized, controlled trial of exercise training in patients with chronic heart failure with NYHA class II, III, or IV symptoms, galectin-3 was associated with NYHA class, lower systolic blood pressure, higher creatinine, higher NT-pro-BNP, and lower maximal oxygen consumption. However, this association diminished after adjustment for NT-pro-BNP [48].

Finally, recent data show that small increases in galectin-3 may confer increased CV risk in the general population, in subjects at risk for heart failure development. Briefly, 7,968 subjects were included in this study from the Prevention of Renal and Vascular End-Stage Disease (PREVEND) cohort (mean age of 50 ± 13 years, median follow-up of ~10 years). Plasma galectin-3 levels correlated very strong with age and sex (Fig. 6.2d) and weakly with a wide range of risk factors of CV disease, including blood pressure, serum lipids, body mass index, renal function, and NT-pro-BNP. After correction for classical CV risk factors (smoking, blood pressure, cholesterol, and diabetes), increased plasma galectin-3 levels independently predicted all-cause mortality in a large community-based cohort [49].

Altogether, these available clinical studies have so far confirmed that plasma galectin-3 levels were significantly upregulated in acutely decompensated heart failure [43, 44, 50, 51], chronic heart failure [46–49], and end-stage heart failure with the need for mechanical circulatory support (MCS) [52]. Furthermore, clinical results from our group further demonstrated the predictive and prognostic value of galectin-3 [46, 47, 49]. A relationship between galectin-3 and cardiovascular (CV) risk factors was investigated in the general population of PREVEND study, and a strong gender-specific interaction was revealed in the correlation between galectin-3 and cardiovascular risk factors [49]. Notably, the established link between plasma galectin-3 and fibrosis was also established in clinical biomarker studies [52] and needs to be explored further in order to develop more efficient strategies to target cardiac remodeling in heart failure leading to fibrosis.

6.5 Conclusion and Mapping of Galectin-3 Pathways

In conclusion, galectin-3 is highly expressed in the fibrotic area of the failing or stressed heart [14, 17, 19, 21, 22], and cardiac fibroblasts and macrophages are the main sources of galectin-3. Further, galectin-3 was shown to activate the TGF-β/Smad3 pathway [22], while macrophages and the inflammatory factor TGF-β demonstrated to elevate galectin-3 expression. Then, galectin-3 inhibition or deficiency was found to not affect macrophage activation and TGF-β expression levels. Therefore, galectin-3 may be considered as an independent participant in macrophage and TGF-β/Smad modulation pathway.

Other experimental animal studies reported that galectin-3 was not only significantly associated with myofibroblast-induced collagen synthesis and deposition but was also markedly correlated with ECM fibrosis markers, such as α-SMA, COL1A1, COL3A1, TIMP, and MMP [53] (Fig. 6.3).

Lastly, relevant clinical studies identified that plasma galectin-3 is significantly correlated with serum extracellular fibrosis turnover biomarkers, like PINP, PIIINP, TIMP, and MMP. Collectively, galectin-3 may be suggested as "culprit

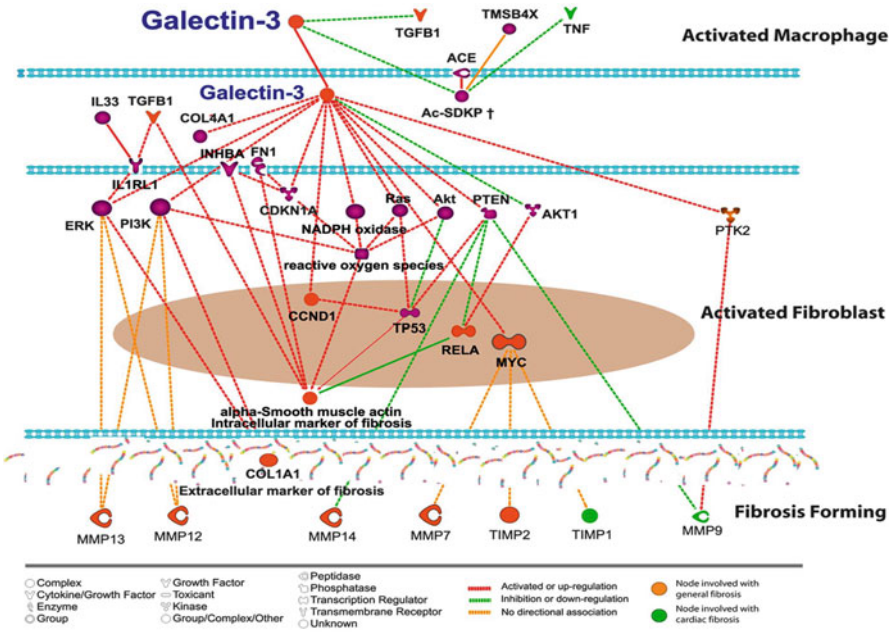

Fig. 6.3 Galectin-3 pathways. The network represents molecular relationships between different gene products. *Node shapes* indicate the functional class of the gene product, whereas *node colors* indicate a role in general fibrosis (*orange*) or cardiac fibrosis (*green*). Edge colors indicate upregulation or activation (*red*), downregulation or inhibition (*green*), or involvement without clear directionality (*yellow*). This figure is reprinted from [53] by de Boer et al. with permission

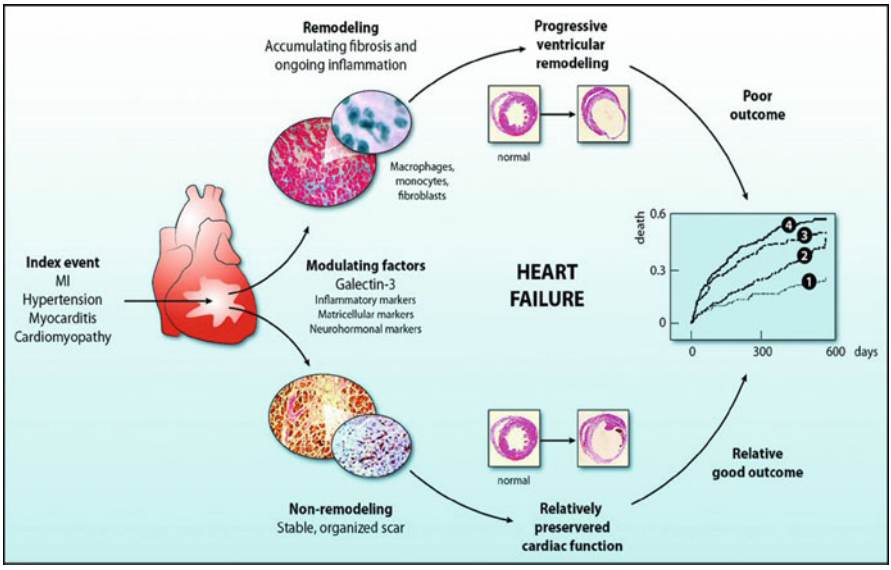

Fig. 6.4 Working scheme representing the sequence of events following an index event (such as myocardial infarction (MI), hypertension, myocarditis, and cardiomyopathy) leading to remodeling and non-remodeling heart failure. The graph within this graphic is taken from de Boer et al. [56] and represents the adjusted Cox regression curves for quartiles of plasma galectin-3 showing the cumulative risk for the combined endpoint, death. The *back circles* with the *white numbers* represent quartile 1 through 4, respectively. Galectin-3 is displayed as a central modulating factor involved in the remodeling process which leads to ongoing damage and eventually poor heart failure outcome. Therapeutically, galectin-3 inhibition could favor non-remodeling heart failure, thereby potentially improving heart failure outcome. Figure reprinted from [56] by de Boer et al. with permission

biomarker" involved in pathophysiology circle of cardiac remodeling and heart failure. A suggested pathway of galectin-3 is displayed in Fig. 6.4 [56].

Data from experimental renal damage and cancer suggest that galectin-3 is a feasible target for therapy. Our pilot data lend support to the notion that also in heart failure galectin-3 may be a target for therapy. More research is warranted herein, specifically, at what stage galectin-3 comes into play and what the ideal window would be for intervention. Most data show that preventative regimen might work, but if fibrosis, once ensued, could be attenuated or reversed is also unknown. From a clinical point of view, this is of utmost importance.

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Chapter 7
A Mitochondriocentric Pathway to Cardiomyocyte Necrosis: An Upstream Molecular Mechanism in Myocardial Fibrosis

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Abstract The pathophysiologic origins of heart failure can be attributed to a pathologic remodeling of myocardium, including necrotic loss of cardiomyocytes and consequent reparative fibrosis. Hypertensive heart disease with concentric left ventricular hypertrophy and fibrosis represents a major etiologic factor accounting for diastolic heart failure. Herein, we focus on molecular mechanisms to the precursor of fibrosis, namely, cardiomyocyte necrosis, whose pathogenic origin resides in a mitochondriocentric signal-transducer–effector pathway. Its major components include intracellular Ca^{2+} overloading of cytosolic and mitochondrial domains, the induction of oxidative stress by these organelles which overwhelms endogenous antioxidant defenses, and the increased opening potential of the mitochondrial permeability transition pore. Novel cardioprotective strategies aimed at preventing the progressive remodeling of the failing heart should target upstream molecular mechanisms that prevent cardiomyocyte necrosis rather than downstream events involving collagen turnover related to fibrosis.

Keywords Mitochondria • Calcium overload • Oxidative stress • Necrosis • Fibrosis • Aldosteronism

Abbreviations

ALDO	Aldosterone
ALDOST	Aldosterone/salt treatment
CHF	Congestive heart failure

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CVF	Collagen volume fraction
DOCST	Deoxycorticosterone/salt treatment
HHD	Hypertensive heart disease
mPTP	Mitochondrial permeability transition pore
MSTE	Mitochondriocentric signal-transducer–effector
PAC	Plasma aldosterone concentration
PTH	Parathyroid hormone
RAAS	Renin–angiotensin–aldosterone system
ROS	Reactive oxygen species
SHPT	Secondary hyperparathyroidism
Spiro	Spironolactone

7.1 Introduction

The congestive heart failure (CHF) syndrome has reached epidemic proportions; its debilitating symptoms and signs account for the number one admitting diagnosis to US hospitals. CHF strains our workforce and drains our health-care economy. Of necessity, improved management strategies have become national priorities.

A pathologic remodeling of myocardium contributes to the failure of this muscular pump during systolic and/or diastolic phases of the cardiac cycle and is related to (a) the loss of contractile parenchyma or cardiomyocytes and (b) their consequent replacement by extracellular matrix or reparative fibrosis. Foci of fibrosis, or microscopic scarring, are found scattered throughout the right and left heart of the explanted failing human heart and are considered the major cause of myocardial remodeling in the cardiomyopathic heart of either ischemic or nonischemic origin [1–4]. Fibrous tissue consists predominantly of type I (75 %) and type III (20 %) fibrillar collagens aligned either in parallel or in series with muscle fibers that adversely influence ventricular function [4–6]. Type I fibrillar collagen has the tensile strength of steel, and therefore, its accumulation adversely impacts myocardial stiffness. Moreover, fibrillar collagen encircling muscle may lead to muscle fiber atrophy [7].

Hypertensive heart disease (HHD) represents a major etiologic factor that accounts for heart failure in which the failing heart may manifest as either diastolic or systolic predominance. The clinical syndrome CHF, with its characteristic signs and symptoms, frequently accompanies either diastolic or systolic heart failure. Its origins are rooted in a salt-avid state mediated by effector hormones of the renin–angiotensin–aldosterone system (RAAS). Concentric left ventricular hypertrophy is a distinguishing feature of HHD, so too is fibrosis, which is found throughout the right and left heart [8–10]. It is not the quantity, rather the quality (structural remodeling), of myocardium that contributes to diastolic heart failure in HHD.

The failing heart's inability to sustain adequate systemic blood flow and organ perfusion elicits adaptive homeostatic responses. In the case of underperfused kidneys, this evokes neurohormonal activation of the RAAS. The attendant effector hormone-mediated retention of salt and water now accounts for the appearance of

the clinical CHF syndrome [11]. Prolonged elevations in circulating angiotensin II and aldosterone each have pathologic consequences on diverse tissues, including the heart as well as skeletal muscle and peripheral blood mononuclear cells (monocytes and lymphocytes) [12–15].

Herein, we focus on the pathologic remodeling of myocardium by reparative fibrosis and molecular mechanisms leading to its precursor, cardiomyocyte necrosis. Ensuing tissue repair at sites of necrosis and resultant scarring preserves the structural integrity of the myocardium, but at the cost of increased stiffness. Cardioprotective strategies aimed at preventing the progressive nature of heart failure with ongoing cardiomyocyte loss and consequent incremental fibrosis should target the upstream molecular mechanisms to prevent necrosis, rather than downstream events related to collagen turnover. In this context, we propose that the specific cascade and the pathogenic origins to necrosis is a mitochondriocentric signal-transducer–effector (MSTE) pathway.

7.2 Animal Model of Aldosteronism

We identified the MSTE pathway to cardiomyocyte necrosis using a rat model of aldosteronism, where plasma aldosterone (ALDO) concentrations (PAC) are raised to those found in human CHF together with 1 % NaCl in drinking water. Aldosterone/salt treatment (ALDOST) allows us to address the pathologic remodeling of the previously normal myocardium that accompanies the secondary aldosteronism of CHF or the primary aldosteronism attendant with autonomous ALDO production associated with adrenal pathology. We have found this remodeling to include cardiomyocyte necrosis and myocardial scarring, a footprint of necrosis. Subcellular and molecular mechanisms responsible for necrosis were then sequentially and systematically investigated.

7.2.1 Cardiac Remodeling in Aldosteronism

Much like the cardiomyopathic heart of ischemic or nonischemic origins, the myocardium in primary aldosteronism includes foci of scarring found scattered throughout the nonpressure-overloaded, nonhypertrophied right and the pressure-overloaded, hypertrophied left ventricle [16]. Cardiac pathology is found at week 4 of ALDOST. It includes multiple foci of microscopic scarring found scattered throughout the right and left atria and ventricles [10, 15, 17]. The accumulation of collagen in the myocardium is a *reparative* fibrosis. It replaces cardiomyocytes lost to necrotic cell death and in so doing preserves the structural integrity of myocardium (reviewed in [18]). In contrast, apoptotic cell death is devoid of inflammatory cell or fibroblast responses and, therefore, is not accompanied by fibrosis.

Myocardial fibrosis can be defined as an increase in its hydroxyproline concentration, an amino acid specific to collagen, and morphologically the volume of myocardium occupied by fibrillar collagen. Furthermore, collagen fiber cross-linking and the relative abundance of type I and III collagens are features of the collagen matrix that determine abnormalities in myocardial stiffness [19]. Fibrosis increases myocardial stiffness leading to diastolic heart failure, also termed heart failure with preserved ejection fraction (reviewed in [20–22]). Diastolic failure is commonly seen with the concentric hypertrophy that accompanies arterial hypertension [23].

7.2.2 Cardiac Remodeling: Role of Hemodynamic Factors

A conspicuous heterogeneity in myofiber size is found in the heart during chronic ALDOST. In the pressure-overloaded left ventricle, myofibers are hypertrophied while they remain normal in diameter in the nonpressure-overloaded right ventricle. Surrounded by fibrillar collagen at sites of scarring, myofibers in either ventricle become atrophic.

In the case of myocardial fibrosis, hemodynamic factors are not directly involved (reviewed in [11]). This conceptual framework and role of a circulating factor is primarily based on the following finding: (a) the presence of fibrosis in the nonpressure-overloaded right atria and ventricle, (b) the absence of fibrosis when a pressure overload on the LV is caused by infrarenal aortic banding without subsequent RAAS activation or when ALDO treatment is combined with a low-Na^+ diet or when 1 % NaCl alone is given, and (c) the prevention of fibrosis with either a small (nondepressor) or large (depressor) dose of Spiro, an ALDO receptor antagonist, which respectively fails to or does prevent hypertension. Furthermore, an intracerebroventricular infusion of a mineralocorticoid receptor antagonist prevents hypertension, but not fibrosis [24], whereas upregulation of ALDO synthase in the heart accounts for increased tissue levels of ALDO, but is not accompanied by fibrosis [25]. Thus, the cumulative evidence gathered to date indicates that the adverse myocardial remodeling during ALDOST is both independent of hypertension and unrelated to plasma or tissue-derived ALDO per se. Therefore, another circulating factor which accompanies aldosteronism must be operative (vide infra).

7.2.3 Hypercalciuria and Hypermagnesuria in Aldosteronism

Metabolic studies demonstrated an early and persistent elevation in urinary and fecal excretion of Ca^{2+} and Mg^{2+} in rats during ALDOST [26, 27]. The marked loss of Ca^{2+} and Mg^{2+} was evident throughout weeks 1–4 of ALDOST and involved both renal and gastrointestinal sites of excretion, each of which are sites of high-density ALDO receptor binding [28]. Hypercalciuria accompanies the short-term treatment of man or animals with a mineralocorticoid plus dietary salt [29–34]. It is present in

patients with primary aldosteronism, where it is accentuated by dietary Na^+ loading [33, 35]. The increment in urinary Ca^{2+} excretion, which occurs in the distal segment of the nephron, is dietary Na^+-dependent. Urinary Mg^{2+} excretion also rises with dietary Na^+ loading in rats treated with a mineralocorticoid, while the hypermagnesuria found in patients with primary aldosteronism is abrogated by either Spiro or surgical removal of diseased adrenal tissue [36–38]. In rats receiving 4 weeks ALDOST, we found Spiro cotreatment to attenuate enhanced urinary and fecal excretion of each cation beginning at week 1 onward [26, 27].

7.2.4 Secondary Hyperparathyroidism

The sustained urinary and fecal loss of Ca^{2+} and Mg^{2+}, which accompanies ALDOST, leads to a progressive fall in plasma ionized concentrations of these divalent cations. Plasma ionized $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ were markedly reduced at week 4, while each of these cations had already begun to fall at weeks 1 and 2 ALDOST. Ionized hypocalcemia and hypomagnesemia each regulate parathyroid hormone (PTH) secretion [39, 40]. Bone and its mineral stores are the primary reserve for these cations with bone resorption facilitated by PTH. Elevations in PTH, elaborated in response to reduced plasma ionized $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$, are expected in the setting of sustained hypercalciuria and hypermagnesuria. We indeed found elevated plasma PTH levels, consistent with secondary hyperparathyroidism (SHPT), during weeks 1–4 ALDOST. Both serum ionized $[Ca^{2+}]_o$ and total plasma Ca^{2+} are reduced in response to deoxycorticosterone/salt treatment (DOCST) [34], together with increased serum PTH and urinary excretion of cAMP, a biomarker of parathyroid activity. SHPT has been reported in patients with primary aldosteronism [38, 41–43] where accompanying aberrations in serum ionized and total Ca^{2+}, together with elevations in PTH, were normalized by either adrenal surgery or Spiro [38, 43].

Our second major finding, and further evidence in keeping with persistent SHPT, was the marked and progressive reduction in bone mineral density and content of tibia and femur that appeared by week 4 ALDOST and became more evident at weeks 5 and 6. This fall in bone mineral density was rapid and accompanied by a corresponding reduction in bone strength. Urinary hydroxyproline, a marker of bone resorption, was increased during ALDOST or DOCST [44], while the hypercalciuria seen with 8 % NaCl loading alone was likewise accompanied by SHPT with a loss of bone Ca^{2+} and Mg^{2+} and increased urinary excretion of various markers of bone resorption [45–47].

7.3 A Molecular Pathway Leading to Cardiomyocyte Necrosis

A molecular mechanism accounting for cardiomyocyte necrosis and subsequent scarring of myocardium found at 4 weeks ALDOST was identified and the pathogenic role of circulating hormone elucidated.

7.3.1 A Proinflammatory Phenotype

Evidence of a proinflammatory phenotype and oxidative stress in the myocardium is found at 4 weeks ALDOST [15, 48–51]. This includes the presence of 3-nitrotyrosine, a by-product of the reaction involving superoxide and nitric oxide; an activation of the $gp91^{phox}$ subunit of NADPH oxidase found in inflammatory cells invading the injured myocardium that contributes to superoxide generation; upregulated redox-sensitive nuclear transcription factor-κB and a proinflammatory gene cascade it regulates that includes intercellular adhesion molecule-1, monocyte chemoattractant protein-1, and tumor necrosis factor-alpha; and increased tissue levels of 8-isoprostane and malondialdehyde, biomarkers of lipid peroxidation [15, 51]. There is also considerable evidence of oxidative stress in blood and urine consistent with the systemic nature of an altered redox state during chronic aldosteronism. This proinflammatory phenotype during ALDOST was prevented by cotreatment with either Spiro or *N*-acetylcysteine, an antioxidant.

7.3.2 Intracellular Ca^{2+} Overloading and SHPT

Our hypothesis for the induction of oxidative stress during ALDOST draws upon Albrecht Fleckenstein's original concept that intracellular Ca^{2+} overloading is an integral pathophysiologic feature leading to myocardial necrosis [52]. In rats receiving 1 and 4 weeks ALDOST, we monitored intracellular Ca^{2+} in cardiomyocytes and mitochondria harvested from the heart. We found increased Ca^{2+} levels in the myocardium during preclinical, clinical, and pathologic stages of ALDOST, accompanied by biomarker evidence of oxidative stress that included increased levels of malondialdehyde and 8-isoprostane and increased H_2O_2 production [14, 27, 51, 53].

The calcium-sensing receptor of the parathyroid glands responds to hypocalcemia with increased secretion of PTH. Accordingly, plasma PTH levels were elevated at weeks 1–4 ALDOST [27] with SHPT as evidenced by bone resorption [26]. We therefore hypothesized that the intracellular Ca^{2+} overloading and induction of oxidative stress that accompanies ALDOST leading to cardiomyocyte necrosis and fibrosis are mediated by the calcitropic hormone, PTH, and not ALDO. This represents an example of the SHPT-associated Ca^{2+} paradox as characterized by Fujita and Palmieri [54]. The elegant studies of Massry and coworkers have demonstrated PTH-mediated intracellular Ca^{2+} overloading of cardiomyocytes that included cardiac myocytes incubated with PTH [55], cells harvested from normal rats receiving a 2-week infusion of PTH, and rats with chronic renal failure having SHPT [56]. In each case, cotreatment with verapamil, a Ca^{2+} channel blocker, prevented the rise in intracellular Ca^{2+}. PTH also regulates cardiomyocyte Ca^{2+} channel opening [57]. Using radiolabeled PTH, Nordquist and Palmieri found that PTH not only penetrates plasmalemma but also localizes within the cytoplasm of renal tubular cells

and predominantly in mitochondria, where its mechanism of transport and function remain to be fully elucidated [58]. Rasmussen and others found PTH to alter mitochondrial $[Ca^{2+}]_m$ and respiration [59–63]. Excessive intracellular Ca^{2+} accumulation in cardiomyocytes and consequent generation of reactive oxygen species (ROS) alter intracellular signaling events, including their perpetuation of intracellular Ca^{2+} overloading via L-type Ca^{2+} channel entry and inhibition of Ca^{2+} efflux by Ca^{2+}-ATPase [64, 65]. A Na^+/Ca^{2+} exchanger involved in regulating Na^+-dependent Ca^{2+} efflux from mitochondria may also be contributory [66, 67].

7.3.3 Mitochondria-Based Oxidative Stress

PTH-mediated intracellular Ca^{2+} overloading is coupled to induction of oxidative stress in diverse tissues that includes cardiomyocytes and their mitochondria. The generation of ROS and reactive nitrogen species appears to overwhelm their rate of detoxification by the cumulative capacity of antioxidant defenses. Together with lost ATP synthesis, Ca^{2+} overloading and oxidative stress of mitochondria lead to a nonphysiologic opening of their mitochondrial permeability transition pore (mPTP). The ensuing osmotic-based structural and functional degeneration of these organelles triggers the downhill final common cell death pathway leading to cardiomyocyte necrosis and subsequent replacement fibrosis [68].

7.3.4 The Cellular–Molecular Cascade to Cardiomyocyte Necrosis

A series of site-directed, sequential pharmacologic interventions targeted along the cellular–molecular cascade was used to block downstream events leading to cardiomyocyte necrosis. These observations collectively validated our hypothesis regarding the pathologic sequelae of events leading to adverse structural remodeling of myocardium in rats with chronic aldosteronism. Various cotreatments were used. They included (1) Spiro, which attenuated the enhanced urinary and fecal losses of these cations to prevent hypocalcemia and hypomagnesemia and thereby ensuing SHPT [27]; (2) a Ca^{2+}- and Mg^{2+}-supplemented diet, together with vitamin D_3 to enhance Ca^{2+} absorption, which prevented hypocalcemia and SHPT [69]; (3) parathyroidectomy prevented SHPT [70] and vascular lesions and the rise in aortic tissue Ca^{2+} content during DOCST [71]; (4) cinacalcet, a calcimimetic that resets the threshold of the parathyroid glands' Ca^{2+}-sensing receptor to prevent SHPT despite hypocalcemia [72]; (5) amlodipine, a Ca^{2+} channel blocker, which prevents intracellular Ca^{2+} overloading [53]; and (6) *N*-acetylcysteine, an antioxidant that abrogated oxidative stress [15].

Taken together, the multitude of evidence gathered to date congruently supports that PTH-mediated intracellular Ca^{2+} overloading is the most tenable mechanism

that leads to the induction of oxidative stress during aldosteronism where ROS, primarily derived from mitochondria in cardiomyocytes and NADPH oxidase in vascular tissue, overwhelm cellular antioxidant defenses. This scenario begs the question whether the overall consequence of an excessive generation of prooxidants or cumulative endogenous antioxidant defenses in combating ROS was compromised.

7.3.5 Subsarcolemmal Mitochondria-Targeted Cardioprotection

The signal-transducer–effector pathway leading to necrotic cell death during ALDOST revolves around intramitochondrial Ca^{2+} overloading, together with an induction of oxidative stress and opening of the mPTP. To further validate this concept, we hypothesized mitochondria-targeted interventions would prove cardioprotective. Accordingly, 8-week-old male Sprague–Dawley rats receiving 4 weeks ALDOST were cotreated with either quercetin, a flavonoid with mitochondrial antioxidant properties, or cyclosporine A, an mPTP inhibitor, and compared to ALDOST alone and untreated, age-/sex-matched controls (see Fig. 7.1). We monitored mitochondrial free Ca^{2+} and biomarkers of oxidative stress, including 8-isoprostane and H_2O_2 production; mPTP opening; total Ca^{2+} in cardiac tissue; collagen volume fraction (CVF) to quantify replacement fibrosis, a biomarker of cardiomyocyte necrosis; and employed TUNEL assay to address apoptosis in coronal sections of ventricular myocardium. Compared to controls, at 4 weeks ALDOST, we found a marked increase in mitochondrial H_2O_2 production and 8-isoprostane levels, a greater propensity for mPTP opening, and higher concentrations of mitochondrial free $[Ca^{2+}]_m$ and total tissue Ca^{2+}, coupled with a fivefold rise in CVF without any TUNEL-based evidence of cardiomyocyte apoptosis. Each of these pathophysiologic responses to ALDOST was prevented by quercetin or cyclosporine A cotreatment. Thus, mitochondria play a central role in initiating the cellular–molecular pathway that leads to necrotic cell death and consequent myocardial scarring. This destructive cycle can be interrupted and myocardium salvaged with its structure preserved by mitochondria-targeted cardioprotective strategies.

7.4 Summary and Conclusions

Intracellular Ca^{2+} overloading accompanies inappropriate (relative to dietary Na^+) elevations in PAC, termed aldosteronism, and leads to a prooxidant/proinflammatory phenotype involving the heart. The dual deleterious consequences of elevated PAC and dietary Na^+ are linked to the induction of oxidative stress via PTH-mediated intracellular Ca^{2+} overloading. The genesis of this SHPT occurs in response to plasma ionized hypocalcemia and hypomagnesemia whose appearance is a consequence of marked urinary and fecal excretory losses of these divalent cations that accompany chronic aldosteronism.

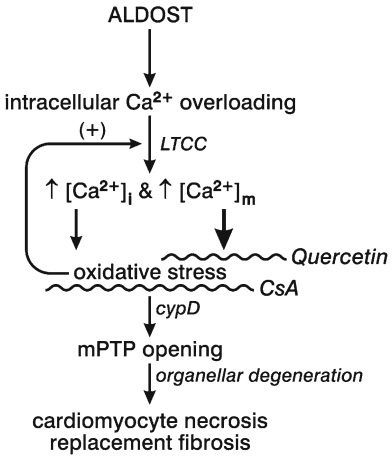

Fig. 7.1 A central hypothesis representing the pathophysiologic sequence of events in chronic aldosterone/salt treatment (ALDOST) that perpetuate cardiomyocyte necrosis with replacement fibrosis (or scarring). Mediated via L-type Ca^{2+} channels (LTCC), intracellular Ca^{2+} overloading of cardiomyocytes, including cytosolic $[Ca^{2+}]_i$ and mitochondrial $[Ca^{2+}]_m$ components, leads to the induction of oxidative stress and generation of reactive oxygen species predominantly by mitochondria (*heavy arrow*) and which feeds back to perpetuate LTCC activity. This Ca^{2+} overloading-mediated redox injury pathway involves the pathologic opening of the mitochondrial permeability transition pore (mPTP)-cyclophilin D (cypD) complex. The ensuing osmotic swelling of these organelles and their degeneration, coupled with impaired mitochondrial generation of high energy phosphates, leads to necrosis and subsequent wound healing with fibrous tissue formation. Quercetin serves as a potent mitochondria-targeted antioxidant, whereas cyclosporine A (CsA) inhibits the opening of the mPTP (Reprinted with permission from Shahbaz AU, Kamalov G, Zhao W, Zhao T, Johnson PL, Sun Y, Bhattacharya SK, Ahokas RA, Gerling IC, Weber KT. Mitochondria-targeted cardioprotection in aldosteronism. *J Cardiovasc Pharmacol*. 2011;57:37–43)

Sustained Ca^{2+} overloading has its pathologic consequences. In the case of cardiac myocytes and their mitochondria, intracellular Ca^{2+} overloading leads to an induction of oxidative stress and opening of the mPTP with ensuing organellar destruction and cellular necrosis with subsequent wound healing response leading to a replacement fibrosis or scarring.

Pharmaceutical and/or nutriceuticals used as targeted interventions of the cellular and molecular pathway leading to excessive intracellular Ca^{2+} accumulation protect against adverse myocardial remodeling and hence are cardioprotective. These cumulative salutary responses raise the prospect that therapeutic interventions, capable of favorably influencing extra- and intracellular Ca^{2+} homeostasis and blocking the MSTE pathway, could potentially optimize the management of the secondary aldosteronism of CHF and patients with primary aldosteronism.

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Chapter 8
The ACE2/Ang (1–7) Pathway in Cardiac Remodeling Due to Pressure Overload

Seyyed M.R. Kazemi-Bajestani, Vaibhav B. Patel, Wang Wang, and Gavin Y. Oudit

Abstract The renin–angiotensin system (RAS) is a critical regulator of cardiovascular and renal functions, primarily through the angiotensin-converting enzyme (ACE)-mediated generation of angiotensin (Ang) II. Ang II mediates majority of its actions through Ang II type I receptor (AT1R) activation. Discovery of homolog of ACE, the ACE2, at the start of the twenty-first century, provided insight into the endogenous negative regulation of RAS. ACE2, a type I transmembrane protein, is a carboxypeptidase which converts Ang II into Ang 1–7. ACE2 has shown blood pressure-lowering effects, largely via reduction of Ang II-mediated pressor effects, along with the suppression of pathological cardiac remodeling. Ang 1–7 acts through its receptor, Mas receptor, and shows antiproliferative, anti-fibrotic, and thus anticardiac remodeling effects. ACE2 also converts Ang I into Ang 1–9, which is recently known to possess antiproliferative effects via AT2R activation. This chapter largely focuses on pathological remodeling as a result of pressure overload, role of Ang II/AT1R axis in the remodeling process, and negative regulation of RAS by ACE2/Ang 1–7/Mas receptor and Ang 1–9/AT2R axis.

Keywords Renin–angiotensin system • ACE2 • Ang 1–7 • Pressure overload • Cardiac remodeling

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8.1 Renin–Angiotensin System: An Essential Regulator of Cardiovascular Function

The renin–angiotensin system (RAS) is an essential regulator of cardiovascular and renal functions. Enhanced angiotensin II (Ang II) formation, as a product of dysregulated RAS, in plasma and several organs including heart and kidney precedes various pathologic cardiovascular processes [1]. Elevated Ang II induces hypertension, myocardial hypertrophy, fibrosis, and diastolic dysfunction, which might be exacerbated by angiotensin-converting enzyme 2 (ACE2) deficiency [2]. The vasoconstrictor effect of Ang II is associated with development of hypertension/pressure overload and its cardiac hypertrophy/remodeling consequences [1]. Furthermore, local expression of Ang II in the heart is a critical mediator of pressure-overload-induced cardiac hypertrophy [3]. Ang II overexpression can also mediate kidney inflammation/fibrosis [4], increase thrombotic potential, augment release of aldosterone from the adrenal cortex, increase central neural secretion of antidiuretic hormone/adrenocorticotropic hormone, and elevate sympathetic tone which may aggravate the induced hypertension [5]. Ang II activates several myocardial hypertrophic/fibrosis signaling pathways, including mitogen-activated protein kinase (MAPK), protein kinase C (PKC)α, PKCβ1, Janus Kinase 2 and signal transducer and activator of transcription 3 (JAK2-STAT3), and extracellular signal-regulated kinases (ERK) 1/2 [2, 6]. Increased inflammatory and fibrotic responses of myocardium to Ang II by increasing type I and III collagen, transforming growth factor (TGF)-β1, and tumor necrosis factor (TNF) α confirm the key role of Ang II in development of inflammation and subsequent fibrosis [7]. Ang II by induction of monocyte chemoattractant protein (MCP)-1 leads to maladaptive cardiac fibrosis [7]. Ang II induces cardiomyocyte apoptosis which is mediated via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation through p38 MAPK activation, decrease in B-cell lymphoma 2(Bcl-2) protein, and caspase activation [8]. In the presence of Ang II, p38 MAPK activation is mediated by reactive oxygen species (ROS)-dependent Ca(2+)/calmodulin-dependent protein kinase (CaMK)II activation [9].

Ang II by enhancement of cardiac inflammation, apoptosis, fibrosis, hypertrophy, and ROS production leads to remodeling and dysfunction. ACE2 is an important enzymatic component of RAS which causes degradation of Ang II into Ang 1–7. Degradation of Ang II by ACE2 can apparently attenuate vascular homeostasis through the continuous control of vascular tone and constitutive inhibition of Ang II-mediated cardiomyopathic effects [1]. ACE2 deficiency augments Ang II-mediated oxidative stress/inflammation and subsequent age-dependent cardiomyopathy [10]. Ang 1–7 can be generated both from Ang I and Ang II and can offset the effects of Ang II [11]. All consequent ligands of RAS may affect through three different receptors: MasR, AT1R, and AT2R. Ang 1–7 function through its receptor, Mas, composes vasodilatory, antiproliferative, and antihypertrophic peptide. Genetic and pharmacological augmentation of ACE2/Ang 1–7/Mas axis seems an endogenous counter-regulatory pathway that results in improvement of heart failure.

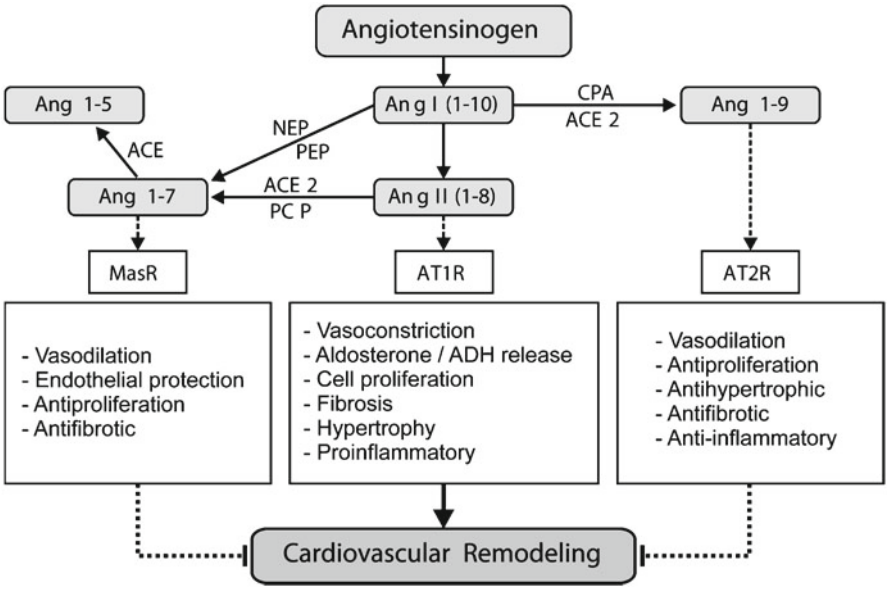

Fig. 8.1 The enzymatic cascade involved in the renin–angiotensin system, key receptor systems, and the biological effects mediated by Ang II, Ang 1–7, and Ang 1–9. *Solid lines*, enzymatic pathways; *broken lines*, peptide agonist interacting with its key receptor. *Bold line*, stimulatory effect; *dotted lines*, inhibitory effects; *ACE2* angiotensin-converting enzyme 2, *ADH* antidiuretic hormone, *Ang* angiotensin, *APA* aminopeptidase A, *AT1R and AT2R* Ang II type 1 and type 2 receptor, respectively; *CPA* carboxypeptidase A, *Mas R* Ang 1–7 receptor, *NEP* neutral endopeptidase, *PCP* prolyl carboxypeptidase (also known as angiotensinase C), *PEP* prolyl endopeptidase

Vasoconstrictive, proliferative, hypertrophic, fibrotic, and proinflammatory effects of Ang II are mediated by AT1R activation [1].

In addition of the effects of ACE2 on conversion of Ang II to Ang 1–7, Ang 1–9 also can be produced in the RAS system through the affects of ACE2 on Ang I [1]. Recently, it has been shown that Ang 1–9 can antagonize pro-hypertrophic signaling in cardiomyocytes via the AT2R activation [12] (Fig. 8.1).

8.2 Pressure Overload Induces Cardiac Hypertrophy/ Remodeling: Role of RAS

Pressure overload results in mechanical stress, which is transduced into intracellular signals that regulate gene expression and protein synthesis in cardiac myocytes [13, 14]. Pressure overload induces cardiac hypertrophy by increasing the expression of proto-oncogenes (c-fos and c-myc) [13]; growth-stimulating substances [15]

enhanced phosphatidylinositol turnover and activation of MAPK subsequent to PKC [16]. Mice with reduced cardiac transcription factor CCAAT/enhancer-binding protein (C/EBPβ) levels showed considerable resistance to cardiac failure upon pressure overload [17]. Downregulation of ERK1/2, protein kinase B (Akt), and glycogen synthase kinase (GSK)-3β is involved in the transition from pressure-overload compensated hypertrophy to heart failure [18].

RAS activation plays a key role in pressure-overload-induced cardiac hypertrophy [19]. Ang II, especially when it is expressed locally, is an essential mediator of pressure-overload-induced cardiac hypertrophy. In a pressure-overload model, local expression of Ang II leads to cardiac hypertrophy by upregulating NADPH oxidase expression and promoting ROS synthesis [20]. Antagonizing Ang II receptors results in regression of hypertensive left ventricular hypertrophy and inhibits the intracellular signaling pathway of stretch-mediated cardiomyocyte hypertrophy [21]. A considerable analogy exists between the signaling pathways that mediate cardiac hypertrophy induced by pressure overload and those induced by Ang II. Either Ang II or pressure-overload mechanical stress activates PKC. PKC activation in these parallel process increases the activity of MAPK, which subsequently induces c-fos mRNA expression. Accumulation of these signaling pathways eventually lead to augmented protein synthesis in cardiac myocytes/hypertrophy [21, 22].

Pressure overload induces mechanical stress, which leads to cardiac hypertrophy through AT1R independently of Ang II [23]. AT1R is considered as a "mechanical sensor" and converts mechanical stress into biochemical signals inside the cells [23]. AT1R is involved in the mechanical stress-induced activation of ERKs and phosphorylation of Jak2 [23]. Mechanical stress evoked by activation of AT1R also induces cardiac hypertrophy through calcineurin, a Ca^{2+}-dependent phosphatase, pathway independent of Ang II effects [24]. Furthermore, activating transcription factor 3 (ATF3) plays a crucial role in development of either Ang II or pressure-overload-induced cardiac hypertrophy [25]. The effects of ATF3 on cardiac remodeling are mediated by negative feedback to the ERK and C-jun-N-terminal kinase (JNK) pathways and regulation of pro-fibrotic cytokines [26] (Fig. 8.2).

Oxidative stress and inflammation play important roles in development of activated RAS-induced cardiac remodeling. Pressure overload combined with ACE2 deficiency results in activation of Ang II-stimulated signaling pathways in myocardium which is associated with increased expression and phosphorylation of $p47^{phox}$, NADPH oxidase activity, and superoxide generation, leading to enhanced matrix metalloproteinase (MMP)-mediated degradation of the extracellular matrix [27]. Pressure-overload-induced Ang II elevation augments cardiovascular inflammation through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and the subsequent production of inflammatory mediators like interleukin (IL)-6 and IL-1β, which leads to exacerbated hypertension [28].

Impaired RAS is associated with hypertension development. Plasma levels of Ang II in hypertensive patients are considerably higher than control individuals [29]. Infusion of Ang II for 14 days leads to significant increase in peak systolic blood pressure over vehicle-infused wild-type mice [2]. According to animal models, it is clear that a pressure overload trigger like transverse aortic constriction is a

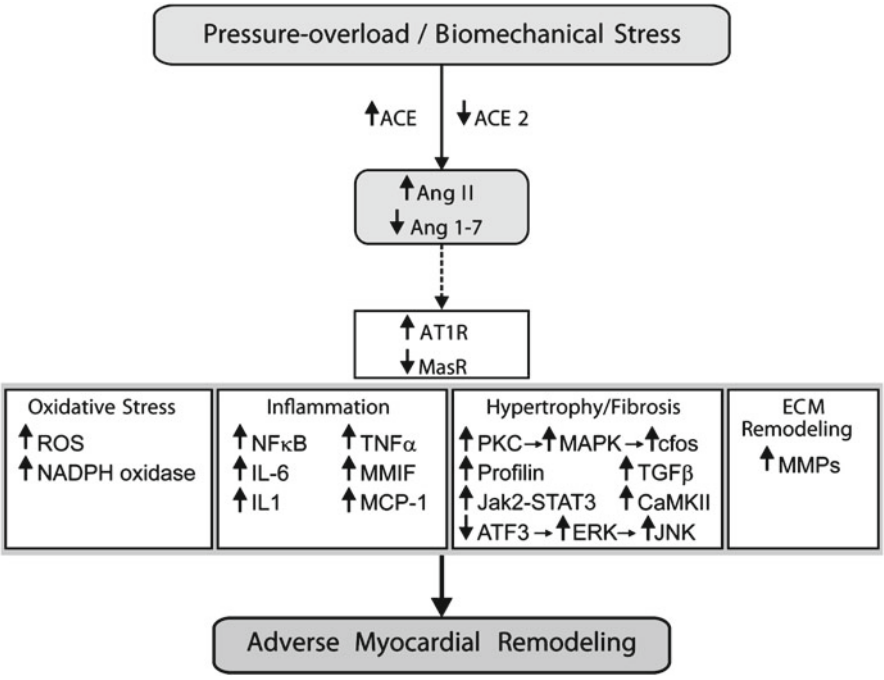

Fig. 8.2 Pressure-overload-induced Ang II signaling pathways which result in adverse myocardial remodeling. *Solid line*, enzymatic pathway; *broken lines*, peptide agonist interacting with its key receptor. *Bold line*, stimulatory effect; *ACE* angiotensin-converting enzyme, *AT1R* Ang II type 1 receptor, *ATF3* Activating transcription factor 3, *CaMKII* Ca^{2+}/calmodulin-dependent protein kinases II, *ERK* extracellular signal-regulated kinase, *IL-6* interleukin-6, *JAK2-STAT* Janus Kinase 2-signal transducer and activator of transcription system, *JNK* C-jun-N-terminal kinase, *MAPK* mitogen-activated protein kinase, *MasR* Ang 1–7 receptor, *MCP-1* monocyte chemoattractant protein 1, *MMIF* macrophage migration inhibitory factor, *MMP* matrix metalloproteinase, *NADPH* nicotinamide adenine dinucleotide phosphate, *NFκB* nuclear factor kappa-light-chain-enhancer of activated B cells, *PKC* protein kinase C, *ROS* reactive oxygen species, *TGFβ1* transforming growth factorβ1, *TNF α* tumor necrosis factor α

primary factor of Ang II increase. However, in humans, it is not apparent that Ang II elevation is a cause or consequence of hypertension. Long-term treatment of the hypertensive patients with Ang II antagonists is associated with lower risk of clinical events [30]. Ang II, as a cause or consequence of hypertension, is a peptide which worsens the cardiovascular status of the patients and may aggravate the cardiac consequences of hypertension.

Various regulator components of RAS have been targeted in recent decades for hypertension treatment. Direct renin inhibition has been proposed to be a comprehensive RAS inhibitor by suppression of the plasma renin activity at its rate-limiting step [31]. For several years, ACE inhibition has been recognized to reduce blood pressure and offset hypertension consequences. ACE inhibitor can inhibit Ang I to

Ang II conversion and therefore prevents the cardiovascular side effects of Ang II. Chronic ACE inhibition decreases the extent of left ventricular hypertrophy and improves cardiac function and survival [32]. ACE inhibition can limit both myocyte and interstitial remodeling despite ongoing cardiac pressure via blunting the induction of collagen I and III, laminin B, and fibronectin at both the mRNA and protein levels [33]. Angiotensin receptor blockers (ARBs) are other group of pharmaceuticals which can modulate RAS in hypertensive patients. Left ventricular hypertrophy can be prevented by ARBs in a pressure-overload model [34]. Gene polymorphism studies about critical components of RAS showed the possible association to the development of hypertension [35, 36] although some studies have not been able to show a strong dependency [37].

8.3 ACE2 Effects on Hypertension: Blood Pressure Reduction and Cardiac Remodeling Suppression

Treatment with ACE2 was found to significantly reduce blood pressure and attenuate cardiac remodeling in various hypertensive models [1, 38]. Reduced ACE2 mRNA and protein expression in hypertensive rats and association of ACE2 impairment with cardiac dysfunction was first reported in 2002 [39]. Generally, the possible mechanism for direct involvement of ACE2 in hypertension attenuation includes Ang II degradation to Ang 1–7. ACE2 antagonizes the pressor, hypertrophic, and pro-oxidative effects of Ang II by decreasing the levels of Ang II [2]. Increased Ang 1–7/Mas results in vasodilation, preserved endothelial function, and cardiac remodeling/fibrosis suppression [2]. Other evidence showed that decrease in the circulatory plasma Ang II was the driving cause of decreased hypertension coupled with a partial contribution from the increased levels of Ang 1–7 [40].

ACE2 not only use RAS substrate but has several other biological substrates. ACE2 as a monocarboxypeptidase can mediate other parallel reactions in apelin and bradykinin family which results in further antihypertensive/remodeling outcomes [1]. Recombinant human ACE2 (rhACE2) reduces Ang II-induced hypertension in mice, by reducing Ang II-mediated activation of enhanced ERK1/2, PKC pathways, and renal fibrosis [2]. ACE2 can decrease Ang II-induced expression of profilin-1 and its subsequent hypertrophic signaling, MAPK, ERK1/2, and JNK, in an Ang 1–7-dependent manner. Overexpression of ACE2 and decreased profilin-1 levels results in suppression of ERK1/2 and JNK phosphorylation in aortas of spontaneously hypertensive rats [41]. In response to pressure overload, activation of MAPK is found to be more in mice with ACE2 deficiency compared to those with intact ACE2. Cardiomyocytes MAPK response to Ang II is considerably enhanced in lack of ACE2 [42]. In a pressure-overload model, ACE2 provides therapeutic benefits against pathological myocardial remodeling as evident by the levels of pathological hypertrophy markers: α-skeletal actin, brain natriuretic peptide, β-myosin heavy chain, and procollagen type Iα1 and procollagen type IIIα1 [2].

In the pressure-overload context, ACE2 is a factor which can attenuate biomechanical stress-induced NADPH oxidase activation, superoxide generation, and enhanced MMP-mediated degradation of cardiac extracellular matrix [27]. It can be concluded that ACE2 is an important enzyme which can decrease the susceptibility of myocardium to mechanical stress. It is important to mention that most of the animal models of pressure overload only investigated the pure effect of pressure overload on RAS system; however, in humans, the process of hypertension is more complex. Hypertension is a multifactorial disease and is due to genetic and environmental determinants [43]. Combination of several affective factors, including atherosclerosis, on hypertension makes the interpretation complicated. RAS predominantly regulates blood pressure, endothelial function, and atherosclerosis, three essential risk factors of cardiovascular diseases and heart failure [1]. The effects of ACE2 on attenuation of these exacerbating factors may attenuate the consequences of hypertension and prevent cardiac remodeling. ACE2 can suppress Ang II- induced inflammation by reducing macrophage migration inhibitory factor [44] and T-lymphocyte-mediated inflammation [4]. ACE2 deficiency contributes to increased proinflammatory cytokines, including IL-6, MCP-1, and vascular cell adhesion molecule-1 [44].

8.4 Ang 1–7/Mas Axis in Pressure-Overload-Induced Heart Failure

Ang 1–7 is produced primarily by the enzymatic breakdown of either Ang I or Ang II. Ang 1–7 can be formed directly from Ang I by endopeptidases including neutral endopeptidase and prolyl endopeptidase. ACE2, as a carboxypeptidase, also can metabolize Ang II from its C terminus and produce Ang 1–7. Ang 1–7 by antagonizing Ang II results in beneficial vasodilation and anticardiac remodeling outcomes [1]. Vasodilator effect of Ang 1–7 in the heart is suggested to be dependent on vasodilator prostaglandins and nitric oxide (NO) release [45]. Evidence from in vivo and ex vivo studies in humans and several animal models shows a key role of Ang 1–7 in blood pressure modulation, probably through a combination of bradykinin and NO signaling [46].

Treatment of cardiomyocytes with Ang 1–7 attenuates Ang II-induced hypertrophy by modulating calcineurin/nuclear factor of activated T-cell signaling cascade via the NO/cyclic guanosine monophosphate pathway [47]. Therapeutic application of Ang 1–7 is limited due to its short half-life and rapid turnover; therefore, ACE2 is a good alternative with acceptable half-life which ultimately leads to Ang 1–7 over production in pathologic conditions [1]. Another limitation for Ang 1–7 supplementation is Mas receptor desensitization and internalization following Ang 1–7 stimulation [48].

Ang 1–7/Mas axis may offset Ang II pathologic effects through other diverse mechanism. Mas is considered to be a physiological antagonist of the Ang II AT1R [49]. Ang 1–7 showed beneficial effects in several experimental models of cardiovascular

disease, including attenuating the pressure-overload-induced heart failure [50]. Ang 1–7 can reduce hypertension-induced cardiac remodeling through a direct effect on the heart. Interestingly, Ang 1–7 showed selective effects on intracellular signaling pathways that mediate cardiac remodeling. Ang 1–7-mediated effects in the heart result in significant decrease in phosphorylation of c-Src and p38 kinase seen after Ang II infusion [50]. Ang 1–7 upregulates MAPK phosphatase dual-specificity phosphatase in association with a decrease in Ang II-mediated phosphorylation of ERK1/2 [51]. Treatment of the spontaneously hypertensive rats with Ang 1–7 showed attenuation of elevated mean arterial pressure and significant increase in MMP-2 mRNA expression. Furthermore, increased tissue inhibitor of metalloproteinases (TIMP-1) mRNA expression in left ventricles of hypertensive rats was significantly attenuated by Ang 1–7 [52]. This novel effect of Ang 1–7 on normalization of expression of pro-fibrotic factors and enhancement of anti-fibrotic factors shows the beneficial effects on recovery of pressure-overload-induced remodeling.

The effect of Ang 1–7 treatment on upregulation of cardiac nitric oxide synthase (NOS) in hypertensive state can be considered as another cardioprotective mechanism of Ang 1–7 against pressure overload [53]. In an ACE2-deficient state, supplementation with Ang 1–7 suppresses the increased NADPH oxidase activity, resulting in rescue of the early dilated cardiomyopathy and normalization of the atrial natriuretic factor, B type natriuretic peptide (BNP), and α-skeletal actin in a pressure-overload model [27]. Interestingly, blocking the Ang II/AT1R axis with irbesartan or enhancing Ang 1–7/Mas receptor axis via Ang 1–7 treatment in pressure-overloaded ACE2-deficient mice showed similar degree of cardioprotection against pressure-overload-induced cardiac remodeling. This illustrated that, there is a marked degree of redundancy in Ang II/AT1R axis and Ang 1–7/Mas receptor axis, counter-regulatory pathways, in ACE2-deficient environment and Ang 1–7 can be a potential therapeutic regimen to counteract cardiac remodeling [54] (Fig. 8.3).

8.5 Ang 1–9/AT2R: Another Valuable Product of ACE2 Activity in RAS

Recently, an interesting role of Ang 1–9 in reduction of cardiac fibrosis and altered aortic contraction in hypertensive rats via the AT2R has been shown [55]. Ang 1–9 supplementation did not affect the blood pressure, cardiac function, or left ventricular mass index; however, Ang 1–9 reduced cardiac fibrosis by 50% through modulating collagen I expression via AT2R mediation and improved NO bioavailability in this model [55]. Application of a selective AT2R inhibitor could significantly increase the MMP-14 gene expression in hypertensive hearts [55]. Activation of intracellular signaling pathway Rho A and its Rho kinase [Rho-associated, coiled-coil containing protein kinase (ROCK)] pathway is a novel mechanism of vasoconstriction and cardiovascular remodeling in hypertensive patients [56]. ROCK inhibition results in reduced systolic blood pressure and increased plasma and aortic ACE2 enzymatic activity, aortic NOS mRNA levels, and Ang 1–9 plasma levels.

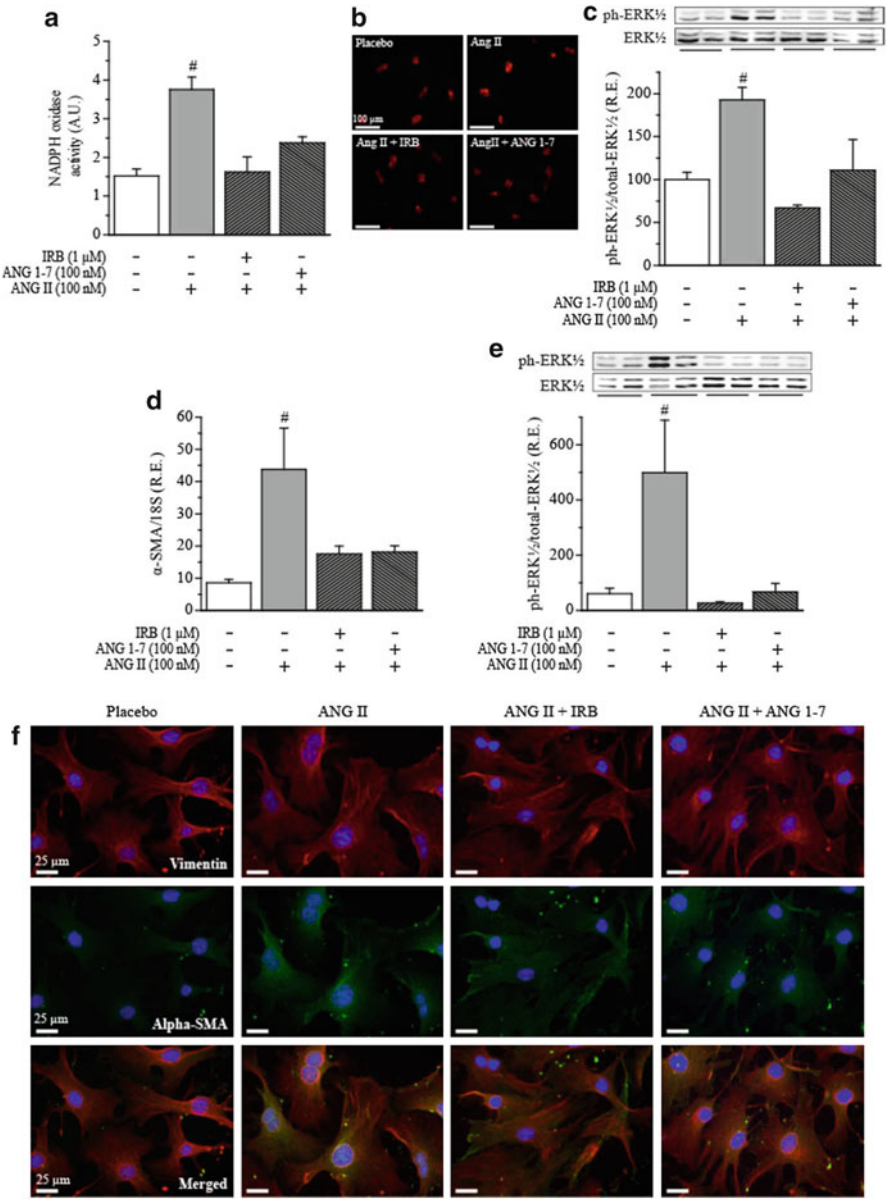

Fig. 8.3 Irbesartan (IRB), AT1R blocker, and Ang 1–7 suppresses oxidant stress, signaling, and remodeling induced by Ang II in ACE2-null cardiomyocytes and cardiofibroblasts isolated from pressure-overloaded hearts. Ang II increases oxidant stress, as evident by increased NADPH oxidase activity (**a**) and DHE fluorescence (**b**) along with phosphorylation of ERK 1/2 (**c**) in isolated cardiomyocytes which were lowered by IRB and Ang 1–7 pretreatment. In cardiofibroblasts, Ang II exposure stimulated mRNA expression of α-SMA (**d**) with increased phosphorylation of ERK1/2 (**e**) resulting in increased immunostaining for α-SMA (**f**) which was prevented by pretreatment with IRB and Ang 1–7 (**d**–**f**). *A.U.* arbitary unit, *R.E.* relative expression, *ERK ½* extracellular signal-regulated kinase 1/2, *α-SMA* alpha-smooth muscle actin. #$p < 0.05$ compared to all other groups (reproduced with permission [54])

ROCK inhibition also leads to decreased aortic mRNA levels of TGF-β1, plasminogen activator inhibitor, and MCP-1 as well as decrease aortic NADPH oxidase activity and ROS production [57]. This increased levels of ACE2 activity in accordance with elevated Ang 1–9 as well as decreased levels of hypertrophic mediators reveal the beneficial effects of Ang 1–9 as an indicator of ROCK inhibition.

The beneficial signaling pathways against cardiovascular remodeling that are affected by Ang 1–7 or Ang 1–9 are similar. It is not clear that which of these peptides will be produced predominantly by ACE2 effects in hypertensive patients. Further studies may elucidate this point that can be very helpful in clinical settings, because then we can augment the ACE2 effects by adding another factor which boosts either Ang 1–9 or Ang 1–7 production. However, in the myocardial infarction model, this comparison has been performed. Administration of Ang 1–9 after myocardial infarction prevents the increase in plasma Ang II levels, left ventricular ACE activities, and cardiac myocyte hypertrophy development. These effects are not mediated by Ang 1–7 or the MasR. In experimental myocardial infarction models, increased Ang 1–9 levels were associated with attenuation of left ventricular hypertrophy even after adjustment for blood pressure reduction [58]. In summary, Ang 1–9, as a product of ACE2 activity, is produced from Ang I. Furthermore, increased Ang 1–9 may have a feedback effect on ACE activity which can prevent Ang II production in parallel. Ang 1–9 supplementation not only neutralizes the pressure-overload-induced fibrosis but also shows anti-ischemia-induced cardiac remodeling. Further studies need to be done to elucidate the effect of combination therapy of ACE2 with either Ang 1–9/AT2R agonists or Ang 1–7/MasR agonists in the pressure-overload models. Their probable feedback effects on the RAS component make the judgment complex at this time.

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Chapter 9
Local Actions of Natriuretic Peptides and Nitric Oxide in Cardiac Remodeling: Implications for Therapy

Michaela Kuhn and Hitoshi Nakagawa

Abstract Cardiac remodeling in response to sustained pressure overload involves cardiomyocyte hypertrophy and contractile dysfunction together with interstitial changes such as fibrosis and diminished capillary density. These changes are modulated by mechanical forces and factors secreted between and even within cells, exerting paracrine and auto-/intracrine actions. Among these factors are the natriuretic peptides atrial and B-type NPs (ANP and BNP, mainly released from myocytes), C-type NP (CNP, possibly secreted from fibroblasts), as well as nitric oxide (NO) (produced by different myocardial cell types). These factors, which signal through specific guanylyl cyclase receptors and intracellular cyclic GMP as second messenger, may act as key local antihypertrophic, antifibrotic, and proangiogenic factors during cardiac remodeling. This chapter will therefore summarize the insights obtained from experimental and clinical studies to highlight (1) natriuretic peptide- and NO-dependent modulation of myocyte growth and secretion of profibrotic factors, (2) the role of direct fibroblast actions of natriuretic peptides and NO during cardiac hypertrophy, (3) potential local proangiogenic actions of ANP and BNP, (4) alterations of these ligand/receptor systems in cardiac remodeling, and (5) potential therapeutical implications.

Keywords Cardiac remodeling • Natriuretic peptides • Nitric oxide • Cyclic GMP

Abbreviations

ANP Atrial natriuretic peptide
BNP B-type natriuretic peptide

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cGKI	cGMP-dependent protein kinase type I
CNP	C-type natriuretic peptide
GC	Guanylyl cyclase receptor
GC	Guanylyl cyclase
NO	Nitric oxide
PDE	Phosphodiesterase

9.1 Introduction

Cyclic guanosine 3′-5′-monophosphate (cGMP) is a ubiquitous intracellular signaling molecule that controls many physiological processes, from cell contractility, secretion, and permeability to cell differentiation, growth, and survival [1]. In mammals, cGMP can be generated from GTP by two families of guanylyl cyclases (GCs), intracellular nitric oxide (NO)-responsive GCs ("soluble" GCs), and transmembrane GCs ("particulate" GCs or pGCs; GC-A, -B, -C, -E, -F) [2]. GC-A, -B, and -C are receptors for extracellular peptide hormones; GC-A binds atrial and B-type natriuretic peptides (ANP with approximately tenfold higher affinity as BNP), GC-B binds C-type natriuretic peptide (CNP), and GC-C is stimulated by the intestinal peptides guanylin and uroguanylin. The activity of the retinal GC-E and GC-F instead is modulated by intracellular calcium-regulated proteins termed guanylyl cyclase activating proteins (GCAPs). These cytosolic proteins and their guanylyl cyclase receptors are specifically expressed in photoreceptor cells and they are essential to phototransduction and vision. In general, cyclic GMP exerts its actions through nucleotide-gated (CNG) ion channels, cGMP-dependent protein kinases (cGKs type I and II), and cGMP-regulated phosphodiesterases (PDEs) [3–5]. These different third messengers of cGMP are differentially expressed in different types of cells and operate via different effector proteins to modulate cell functions. Intracellular cGMP concentrations are controlled by the activities of these cGMP-forming receptors, as well as by the activity of cGMP-hydrolyzing phosphodiesterases (mainly PDE type 5; less PDEs 1, 2, and 9) [6].

Dysfunctions at various steps of the cGMP signaling cascade have been implicated in cardiovascular, neuronal, and skeletal diseases such as arterial hypertension, cardiac hypertrophy and insufficiency, metabolic syndrome, retinal degeneration, Alzheimer's disease, schizophrenia, and skeletal deformities [2, 7, 8]. Drugs that increase the intracellular cGMP concentration are used in the clinics, for instance, the NO-releasing organic nitrates for the treatment of angina pectoris or the PDE5 inhibitor sildenafil (Viagra®) for erectile dysfunction and pulmonary hypertension. (Pre) clinical studies showed that PDE5 inhibitors inhibit the immunosuppressive effects of inflammation; improve antitumor immunity, for example, in melanoma; and possibly improve cardiac function in patients with cardiac insufficiency. Hence, the cGMP signaling system comprises several potential targets for novel pharmacological therapies.

The critical role of the NO/sGC/cGMP and ANP/BNP/GC-A/cGMP systems in the regulation of arterial blood pressure is known for a long time. Importantly, a

recent genetic study showed that even subtle expression changes in components of these pathways (e.g., sGC, ANP, BNP) significantly influence blood pressure and cardiovascular disease risk in humans [9]. The main hypotensive paracrine action of endothelial NO is vasodilatation. In contrast, the cardiac peptides ANP and BNP exert much more pleiotropic hypotensive and hypovolemic endocrine actions which include the following: stimulation of renal function, vasodilatation, mildly enhanced endothelial albumin permeability in the microcirculation of the skin and skeletal muscle, increased fluid efflux from the intravascular to the lymphatic system within the spleen, inhibition of the renin–angiotensin–aldosterone (RAA) system by direct actions on juxtaglomerular cells and the adrenal glomerulosa, and central nervous effects that decrease salt appetite and water drinking [2, 8, 10].

While ANP and BNP expression is found primarily in the heart, CNP expression is more diffuse. Expression has been described in vascular endothelial cells, growth plate cartilage, uterus, ovaries, brain, and other tissues [11]. CNP does not circulate in the blood in appreciable amounts and therefore may act locally. The GC-B receptor is also expressed in many different tissues, in particular in vascular endothelial and smooth muscle cells, regions of brain and bone, and at high density in fibroblasts [11]. The most critical functions of CNP/GC-B have been unmasked by the severe consequences of genetic dysfunctions of this ligand/receptor system. Ablation of CNP, GC-B, or cGMP-dependent protein kinase II (PKG II) in mice all resulted in severe dwarfism as a result of impaired endochondral ossification [12–14]. In humans, homozygous GC-B mutations are the cause of acromesomelic dysplasia, Maroteaux type (AMDM), a severe form of autosomal recessive, short-limbed dwarfism with characteristic skeletal deformities [15]. These and other studies demonstrated that C-type "natriuretic peptide" in fact is unlikely to regulate renal sodium excretion under physiological conditions. Instead, it exerts important autocrine/paracrine GC-B-mediated modulatory effects on cellular proliferation and differentiation within the heart, during angiogenesis and fundamentally during bone formation.

Regarding the cardiovascular system, it is widely accepted that an *acute* increase in the intracellular cGMP concentration has beneficial short-term effects such as vasodilatation and inhibition of platelet aggregation, and the molecular mechanisms of these cGMP effects have been worked out in great detail. However, the long-term actions of cGMP on cell growth, differentiation, viability, and associated (patho)physiological processes are less well understood and controversially discussed. As described below, in experimental studies, cGMP *inhibits* pathological cardiomyocyte hypertrophy and proliferation/differentiation of cardiac fibroblasts. On the other hand, cGMP *stimulates* the regenerative proliferation of vascular endothelia or insulin-secreting pancreatic β-cells, and it protects cardiomyocytes from ischemic apoptosis. Hence, obviously natriuretic peptides and NO, via their cGMP-forming receptors, can exert opposite effects on cell growth and survival depending on the cell type, and even in the same cell, they might inhibit growth/proliferation or prevent cell death depending on the cGMP generators, effectors, and cGMP concentrations involved. The exact intracellular cascades that mediate these effects or that provoke alterations of cGMP signaling during diseases are only partly known.

The chapter focuses on the complex actions of natriuretic peptides and NO and their shared second messenger cGMP in the control of cell growth, differentiation, and survival in the heart, especially during the development of cardiac diseases provoked by pressure overload or after tissue ischemia. A great challenge in understanding cGMP's pleiotropic activities within and outside the heart is to improve our knowledge of the intracellular compartmentalization of cGMP signaling complexes and their functional relevance. Some answers to these questions have been provided by studies in various transgenic mouse models with conventional and conditional, cell-restricted deletions of specific cGMP generators (sGC and membrane GCs such as GC-A and GC-B), cGMP effectors (CNG channels, cGMP-dependent protein kinases), and their downstream targets. These mouse models were instrumental to getting new insights into cytoprotective versus deleterious cGMP signaling. The results of this experimental research could also identify new therapeutical indications (e.g., prevention of cardiac remodeling and diastolic dysfunction, improvement of postischemic angiogenesis) for known drugs that modulate the cGMP pathway, such as the NO-independent stimulators and activators of sGC or PDE5 inhibitors. Therefore, this chapter reviews the cardiac functions of sGC and the membrane GC receptors for natriuretic peptides (ANP, BNP, CNP), with special focus on the insights gained to date from genetically modified mice, the role of alterations of these ligand/receptor systems in cardiac remodeling, and potential therapeutical implications.

9.2 Monogenetic Mouse Models Indicate That Local Cardiac Functions of Natriuretic Peptides and NO Can Counteract Hypertensive Cardiac Remodeling

The cardiac pathological response to sustained pressure overload involves cardiomyocyte hypertrophy and contractile dysfunction along with interstitial changes such as fibrosis and altered capillary density. These changes are orchestrated by mechanical forces and factors secreted between and even within cells, exerting paracrine and auto/intracrine actions. Among these factors are the natriuretic peptides ANP and BNP (mainly released from myocytes), CNP (possibly secreted from fibroblasts), as well as nitric oxide (NO) (produced by different myocardial cell types), which may act as key local antihypertrophic, antifibrotic, and proangiogenic factors during cardiac remodeling. This section will therefore summarize the insights obtained from studies in genetic mouse models with global or conditional, cell-specific deletion of GC-A, GC-B, NO synthases, or cGMP-dependent protein kinase type I (cGKI) to highlight (1) natriuretic peptide- and NO-dependent modulation of myocyte growth and secretion of profibrotic factors (such as transforming growth factor beta, TGF-β, and connective tissue growth factor, CTGF), (2) the role of direct fibroblast actions of natriuretic peptides and NO during cardiac hypertrophy, and (3) potential local proangiogenic actions of ANP and BNP.

9.2.1 Atrial and B-Type Natriuretic Peptides and Their Common Guanylyl Cyclase-A Receptor

During chronic hemodynamic overload, the expression levels of ANP and especially BNP in the cardiac ventricles significantly increase [16]. Studies with cultured neonatal rat cardiomyocytes and cardiac fibroblasts suggested that NPs in this situation may act not only as circulating endocrine factors but also as local antihypertrophic (ANP) and antifibrotic (BNP) cardiac factors [17]. Indeed, mice with global deletion of ANP ($ANP^{-/-}$) or its GC-A receptor ($GC\text{-}A^{-/-}$) not only have chronic arterial hypertension and hypervolemia but also exhibit very pronounced cardiac remodeling [18, 19]. To test the hypothesis that NPs locally modulate cardiomyocyte growth in vivo, we generated mice with selective deletion of GC-A in cardiomyocytes through Cre-Lox recombination [20]. Already under resting conditions, these mice exhibit mild cardiomyocyte hypertrophy and elevation of known hypertrophy-marker genes. In addition, they respond to pressure overload (provoked by surgical transverse aortic constriction) or systemic Angiotensin II administration with exacerbated cardiac hypertrophy and fibrosis [20–23]. These observations were in line with studies of Patel and colleagues [23], showing that cardiac overexpression of a dominant-negative form of GC-A in transgenic animals also resulted in a modest accentuation of the hypertrophic response to pressure overload, together with a dramatically increased cardiac fibrotic response. Intriguingly, at difference to the $ANP^{-/-}$ mice, the cardiac phenotype of mice lacking the BNP gene was fibrosis, without cardiomyocyte hypertrophy [24]. Extending these observations, two later reports demonstrated that, in cultured cardiac fibroblasts, BNP stimulates selective matrix metalloproteinases that degrade collagen and suppress profibrotic extracellular matrix genes activated by TGF-ß [25, 26]. Hence, endogenous BNP might participate in an intramyocardial myocyte–fibroblast communication, modulating the secretory activity of fibroblast and extracellular matrix production.

Within the heart, the GC-A receptor is not only expressed in cardiomyocytes and fibroblasts but also and even at much higher density in coronary endothelial cells [27]. Various in vitro studies showed that ANP and BNP can stimulate proliferation and migration of cultured endothelial cells (such as human umbilical vein endothelial cells, HUVEC) and promote the migration and differentiation of endothelial progenitor cells (EPC), suggesting that these peptides exert proangiogenic actions [28, 29]. Postnatally, (patho)physiological angiogenesis is critical for tissue regeneration after ischemia but also during adaptive tissue growth. In particular, myocyte hypertrophy and angiogenesis are coordinately regulated during adaptive cardiac growth, which is at least in part due to the secretion of proangiogenic factors by cardiac myocytes [30]. Disruption of the balanced growth of myocytes and endothelia leads to contractile dysfunction and heart failure [30]. Based on these reports, we hypothesized that ANP and/or BNP, released from hypertrophic ventricular cardiomyocytes, stimulates proangiogenic responses of neighboring capillary endothelial cells. To follow this hypothesis, we generated mice with conditional, endothelial-restricted deletion of the GC-A receptor. Notably, these mice responded to pressure

overload (transverse aortic constriction) with myocardial capillary rarefaction, fibrosis, and diastolic dysfunction [31], suggesting that endogenous ANP and BNP indeed function as stress-responsive local regulators of angiogenesis in the murine hypertrophic heart.

These experimental studies taken together support an important autocrine/paracrine role for the cardiac ANP–BNP–GC-A signaling pathway, which moderates cardiomyocyte growth and fibroblast proliferation/differentiation, mildly stimulates coronary angiogenesis, and reduces extracellular matrix production. Thereby, ANP and BNP are "best friends of the heart," attenuating cardiac remodeling in chronic pressure overload or tissue ischemia [32].

9.2.2 C-Type Natriuretic Peptide and its cGMP-Forming Guanylyl Cyclase B Receptor

Whereas the (patho)physiological cardiac roles of ANP/BNP and their GC-A receptor have been widely studied, the cardiovascular functions of the CNP/GC-B system are less clear. Severe bone abnormalities and premature death of mice with global ablation of CNP or GC-B impeded detailed cardiovascular phenotyping [14]. To study the cardiac effects of endogenous CNP, Langenickel et al. [33] generated a transgenic rat model overexpressing a dominant-negative mutated GC-B receptor in cardiomyocytes. Already under baseline conditions, these animals displayed age-dependent but blood pressure-independent cardiac hypertrophy and tachycardia. These cardiac alterations were exacerbated by chronic cardiac volume overload. In line with these results, cardiomyocyte-restricted overexpression of CNP attenuated cardiac hypertrophy and inflammation induced by myocardial infarction in mice [34]. In addition to these modulating effects on cardiomyocyte growth, CNP might modulate myocyte contractile–relaxation functions. In isolated myocytes, the CNP/GC-B/cGMP pathway, via cGMP-dependent protein kinase I (cGKI), stimulated phosphorylation of the sarcoplasmic reticulum (SR) regulatory protein phospholamban, thereby enhancing $[Ca^{2+}]_i$ handling and contractility. In contrast, ANP had no effect [35].

Notably, the expression levels of CNP and of its cGMP-forming GC-B receptor are upregulated in failing hearts [36, 37]. Cardiac fibroblasts might be a local source for CNP in this situation [36]. Hence, endogenous CNP might participate in intramyocardial fibroblast–myocyte communication during chamber remodeling, modulating myocyte growth and contractility as well as inflammatory infiltration.

9.2.3 The Nitric Oxide/GC System

Cardiomyocytes express both the endothelial (NOS3) and neuronal (NOS1) isoforms of NO synthases [38]. NOS3 localizes to caveolae, where compartmentalization

with ß-adrenergic receptors and L-type Ca^{2+} channels allows NO to attenuate ß-adrenergic inotropy. NOS1, however, is targeted to SR, and NOS1-derived NO can stimulate SR Ca^{2+} release via activating the ryanodine receptor. Mice with global deletion of either NOS1 ($NOS1^{-/-}$) or NOS3 ($NOS3^{-/-}$) both develop age-related cardiac hypertrophy by 5 months, although only NOS3-deficient mice are hypertensive. These data suggest that NOS1-derived and NOS3-derived intracrine NO mediates opposite effects on cardiac contractile functions, but similar inhibitory effects on pathological cardiomyocyte growth [39]. These intracrine effects of NO in cardiomyocytes might involve cGMP production by sGC but also cGMP-independent signaling pathways, such as S-nitrosylation of target proteins [39].

9.3 Intracellular Pathways Mediating the cGMP-Dependent Cardiac Effects of Natriuretic Peptides or Nitric Oxide

9.3.1 Cardiomyocytes

The downstream signaling pathways which transduce the raises in myocyte cGMP levels in changes of myocyte growth and contraction/relaxation functions are much less clear than that leading to increased cGMP levels. At least two cGMP-stimulated proteins (as third messengers) are expressed in myocytes: cGMP-dependent protein kinase type I (cGKI) and phosphodiesterase (PDE) 2, a dual substrate esterase, which appears to hydrolyze cGMP under resting conditions but targets cAMP in the presence of ß-adrenergic stimulation [6]. Studies in cultured neonatal cardiomyocytes indicated that *cGKI* is the main third messenger activated by cGMP in cardiomyocytes, mediating antihypertrophic effects of locally produced natriuretic peptides or nitric oxide [40]. However, conclusive in vivo studies about the cardiac role of cGKI were missing for a long time because the cardiac expression level of this kinase is rather low. Even more, studies of cardiac hypertrophy in mice with global deletion of cGKI were hampered by their severe systemic phenotype, with gastrointestinal dysfunction and early lethality [41]. To circumvent these limitations, recently we generated mice with cardiomyocyte (CM)—restricted deletion (KO) of cGKI [35]. Absence of cGKI in myocytes did not alter baseline cardiac growth and function or cardiac hypertrophic and contractile responses of mice to chronic ß-adrenergic stimulation with isoproterenol. However, CM cGKI KO mice reacted to chronic Angiotensin II administration or chronic pressure overload (induced by surgical aortic constriction) with dilated cardiomyopathy, which was never observed in respective control littermates with unaltered cGKI expression levels. Overall, our data indicated that at least in mice, cGKI activation is involved in myocyte processes which prevent dilated cardiomyopathy and cardiac dysfunction in response to sustained pressure overload and/or neurohumoral stressors such as Angiotensin II.

Several proteins centrally involved in cardiomyocyte calcium transients and electromechanical coupling have been shown to be regulated by cGKI, such as the L-type Ca^{2+} channel (inhibition), phospholemman (inhibition), phospholamban (inhibition, resulting in enhanced SR calcium recycling by SERCA), troponin I (diminished Ca^{2+} sensitivity of sarcomeric myofilaments), and titin (improved ventricular diastolic distensibility) [42]. However, this section will focus on three specific targets of cGKI which are critically and primarily involved in the regulation of pathological growth responses of cardiomyocyte to hormones signaling via $G_{q/11}$-coupled receptors, such as Angiotensin II, endothelin-1, or catecholamines: the regulator of G protein signaling (RGS)-2 and the transient receptor potential canonical channels TRPC3 and TRPC6.

RGS-2 is a GTPase-activating protein for $G_{q/11\alpha}$ subunits. RGS-2 deficiency is linked to arterial hypertension in humans and mice. Causative mechanisms are not fully understood but possibly involve increased peripheral vascular resistance [43]. RGS-2 is specifically phosphorylated and thereby activated by cGKI [44, 45]. For instance, in vascular smooth muscle cells, the NO/sGC/cGMP/cGKI pathway enhances the association of RGS-2 with the plasma membrane and thereby attenuates the calcium and vasoconstrictory effects of vasopressin or endothelin [44, 45]. Similarly, in cardiac myocytes, cGMP/cGKI-mediated modulation of RGS-2 and subsequent inhibition of $AT_1/G_{q/11\alpha}$-signaling contribute to the counterregulation of the calcium raising—and hypertrophic responses to Angiotensin II by natriuretic peptides [22, 46].

Studies in neonatal rat cardiomyocytes and in transgenic mice have indicated that growth hormones acting via $G_{q/11\alpha}$-coupled receptors, such as Angiotensin II, activate the Ca^{2+}-permeable cation channels *TRPC3/C6* through phospholipase C (PLC)/DAG signaling pathways [22, 47]. In neonatal rat and adult mouse, cardiomyocyte activation of TRPC3/C6 ultimately leads to activation of L-type Ca^{2+} channels and pathologically increased $[Ca^{2+}]_i$, which ultimately translates into cardiomyocyte hypertrophy [22]. cGMP-stimulated, cGKI-mediated inhibitory phosphorylation of TRPC3 and TRPC6 provides another mechanism mediating the specific counterregulation of the calcium and hypertrophic responses of cardiomyocytes by natriuretic peptides and NO [48, 49].

9.3.2 Fibroblasts

Experimental observations in vitro/in vivo indicated that natriuretic peptides, via GC-A/cGMP/cGKI signaling, inhibit the stimulatory effects of Angiotensin II and TGF-β both on fibroblasts [50] and also on cardiomyocyte secretion of profibrotic factors such as CTGF, thereby moderating interstitial fibrosis accompanying hypertensive heart disease [35]. In particular, molecular studies by Li et al. [50] showed that the cGMP pathway interferes with downstream signaling from TGF-β in cardiac fibroblasts by cGKI-mediated phosphorylation of the transcription factor Smad3. The specific phosphorylation of two amino acids (S309 and T368) prevents

the heterodimerization of Smad3 with Smad4 and thereby disrupts their nuclear translocation, resulting in repression of transcriptional activation. Ultimately this signaling cascade attenuates the stimulatory effects of TGF-β on fibroblast proliferation, myofibroblast transformation, and synthesis of extracellular matrix proteins.

9.3.3 Endothelial Cells

The intracellular pathways which link the activation of ANP/BNP/GC-A/cGMP signaling to stimulation of coronary endothelial proliferation and migration can be assumed only based on in vitro/in vivo studies with peripheral endothelia, for example, HUVECs. Intriguingly, based on these studies, cGKI, which mediates growth-inhibiting effects of cGMP in cardiac myocytes and fibroblasts, elicits growth-promoting effects in endothelial cells and their progenitors [51–53]. One effect of endothelial NP/cGMP/cGKI signaling is the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), which is associated with focal adhesion sites and adherens junctions [31, 54]. This pathway was shown to be important for reorganization of the actin cytoskeleton and endothelial tube formation [29]. Members of the mitogen-activated protein kinase (MAPK) family, including ERK1/2 and p38 MAPK and the serine/threonine kinase Akt/PKB, are also important mediators of the endothelial growth and migration responses to natriuretic peptides [28]. Taken together, these in vitro data suggest that activation of VASP, MAPK/ERK, and Akt/PKB cooperates in the NP/GC-A/cGKI-dependent stimulation of endothelial cell proliferation and migration, processes which are required for coronary angiogenesis accompanying adaptive cardiac hypertrophy.

9.4 Chronic Cardiac Hypertrophy Is Accompanied by Impaired Signaling of Natriuretic Peptides and Nitric Oxide

9.4.1 Altered Cardiac NP/GC-A Signaling

Recent genetic studies examined the association of common variants at the human *ANP* and *BNP gene* loci with circulating concentrations of ANP/BNP and arterial blood pressure [9, 55]. The results demonstrate that genetically determined small variations in natriuretic peptide concentrations correlate with significant inverse changes in blood pressure, emphasizing the critical role of these hormones in the maintenance of chronic arterial blood pressure homeostasis. Indeed, gene polymorphisms of ANP or GC-A have been associated with essential hypertension and also with hypertensive cardiac hypertrophy [56–59].

Apart from these genetic variations, functional alterations of ANP, BNP, or GC-A might also be involved in cardiovascular diseases, especially in the aggravation of hypertensive cardiac hypertrophy. Patients with heart hypertrophy/insufficiency have markedly elevated plasma levels of ANP and BNP, with these levels being highly related to the severity of the disease [60]. However, several observations implicate that despite these high hormone levels, patients with heart failure of different etiologies may be in fact in a state of NP deficiency. For instance, mass spectrometry analyses revealed that most of the immunoreactive BNP in heart failure is proBNP, which possesses much lower activity than the processed 32-amino acid hormone [61]. In addition, experimental together with clinical studies have shown that chronic high NP concentrations lead to homologous desensitization of their receptor, which is due to a complex pattern of GC-A (de)phosphorylation [2, 62, 63]. Even more, in this situation of impaired cGMP formation, ANP or BNP can stimulate a cGMP-independent signaling pathway of the GC-A receptor in cardiac myocytes, ultimately resulting in pathologically elevated intracellular Ca^{2+} levels. This pathway involves the allosteric activation of TRPC3/C6 channels by GC-A which forms a stable complex with TRPC3/C6 channels. The concomitant rise in myocyte $[Ca^{2+}]_i$ can aggravate cardiac hypertrophy and possibly increase the propensity to arrhythmias [64].

9.4.2 Diminished Intracrine Production and Activity of Myocyte NO

In cardiomyocytes, NOS3 (endothelial NO synthase) is colocalized with the cGMP-producing sGC receptor in caveolin-3 containing lipid raft microdomains. Studies in rats and mice have shown that pressure overload-induced cardiac hypertrophy is accompanied both with diminished NOS activity through functional uncoupling of the enzyme [65] and with depressed NO/heme-dependent activation of the NO receptor, sGC, which is due to enhanced oxidation [66]. The latter apparently is linked to translocation of sGC out of the Cav3 microdomain of hypertrophied cardiomyocytes, which seems to favor sGC oxidation and reduces cGMP formation in response to NO [66].

9.4.3 Enhanced Myocardial Expression and Activity of PDE5

Lastly, several experimental and clinical studies have shown that the cGMP-degrading phosphodiesterase (PDE) 5 is induced in left and right hypertensive heart disease [67–69]. This could hasten inactivation of cGMP formed by NP/GC-A, CNP/GC-B, or NO/sGC signaling.

9.5 Implications for the Treatment of Hypertensive Heart Disease

The here presented studies in genetic mouse and rat models with global or conditional GC-A, GC-B, NO synthase, or cGKI deletion suggest that an impairment of the myocardial effects of natriuretic peptides or NO might aggravate and accelerate the progression of hypertensive cardiac remodeling and heart failure. As mentioned above, several experimental and clinical observations implicate that despite high endogenous levels of NPs or NO, patients with heart failure of different etiologies may be in fact in a state of hormone and cGMP deficiency, due to the production of inactive hormones, dysfunction of the respective cGMP-forming guanylyl cyclase receptors, or enhanced cGMP degradation. These findings suggest that synthetic natriuretic peptides, NO donors or mimetics, their analogs, or drugs preventing cGMP degradation could be effective in preventing the progression of cardiac remodeling and dysfunction.

9.5.1 Synthetic Natriuretic Peptides and Their Analogs

Mainly for "patent and historical reasons," Anaritide® (synthetic ANP) is clinically used in Japan, Neseritide® (synthetic BNP) in the USA, and Uralitide® (synthetic urodilatin, the renal isoform of ANP) in Europe [70, 71]. Anaritide® has been used for the treatment of different cardiovascular, renal, and metabolic diseases [72], but large placebo-controlled clinical trials demonstrating its clear efficacy are missing. Presently, the clinical use of synthetic BNP (Neseritide®) and synthetic urodilatin (Uralitide®) is restricted to the treatment of acute heart failure [73]. This indication is based on the renal natriuretic and venodilatory actions of the peptides and their counterregulatory effects on the renin–angiotensin–aldosterone system. Due to these acute effects, NPs diminish systemic fluid retention and cardiac pre- and afterload. However, based on the results of the recent Acute Study of Clinical Effectiveness of Neseritide® in Decompensated Heart Failure Trial (ASCEND-HF), the benefit of short-term NP administration in these patients remains questionable [73]. On the other hand, experimental studies have shown that long-term ANP or BNP administration by local adenoviral-driven expression can prevent the development of hypertensive heart disease in rats [74]. Of course, long-term administration of synthetic NPs or their modified analogs (chimeric peptides) to patients remains challenging due to their short in vivo half-life and peptidergic structure. However, novel technologies are being developed to allow oral or subcutaneous delivery of proteins, making the long-term use of these hormones feasible. In particular, a recent case report showed the efficacy of subcutaneous BNP administration in the treatment of a patient with essential hypertension [75]. Based on the here presented experimental observations, the development of strategies for long-term therapeutical administration of synthetic ANP or BNP or their synthetic modified analogs (such as the novel

chimeric peptides CD-NP and CU-NP [76]) could represent a novel, elegant therapeutical approach to prevent the progression of hypertensive heart disease and cardiac dysfunction if used early in the course of hypertension.

9.5.2 NO-Independent Activators and Stimulators of SGC

Activation of sGC has traditionally been achieved with nitrovasodilators. However, long-term application of these drugs is associated with the development of tolerance and potentially deleterious cGMP-independent actions. Even more, as already mentioned, in hypertensive heart disease, sGC–cGMP signaling is impaired due to reduced NO bioavailability and an alteration in the redox state of SGC, making it unresponsive to NO. Accordingly, increasing directly the activity of sGC is an attractive pharmacologic strategy. With the development of two novel classes of drugs, sGC stimulators (BAY 41-2272 and riociguat, which stimulate the enzyme synergistically with NO) and sGC activators (cinaciguat), the hypothesis that restoration of NO–sGC–cGMP signaling is beneficial in patients with cardiac hypertrophy/heart failure can now be tested [77]. Characterization of these agents in preclinical and clinical studies has begun with investigations suggesting both hemodynamic effects and organ-protective properties independent of hemodynamic changes. Both sGC stimulators and activators had antihypertensive actions and attenuated cardiac hypertrophy and fibrosis in rodent models of systemic arterial hypertension induced by Angiotensin II infusion or surgical aortic constriction [78–80]. However, clinical studies with the intravenous sGC activator cinaciguat for treatment of acute heart failure (COMPOSE program) were terminated early due to an excess of nonfatal adverse hypotension without improvements in dyspnea or cardiac index (please see a recent comprehensive review by Gheorghiade et al. [81]). However, given the preclinical anti-remodeling properties, the possibility of sGC stimulators and activators improving outcomes in chronic heart failure remains. An oral formulation at a dose with low risk of hypotension would be desirable and would make these agents applicable to the outpatient setting where they could be tested for effects on the progression of heart failure and subsequent rehospitalization.

9.5.3 Inhibitors of Phosphodiesterase 5

Cyclic GMP formed by natriuretic peptides or NO is hydrolyzed by members of the phosphodiesterase (PDE) family, mainly PDE5 and possibly PDE9, as well as by the dual substrate, cGMP/cAMP hydrolyzing PDEs 1 and 2. PDE5 remains the best characterized, and its inhibition by drugs such as sildenafil (Viagra®) is clinically used to treat erectile dysfunction and pulmonary hypertension [82]. In particular, many small clinical studies and a large randomized controlled (Super-1) trial have demonstrated the safety and efficacy of sildenafil in improving mean pulmonary

artery pressure, pulmonary vascular resistance, cardiac index, and exercise tolerance in patients with pulmonary arterial hypertension [83]. Despite its expression in cardiomyocytes, the role of PDE5 within the heart has long been considered minimal. PDE5 expression and activity in the heart are two orders of magnitude lower than in the lungs. Nevertheless, experimental studies have indicated potent direct cardiac anti-remodeling effects from selective PDE5 inhibitors, ameliorating cardiac ischemia/reperfusion [84] and effectively preventing and even reversing *left* and *right* ventricular hypertrophy, fibrosis, and dilatative cardiomyopathy in mice subjected to constant pressure overload induced by surgical aortic or pulmonary arterial constriction [85]. Based on these and other data, the National Institutes of Health initiated a multicenter, placebo-controlled phase 3 trial (RELAX; http://clinicaltrials.gov/ct2/show/NCT00763867) to test the utility of sildenafil for treating heart failure patients with a preserved ejection fraction (diastolic heart failure). These patients commonly display systemic arterial hypertension and substantial ventricular hypertrophy. The first results of this study will be available in 2013.

9.5.4 Rescue/Resensitization of Natriuretic Peptide Receptors

As another important aspect, this book chapter emphasizes that cardiac hypertrophy/insufficiency is accompanied by dephosphorylation/desensitization of the GC-A and (less) GC-B receptors for natriuretic peptides [62, 86] and oxidation/dysfunction of the sGC receptor for NO [66]. Of note, the enzymes regulating the activity and responsiveness of these guanylyl cyclase receptors are largely unknown. Therefore, clarification of the responsible receptor or postreceptor defects and identification of proteins that regulate the activity of GC-A, GC-B, and sGC in cardiomyocytes, fibroblasts, and endothelial cells may have important pathophysiological and therapeutical implications. This could lead to the development of drugs that could revolutionize the treatment of some forms of cardiac disease.

9.6 Conclusions and Future Directions

Although the observations in rodent systems cannot be directly extrapolated to human beings, the application of gene-targeting technology in mice has provided valuable information regarding the cardiac (patho)physiology and diverse local functions of natriuretic peptides, NO, their guanylyl cyclase receptors, and cGMP signaling in different types of myocardial cells. They also pointed out the potential implications of specific dysfunctions in these systems for human diseases. In fact, as reviewed in this chapter, specific links between polymorphisms or mutations in genes coding for NPs and NO and their receptors and human polygenetic diseases (essential hypertension and hypertensive heart disease) were very recently found and reemphasized the value of genetic mouse models—and in general the important physiological role of the second messenger cGMP in all types of cells of the heart.

Although our general knowledge about cGMP formation and its cellular effects has substantially increased in the last years, critical gaps remain. The gaps mostly concern the posttranslational modifications and regulation of the guanylyl cyclase receptors and the distal signaling pathways that mediate the positive versus negative effects of cGMP on cell growth and survival, for instance, in endothelial cells and cardiomyocytes. These questions are particularly difficult to address, but the answers are needed to understand and potentially improve repair mechanisms in the diseased state, such as cardiac and vascular remodeling and ischemic heart disease.

One of the major open questions concerns the subcellular compartmentalization of cGMP formation and signaling. sGCs are thought to generate global cytoplasmic cGMP signals, whereas transmembrane GCs (GC-A, GC-B) possibly establish local membrane-associated cGMP microdomains, especially in cardiomyocytes. Moreover, our recent observation of distinct effects of ANP (via GC-A) and CNP (via GC-B) on myocyte calcium homeostasis or contractile functions suggests that even different membrane-bound guanylyl cyclase receptors (GC-A and GC-B) generate different cGMP microdomains [35]. Hence, a major focus of future experimental and clinical studies in cardiovascular medicine will be to dissect the role of global versus local cGMP and associated signaling complexes in the control of cardiac myocyte, fibroblast, and endothelial cell growth, survival, and differentiation. This knowledge will be central to the development of new therapeutical strategies.

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Chapter 10
Modulating G Protein-Coupled Receptors to Effect Reverse Cardiac Remodeling

Cinzia Perrino and Howard A. Rockman

Abstract G protein-coupled receptors (GPCRs) represent the largest known family of transmembrane receptors and therapeutic targets in cardiovascular medicine, accounting for a large number of marketed cardiovascular pharmaceuticals. Traditionally, GPCR stimulation promotes G protein signaling and, to limit unrestrained stimulation, activation of G protein-coupled receptor kinases (GRKs), leading to agonist-dependent receptor phosphorylation. In turn, GPCR phosphorylation promotes β-arrestin binding to the receptors, which sterically prevents further G protein signaling and scaffold receptors to the internalization machinery. However, novel aspects of GPCR signaling have been recently appreciated, including G protein modulators, G protein-independent pathways, and GRK adrenal modulation of adrenergic drive. Since all currently used drugs have been developed using assays only testing G protein-dependent effects, the discovery of such novel signal transduction pathways might represent an important opportunity to identify additional therapeutic approaches to reverse or prevent cardiac remodeling and failure.

Keywords G proteins • Receptors • Cardiac remodeling • Arrestins • Hypertrophy • Heart failure • Signaling

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10.1 Introduction

Heart failure is a common final phenotype of many forms of heart disease. Initiated by structural and molecular remodeling of the heart, it frequently leads to contractile dysfunction. Despite optimal treatment, heart failure is a relentless, progressive disease, and novel therapeutic approaches are being continuously explored. Molecular, cellular, and interstitial changes are critical in the development of cardiac remodeling and failure. Among these signals, those activated by plasma membrane G protein-coupled receptors (GPCRs, also known as seven-transmembrane receptors) have been undoubtedly the most well investigated, currently accounting for the majority of prescriptions for cardiovascular diseases.

To date, there are nearly 200 GPCRs with known cognate ligands and a much larger number of "orphan" receptors, i.e., receptors without a known "parent" ligand [1]. A substantial number of orphan receptors are predicted to be expressed in the heart [2]. Among cardiovascular GPCRs, the most commonly investigated and clinically targeted include the nine different adrenergic receptors ($\alpha_{1A,\ 1B,\ 1D}$; $\alpha_{2A,2B,\ 2C}$; and $\beta_{1,\ 2,\ 3}$ARs), followed by the angiotensin II (AT_1R, AT_2R), endothelin (ET-A, ET-B), muscarinic (M2, M3), dopamine, adenosine, glucagon, prostanoid, bradykinin, histamine (H_1, H_2, H_3), NPY (Y_1, Y_2), opioid (μ, κ, δ), and serotonin receptors [3]. Undoubtedly, the most extensively examined cardiac GPCR is the β-adrenergic receptor (βAR), which preferentially couples to the G_s–adenylyl cyclase–cAMP signaling cascade [4].

In response to cardiac stress or hemodynamic overload, several neurohumoral adaptations occur, including the activation of the renin–angiotensin–aldosterone and the sympathetic nervous systems, leading to heightened and continuous GPCR stimulation in the heart. Although neurohumoral activation contributes to the maintenance of perfusion of vital organs, it causes impaired GPCR signaling and several maladaptive intracellular consequences, leading to cardiac remodeling and eventually failure [5]. Indeed, βAR blockers, angiotensin receptor blockers, and angiotensin-converting enzyme inhibitors have been shown to reverse cardiac remodeling, reduce cardiac hypertrophy, slow the progression of heart failure, and improve survival in heart failure patients [6–11]. Importantly, all currently used drugs have been developed using assays only testing "conventional" GPCR signaling. Thus, the recent discovery of novel GPCR signal transduction pathways might represent an important opportunity to identify additional therapeutic approaches to reverse or prevent cardiac remodeling and failure.

10.2 Old and New Paradigms in GPCR Signaling

10.2.1 Classical G Protein-Dependent Signaling

Activation of all types of GPCRs results in G protein-mediated generation of second messengers and/or activation of ion channels (Fig. 10.1) [4]. By inducing

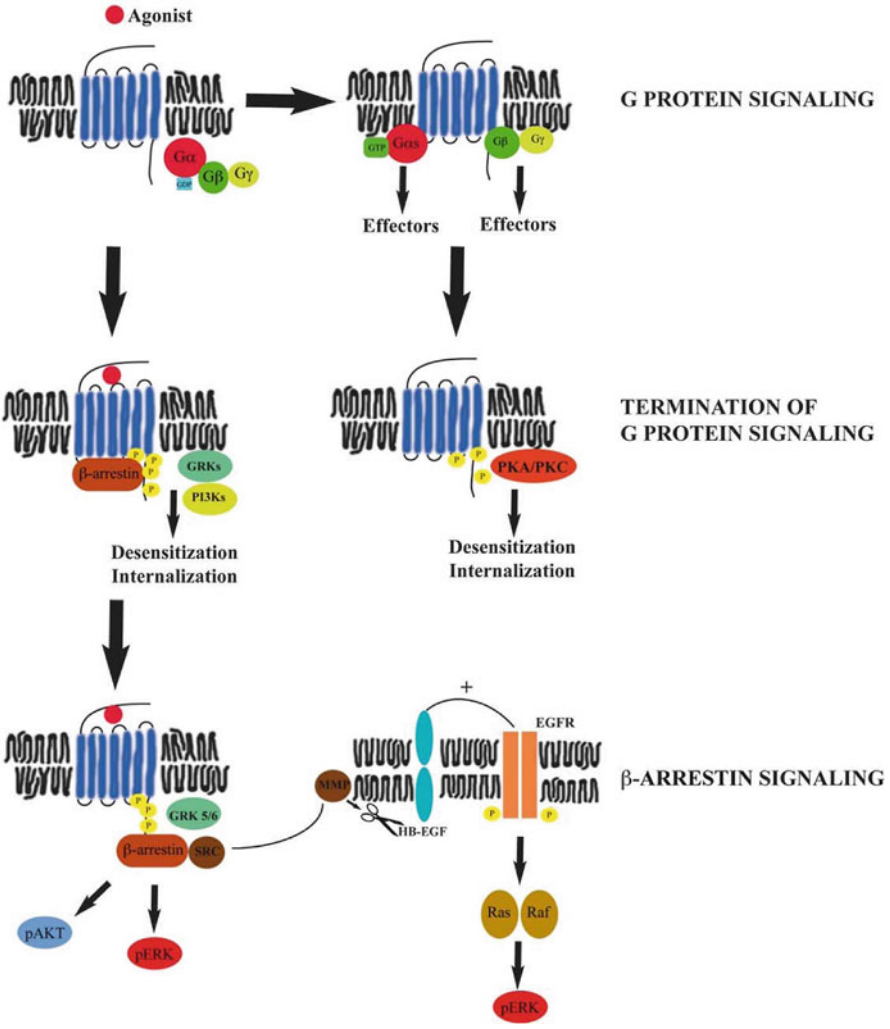

Fig. 10.1 Schematic representation of signaling pathways activated by GPCRs. In response to agonist stimulation, GPCRs undergo a conformational change, leading to the activation and dissociation of G protein subunits and subsequent activation of downstream effectors ("classical" G protein signaling). In the presence of continuous agonist stimulation, termination of G protein signaling is obtained through receptor phosphorylation by second messenger-activated kinases (e.g., protein kinase A or protein kinase C) or by G protein-coupled receptor kinases (GRKs). GPCR phosphorylation by GRK 5 or 6 promotes β-arrestin-dependent signaling that further stimulates a plethora of downstream signaling pathways (MAPKs, PI3K, AKT, EGFR, etc.). Abbreviations: *MMP* matrix metalloproteinase, *HB-EGF* heparin-binding epidermal growth factor, *EGFR* epidermal growth factor receptor

conformational changes in the receptor, agonist stimulation allows interaction of the activated receptor with the cognate heterotrimeric G proteins and promotes the dissociation of G proteins into G_{α} and $G_{\beta\gamma}$ subunits. Activated G_{α} subunits couple with an effector, an enzyme, or an ion channel [12]. Dissociated $G_{\beta\gamma}$ subunits also target a wide range of signaling pathways involved in desensitization, downregulation, apoptosis, and ion channel activation [12].

In the presence of continuous agonist stimulation, a rapid dampening of receptor responsiveness (i.e., receptor "desensitization") is obtained through receptor phosphorylation by second messenger-activated kinases (e.g., protein kinase A or protein kinase C) in a process known as heterologous desensitization or by a different family of serine/threonine GPCR kinases named GRKs (Fig. 10.1) [13]. Phosphorylation mediated by GRKs is promoted by occupancy of the receptor by agonist and triggers an "agonist-specific" or homologous desensitization. Of the seven known GRKs, only GRKs 2, 3, and 5 are appreciably expressed in the heart, and GRK2/GRK5 are the most abundant [13]. Membrane targeting of all the GRKs is critical to their function and is conferred by the C-terminal tail domain. While GRKs 2 and 3 are primarily cytoplasmic and are targeted to the plasma membrane by binding $G_{\beta\gamma}$ subunits through their C-terminal pleckstrin homology domain, GRK5 constitutively binds phospholipids and displays preferential membrane localization [13]. Although individual GRKs might promote specific patterns of GPCR phosphorylation that might affect downstream signaling (see below) [14], the phosphorylation signal invariably determines the uncoupling of the receptor from its signal-transducing G protein and enhances its affinity for a family of cytosolic proteins known as the β-arrestins [15]. GRK-mediated GPCR phosphorylation and binding of β-arrestin to agonist-occupied receptor facilitate the interaction of the receptor with proteins of the clathrin-coated pit machinery to promote internalization into intracellular compartments.

After internalization, GPCR trafficking is regulated by the variable binding affinity to the different β-arrestin isoforms and their relative intracellular localization [16]. According to the differential affinities for β-arrestins, GPCRs can be divided into two major classes: class A receptors (such as β_1ARs and D_1 receptors), binding β-arrestin 2 with greater affinity than β-arrestin 1 and losing their interaction during internalization, and class B receptors (such as AT_{1A} receptors), binding β-arrestins 1 and 2 with equal affinity and the interaction remaining intact during internalization [17]. Importantly, while β-arrestin 1 can be found in both the cytoplasm and the nucleus, β-arrestin 2 is localized only in the cytoplasm [17].

Internalized receptors can be sorted for degradation to lysosomes or for recycling to acidified vesicles where they are dephosphorylated and recycled back to the plasma membrane [18]. It has been previously suggested that, for some GPCRs such as βARs, a continuous equilibrium between internalization and recycling to the sarcolemma exists in vivo under physiological conditions [19]. Consistent with these assumptions, failing human hearts are characterized by large intracellular pools of βARs within endosomal compartments [20], and these findings are consistent with similar observations in animal models of heart failure [21]. Interestingly, changes in environmental conditions associated with reverse cardiac remodeling

(e.g., changes in cardiac load by left ventricular assist devices or neurohumoral stimulation) seem to promote reverse trafficking of "sequestered" βARs back to the plasma membrane [20]. Although it is not yet known how long βARs reside within the endosomal compartments with continuous agonist stimulation, the mechanisms for net loss of cell surface receptors (i.e., receptor downregulation) are due to a decrease in receptor synthesis, a destabilization of receptor messenger RNA, and an increase in receptor degradation.

After removal of the agonist, the attenuated GPCR responsiveness is reversed in a process known as "resensitization," which primarily involves dephosphorylation of the receptors by the serine–threonine protein phosphatase 2A (PP2A) [22]. Compared to desensitization and internalization, much less is currently known about the resensitization and dephosphorylation processes. Although sequestration into vesicles seems not to be required for desensitization, it appears to be important for resensitization, since agents blocking sequestration do not resensitize [22]. These data led to a model proposing that sequestration of the receptors into internalized vesicles might be required for their dephosphorylation within an acidic environment [22]. However, this view has been recently challenged (see below) [23].

10.2.2 Role of Phosphoinositide 3-Kinases in GPCR Function

Several recent studies have highlighted the important role of the family of protein and lipid kinases named phosphoinositide 3-kinases (PI3Ks) in the modulation of GPCR internalization and signaling at multiple levels and with different molecular mechanisms. Firstly, it has been demonstrated that Gβγ and phosphatidylinositol 3-kinases (PI3Ks) physically interact in the cytosol through the helical phosphoinositide kinase domain (PIK) of PI3Ks [24]. Upon agonist binding and release of $G_{\beta\gamma}$ subunits, Gβγ mediates PI3K membrane translocation, wherein it generates phosphoinositides regulating receptor internalization [25]. PI3Kγ inhibition by overexpressing a kinase-dead mutant markedly attenuates βAR endocytosis [26, 27], demonstrating that production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the receptor complex is necessary for efficient receptor endocytosis. In particular, PIP_3 is needed for AP-2 adaptor recruitment at the plasma membrane and for the consequent organization of clathrin-coated pits [24]. Subsequent studies have also shown that overexpression of the minimal PIK domain of PI3Ks disrupts the complex between GRK2 and endogenous PI3Ks, resulting in the targeted reduction of PI3K activity at the site of agonist-occupied receptors and inhibition in the formation of cytosolic aggregates following agonist stimulation [24]. Since the PIK domain is conserved in all PI3K isoforms, GRK2 will complex with either PI3Kα or PI3Kγ depending on the availability of the isoform in a given tissue, in heart tissues the predominant isoforms being PI3Kα, β, and γ [28].

Interestingly, it has been recently shown that the cytoskeletal protein non-muscle tropomyosin is a substrate for PI3K protein kinase activity [29]. A constitutively dephosphorylated tropomyosin mutant blocked agonist-dependent βAR internalization,

while a tropomyosin mutant mimicking constitutive phosphorylation reversed the defective βAR internalization induced by PI3K inhibition [29], suggesting that non-muscle tropomyosin might exert an important role in GPCR internalization and that its phosphorylation by PI3K might represent a crucial event in its regulation.

In addition to its effects on βAR internalization and trafficking, PI3Kγ might also negatively regulate βAR resensitization by phosphorylating I2PP2A, an endogenous inhibitor of PP2A, and thus modulating PP2A activity [23]. Indeed, I2PP2A phosphorylation by PI3Ks might result in enhanced binding to PP2A catalytic subunit, inhibiting its phosphatase activity and promoting βAR desensitization [23]. These studies suggest that GPCRs might be resensitized at the plasma membrane without necessarily undergoing internalization as previously postulated, and this process might be regulated by PI3Kγ.

Finally, recent evidence suggests that PI3Kγ might also integrate PIP_3 and cAMP signaling in the heart [30]. In contrast to mice with genetic deletion of PI3Kγ ($PI3K\gamma^{-/-}$), mice carrying a targeted mutation in the PI3Kγ gene causing loss of the kinase activity ($PI3K\gamma^{KD/KD}$) displayed normal basal cardiac contractility or cAMP levels and, after pressure overload, were protected from pathological cardiac remodeling [31]. Indeed, $PI3K\gamma^{KD/KD}$ mutants can still regulate intracellular cAMP levels by interacting with the phosphodiesterase PDE3B, and this interaction, likely based on the formation of a large multiprotein complex, does not require the kinase activity of PI3Kγ [31]. In addition, PI3Kγ directly binds protein kinase A (PKA), the main effector molecule of βAR signaling, and a recognized activator of PDE3B [30]. PI3Kγ-anchored PKA activates PDE3B to enhance cAMP degradation and phosphorylates p110γ to inhibit PIP_3 production, providing local feedback control of PIP_3 and cAMP signaling events in the heart.

10.2.3 G Protein-Independent, Beta-Arrestin-Mediated Signaling

In addition to the well-known role in GPCR desensitization, internalization, and trafficking, β-arrestins and GRKs participate in further signal propagation, assembling macromolecular complexes in the receptor environment, activating different signal transduction pathways, and even regulating other receptor families, such as tyrosine kinase receptors (Fig. 10.1).

While it has been known for several years that GPCRs activate mitogen-activated protein kinases (MAPKs), it has now been well documented that GPCRs can also utilize β-arrestins to activate a signaling [32–35]. The kinetics of ERK activation, as well as its cellular distribution, are quite distinct from those of ERK phosphorylation stimulated by conventional G protein-mediated processes [36]. Indeed, while G protein activation leads to rapid (2–5 min) and both nuclear and cytoplasmic ERK activation, β-arrestin-mediated ERK activation has different kinetics, with a slower and more prolonged pattern of ERK activation, only in the cytoplasm [33, 34].

It has been recently disclosed that β-arrestin-mediated ERK signaling following catecholamine stimulation is also dependent, at least in part, on transactivation of the

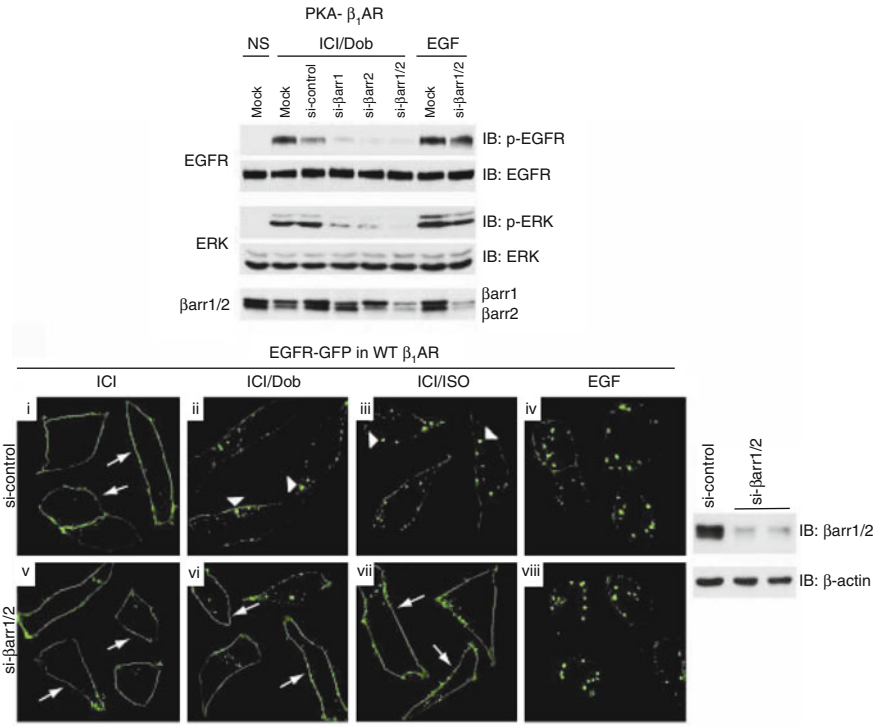

Fig. 10.2 β-arrestins are required for β_1AR-mediated transactivation of the epidermal growth factor receptor. *Top*. HEK293 cells stably expressing β_1AR mutants lacking PKA phosphorylation sites (PKA$^-$ β_1AR) were transfected with FLAG–EGFR alone (Mock) or with siRNAs targeting β-arrestin1 (si-βarr1), β-arrestin2, β-arrestin1/2, or scrambled siRNA (si-control). Cells were then treated with the β_2AR-specific antagonist ICI-118,551 (ICI), ICI + dobutamine (ICI/Dob), or epidermal growth factor (EGF), and phospho-ERK/phospho-EGFR levels were assessed. *Bottom*. HEK293 cells stably expressing wild-type (WT) β_1AR were transfected with EGFR–GFP and si-control or si-βarr1/2 to knock down expression (*right panel*). In the absence of agonist, EGFR–GFP was located at the membrane (i and v, *arrows*), while EGF stimulation induced EGFR–GFP redistribution into aggregates (iv and viii). Treatment of si-control-transfected cells with dobutamine (Dob) or isoproterenol (ISO) also resulted in redistribution of EGFR into aggregates (ii and iii, *arrowheads*), an effect that was diminished in si-βarr1/2-transfected cells, where EGFR–GFP remained at the membrane (vi and vii, *arrows*) (Reproduced with permission from [39])

epidermal growth factor receptor (EGFR), which then mediates ERK activation via the canonical Ras pathway [37]. Multiple mechanisms seem to connect GPCRs to EGFR, including the formation of GPCR–EGFR signaling complexes [38] and the matrix-metalloproteinase-mediated release of heparin-binding epidermal growth factor (HB-EGF) to stimulate EGFR phosphorylation, internalization, and initiation of ERK signaling (Figs. 10.1 and 10.2) [40, 41]. In addition to ERK activation, several GPCRs have been also shown to activate the PI3K–AKT pathways in a β-arrestin-dependent manner [42]. Interestingly, β-arrestin-dependent activation of both ERK

and PI3K–AKT seems to cooperatively promote phosphorylation and inactivation of the proapoptotic protein BAD (BCL2-associated agonist of cell death) [43].

It has been only recently appreciated that the different GRK isoforms, while phosphorylating GPCRs at specific sites, create a sort of "bar code" responsible for the initiation of specific signaling via the alternative β-arrestin-biased pathways [44]. Indeed, while inhibition of GRK 5 or 6 attenuates β-arrestin-mediated ERK activation via the β1AR and $AT1_A$ receptor, this latter remains unaffected (or may even increase) upon GRK 2 or 3 knockdown (Figs. 10.1 and 10.3) [14, 45]. Furthermore, GRK 5 or 6 overexpression seems to promote β-arrestin-dependent ERK signaling after $AT_{1A}R$ or β_2AR in vitro stimulation [41, 46]. Taken together, these data suggest that GPCR phosphorylation by the different GRKs together with β-arrestin binding is a critical factor in directing receptor signaling towards G protein-dependent or G protein-independent pathways.

10.2.4 GPCR Oligomerization

According to the "traditional" paradigm, monomeric GPCRs interact with single heterotrimeric G protein units, and, in response to agonist-mediated conformational changes in the receptor, G protein activation occurs, activating/inhibiting effector molecules. However, a growing body of evidence shows that many, if not all, GPCRs might exist as homo- and/or heterodimers [47–49]. Although the exact nature of the dimer interface is still unknown, several distinctive dimerization interfaces have been described within the transmembrane helices, the NH_2-terminal or the COOH-terminal [50–52]. Two basic models of interaction have been proposed (but not yet demonstrated): one in which dimerization occurs simply by superficial contact between monomers, and another in which several domains from different monomers are tangled between each other [48]. Importantly, heterodimerization between GPCRs might induce intracellular responses that differ from those activated by homogeneous populations of GPCRs, thereby generating previously unrealized diversity of function (Fig. 10.4) [48]. Furthermore, the development of cell- or tissue-specific GPCR populations might be extremely clinically relevant, since it might allow the development of agents specific for a certain heterodimer and regulate its signaling only in certain tissues.

In the heart, accumulating data regarding the existence of direct interactions among adrenergic receptors, and between adrenergic receptors and other GPCR families indicate a much more complex role for each receptor in regulating cardiac function. β_1AR and β_2AR homo- and heterodimers have been shown to be constitutive [53, 54] or agonist dependent [55]. When expressed in cells, β_1AR–β_2AR heterodimers activate specific intracellular responses compared with the respective homodimers [56], and this is particularly important, since cardiomyocytes express both β_1 and β_2ARs, and their expression levels can be differently regulated in response to cardiac overload and remodeling, leading to different proportions of homo- or heterodimers. Furthermore, βARs have also been shown to heterodimerize

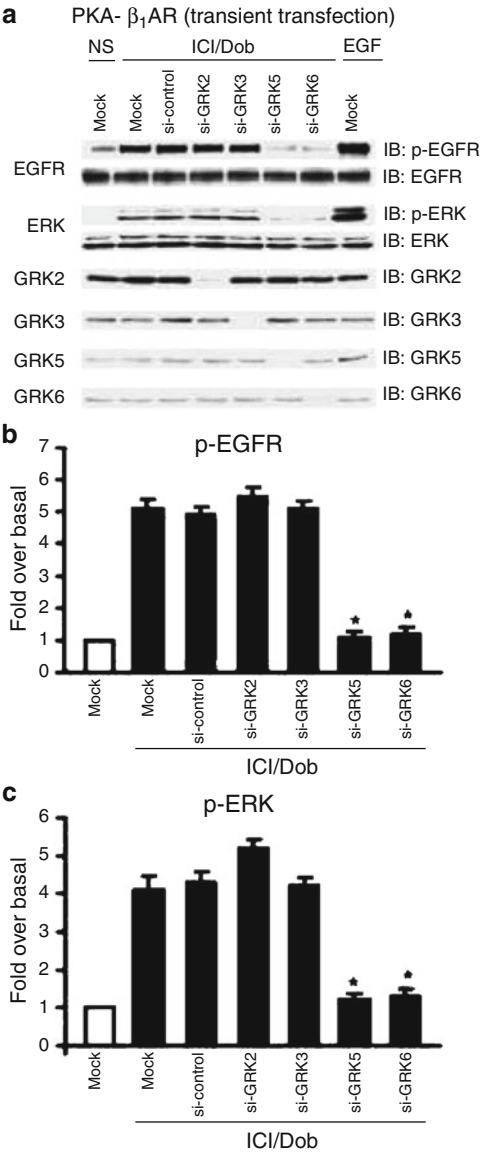

Fig. 10.3 GRK5 and 6 are required for β-arrestin-mediated EGFR transactivation. (**a**) HEK293 cells transiently expressing β_1AR mutants lacking PKA phosphorylation sites (PKA$^-$ β_1AR) were transfected with FLAG–EGFR alone (Mock) or with siRNAs targeting ubiquitous GRKs (si-GRK2, si-GRK3, si-GRK5, and si-GRK6) or a scrambled siRNA sequence (si-control). Summary of multiple independent experiments showing significant inhibition of EGFR transactivation (**b**) and ERK1/2 activation (**c**) upon dobutamine (Dob) stimulation in the cells transfected with siRNA targeting GRK5 or 6; $^*P<0.001$ versus Dob-stimulated Mock, si-control, si-GRK2, and si-GRK3 (reproduced with permission from [39])

with α_{2A}ARs [57], suggesting that GPCRs might display variable activation of signaling pathways depending on the dimer partner and/or subcellular segregation.

In addition to the interaction between same family members, interfamily interactions between endogenous βARs and AT_1Rs were recently demonstrated in the heart [58]. Interestingly, the blockade of one of the two receptors in complex was sufficient to inhibit signaling and trafficking of both receptors. Indeed, treatment of murine myocytes with a βAR blocker inhibited angiotensin/Gq coupling and contractility,

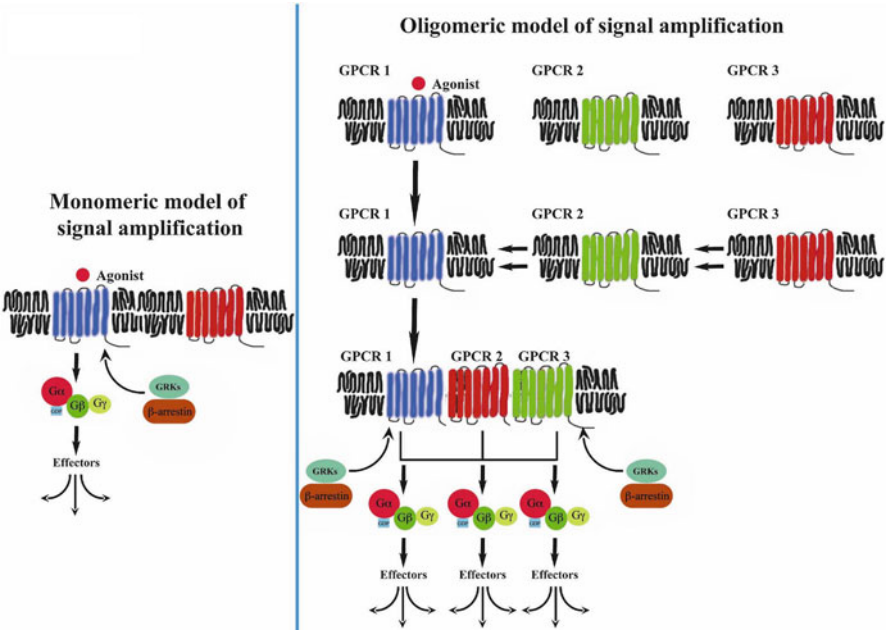

Fig. 10.4 Schematic representation of signaling pathways activated by GPCR oligomerization. According to the traditional paradigm, a single agonist activates a single receptor, its cognate G protein, and effectors (monomeric model of signal amplification, *left*). Ligand binding to one receptor might promote the formation of an oligomeric complex (*right*), leading to novel, unexpected intracellular responses that differ from those activated by homogeneous populations of GPCRs

while treatment of mice with a selective angiotensin receptor blocker attenuated heart rate response to a βAR agonist [58]. Whether, and how, receptor ligands might affect the dimerization process, and whether pharmacology of monomeric receptors is distinct from that of homodimers, is still not clear.

10.3 Novel Molecular Approaches to Modulate GPCR Signaling and Cardiac Remodeling

10.3.1 Deorphanization of Cardiac GPCRs

It has been postulated that among cardiac GPCRs, approximately one third may be "orphan" receptors, with no currently known endogenous agonists [2]. Very little information is currently available regarding the expression, regulation, and physiological or pathophysiological functions of such orphan receptors, and this might be a fertile

area for future research, leading to the discovery of new cardiovascular drugs to ameliorate cardiac remodeling [3].

Orphan GPCR research has been previously used to identify novel hormone, peptide, and neurotransmitter ligands [59, 60]. Recently, large compound libraries have been used to identify chemicals that interact with orphan GPCRs that may have therapeutic potential [61]. Recently, a "reverse" approach has been undertaken to shed light on the pathophysiological role of GPR22 in cardiac remodeling [62]. GPR22 is expressed at high levels in the heart, signaling through G_i/G_o activation. In response to TAC, cardiac pathological remodeling and dysfunction were enhanced in GPR22 knockout mice ($GRP22^{-/-}$) compared to their wild-type littermates, suggesting that this orphan receptor might exert an important role in cardiac remodeling. Although GRP22 downstream kinases are still not known and will be subject for future research, one of the most intriguing aspects of these studies is the search for novel, unrecognized endogenous ligands. These exciting studies might identify novel therapeutic targets and a novel strategy in orphan GPCR research, by defining a phenotype of an orphan receptor without immediately deorphanizing it.

10.3.2 Modulators of G Protein Signaling

In response to excessive GPCR stimulation, perturbations in G protein signaling also occur and are major contributors to pathological cardiac remodeling [63]. A long-standing strategy to target GPCR signaling at the level of the G proteins has been to mitigate Gβ-γ signaling, initially using large peptide inhibitors and more recently small molecule inhibitors [64]. However, the magnitude and duration of G protein activation are mainly regulated by the rate of GTP hydrolysis by Gα subunits, since the resulting GDP-bound inactive Gα reassociates with the Gβ-γ dimer and can enter a new activation cycle. Thus, the recent discovery of a new large family of proteins regulating G protein signaling (RGS proteins) and capable of decreasing amplitude and duration of both Gα- and Gβ-γ-mediated signals might represent novel additional therapeutic targets [65, 66]. According to sequence homology within the RGS domain and other non-RGS domains, the superfamily of RGS proteins can be divided into subfamilies, and several of these canonical RGS protein isoforms are expressed in the myocardium [67]. So far, several different strategies have been undertaken to modulate RGS expression or function in vitro: inhibitory peptides or competitive antibodies have been used to disrupt the Gα–RGS interface, and RNAi, ribozymes, or antisense oligonucleotides have been used to reduce RGS expression [67]. In addition, gain and loss of function RGS in vivo models [67] and an RGS-insensitive Gα model have been produced [68]. Undoubtedly, the R4 is one of the best characterized subfamilies in the heart, with RGS4 being the prototypical member [67]. RGS4 transgenic mice exhibited no basal phenotype, but were characterized by rapid heart failure and increased mortality in response to pressure overload [69] while transiently ameliorated cardiac dysfunction in $G\alpha_q$ transgenic mice [70].

More recently, RGS2 has been shown to modulate cardiac remodeling in response to pressure overload, since mice with global genetic deletion of RGS2 were characterized by enhanced $G\alpha_q$ signaling, cardiac hypertrophy, and accelerated dysfunction after transverse aortic constriction [71]. Similarly, RGS5 has been demonstrated to reduce cardiac hypertrophy and fibrosis in response to pressure overload [72]. Therapeutic benefits could be derived from inhibition or enhancement of RGS protein function, depending on the nature of the targeted isoform, its regulatory function, and the cellular and pathophysiological context [67]. Importantly, closely related RGS isoforms with similar sequence and structure seem to have different responsiveness to the same inhibitors, suggesting that RGS inhibitors might have the advantage of increasing the specificity of exogenous GPCR agonists by targeting specific RGS proteins. In contrast, enhancing RGS protein function could be beneficial in settings where reduction in RGS protein expression or activity is associated with pathophysiological consequences. Although RGS proteins are clearly promising targets for therapeutic development, much work still must be done to develop strategies that can eventually be successfully used in vivo.

10.3.3 Cardiac or Adrenal GRK2 Inhibition

The chronic elevation in circulating catecholamine (CA) levels under conditions of heart failure is considered a crucial event in the development of pathological cardiac remodeling and contractile dysfunction, causing several molecular abnormalities in the heart. Upregulation of GRK2 contributes significantly to βAR dysfunction and ventricular pathological remodeling [4, 13], and a large number of previous studies have shown that cardiac GRK2 inhibition effectively prevents cardiac dysfunction and ameliorates survival in HF models (recently reviewed in [13]). However, recent studies have shown that, in addition to the myocardium, GRK2 is upregulated in the adrenal gland in animal models of HF, leading to enhanced CA release via desensitization/downregulation of α_2ARs in chromaffin cells [73]. Adrenal GRK2 seems to be not only an important trophic factor for the adrenal gland in health but also a major driving force behind adrenal hypertrophy and hyperfunction in HF. Interestingly, adrenal-restricted GRK2 deletion indeed leads to a significant reduction of circulating CAs in vivo in post-myocardial infarction HF, and the resultant reduction of both circulating norepinephrine and epinephrine prevents the deterioration of cardiac function and βAR signaling. The beneficial effects of adrenal-specific GRK2 deletion also include downregulation of cardiac GRK2, an important indicator of cardiac dysfunction in HF, even without a dramatic reduction in CA levels, which could be detrimental for cardiac hemodynamic support of the circulation under critical conditions. In addition, adrenal GRK2 inhibition via adenoviral-mediated in vivo gene therapy using a GRK2 inhibitory peptide decreased circulating CAs and improved cardiac inotropic reserve and function in rats with already established HF [74]. These results suggest that lowered GRK2 expression and

activity in the adrenal gland might have a significant beneficial effect on cardiac remodeling by exerting an indirect sympatholytic effect on CA secretion from the adrenal medulla [75] and that reduction of sympathetic outflow via adrenal GRK2 inhibition might represent a novel attractive therapeutic strategy in the prevention of cardiac pathological remodeling [76, 77].

10.3.4 Targeted PI3K Displacement

Cardiac remodeling induced by various pathological causes is consistently associated to profound βAR abnormalities and activation of both GRK2 and PI3Ks [4, 20, 26, 78]. Several lines of evidence now suggest that selective inhibition of receptor-localized PI3K activation ameliorates βAR dysfunction and cardiac remodeling in different experimental settings [20, 26, 78]. Transgenic cardiac overexpression of the kinase-dead PI3K mutant in mice to reduce GRK2-associated PI3K activity preserved βAR levels and function after chronic pressure overload [28]. Similarly, receptor-localized inhibition of GRK2-associated PI3K activity preserved βAR signaling, improved cardiac function, and prolonged survival in a murine model of severe heart failure induced by cardiac-specific calsequestrin overexpression [27]. Interestingly, in a mouse model of intermittent pressure overload characterized by a mild hypertrophic response but profound molecular, structural, and functional abnormalities, selective inhibition of receptor-localized PI3K prevented βAR abnormalities, vascular rarefaction, and diastolic dysfunction while not remarkably affecting cardiac remodeling [79].

Consistent with in vitro studies showing that PIP_3 generation by PI3K is required for efficient internalization of βARs [24] in pigs with pacing-induced heart failure or left ventricular samples from failing human hearts, βARs underwent redistribution to intracellular compartments with downregulation from the plasma membrane, and this was accompanied by a significant increase in membrane-targeted PI3K activity [21]. Similar to these findings in failing hearts, chronic isoproterenol (ISO) infusion in mice induced a rapid redistribution of βARs into endosomal compartments [21]. Importantly, in mice with transgenic overexpression of the PIK domain of PI3K, the redistribution of βARs into early and late endosomes was inhibited despite prolonged ISO stimulation, resulting in normal βAR plasma membrane levels and ability to couple [21]. These data indicate that disruption of the GRK2/PI3K complex by overexpressing the PIK domain peptide alters the intracellular trafficking of βARs following prolonged agonist stimulation by preventing membrane translocation of PI3Ks and βAR-targeted generation of PIP3.

Very recently, pharmacological inhibitors of PI3Kγ have been shown to reduce arterial blood pressure levels by a vasorelaxant Akt/L-type calcium channel mechanism [80] and ameliorate cardiac remodeling in response to pressure overload [30]. Importantly, the molecular approach of displacing the kinase activity of PI3K from GRK2 might selectively restore agonist-dependent βAR signaling without inducing chronic βAR stimulation or affecting total PI3K activity [21].

10.3.5 GPCR Biased Ligands

For both AT_{1A}Rs and β_1ARs, it is well known that chronic G protein signaling is detrimental in cardiac remodeling, whereas accumulating experimental evidence now suggests that β-arrestin-mediated signaling, in particular EGFR transactivation, exerts beneficial effects in cardiac remodeling [39, 81]. The physiological relevance of this pathway in cardioprotection was first demonstrated using transgenic mice overexpressing wild-type β_1ARs or mutant β_1ARs lacking GRK phosphorylation sites (β_1AR GRK^-). Under conditions of chronic catecholamine stress, β_1AR GRK^- mice were unable to induce β-arrestin-mediated EGFR transactivation, and, compared to mice expressing wild-type β_1ARs or mutant β_1ARs lacking PKA phosphorylation sites, these mice showed increased cardiomyocyte apoptosis and left ventricular dysfunction [39].

Recently, it has been also shown that mechanical stretch triggered a conformational change in AT_{1A}Rs, leading to β-arrestin recruitment to the receptor in the absence of ligand and antiapoptotic ERK signaling in the absence of detectable G protein activation [81]. Interestingly, hearts from mice lacking β-arrestin or AT_{1A}Rs displayed blunted ERK and Akt activation, impaired transactivation of the EGFR, and enhanced myocyte apoptosis [81], suggesting that the heart responds to acute increases in mechanical stress by activating β-arrestin-mediated cell survival signals.

Based on these studies, it is now becoming increasingly clear that distinct conformations of activated GPCRs might preferentially allow binding of β-arrestin to promote downstream protective signaling in the absence of deleterious excessive G protein activation [82]. It is conceivable that specific ligands might differentially or even exclusively activate one or another signaling mechanism by inducing specific conformations of a wild-type endogenous receptor, suggesting that it should be possible to develop drugs that can activate one or the other pathway preferentially or alternatively (Fig. 10.5). This assumption led to the hypothesis of "biased agonism," which is a property of the ligand–receptor complex, whereby a ligand or a receptor may be biased towards a particular signaling pathway. Thus, "biased ligands" or "biased receptors" are those that preferentially exploit either β-arrestin or G protein signaling pathways [36]. Since many of the currently available therapies to treat heart failure, such as angiotensin receptor blockers or βAR blockers, block both G protein- and β-arrestin-mediated signaling, this concept, also described as "functional selectivity" or "collateral/pluridimensional efficacy," might have major clinical implications for pharmacological therapeutics targeting GPCRs to effect reverse cardiac remodeling in the near future [15].

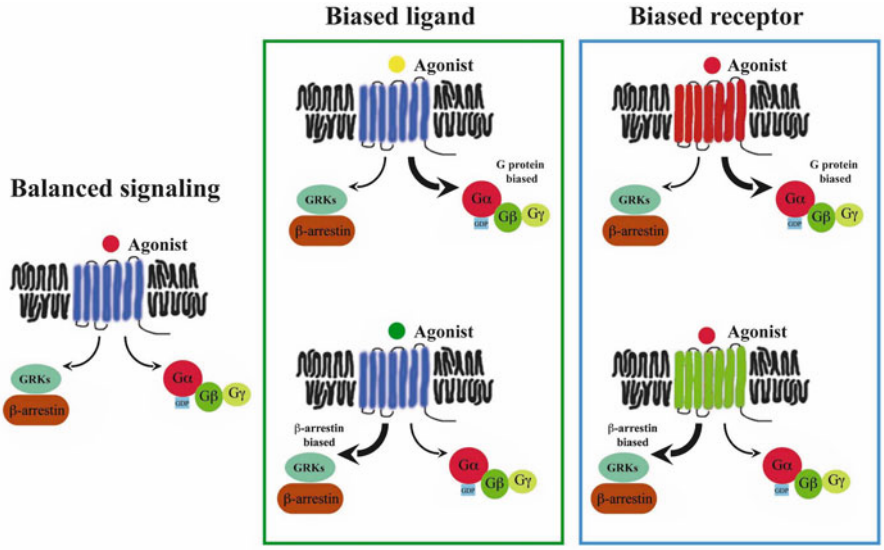

Fig. 10.5 Balanced or biased signaling of G protein-coupled receptors. Binding of a ligand results in activation of G protein signaling and β-arrestins, as well as desensitization and internalization (balanced signaling, *left panel*). Two possible mechanisms underlying biased signaling are also schematically represented. In the case of a biased ligand, binding of a ligand (G protein biased or β-arrestin biased) to an unbiased receptor results in a biased response. In the case of a biased receptor, binding of an unbiased ligand to the biased receptor (G protein biased or β-arrestin biased) might also result in a biased response

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Chapter 11
Role of Inflammation and Matrix Proteinases in Cardiac Remodeling Following Stress and Injury

Davy Vanhoutte and Stephane Heymans

Abstract Whether the myocardial damage is initially caused by ischemia or viral infection of the heart, a tightly regulated inflammatory response is provoked in an attempt to restore cardiac homeostasis and to preserve its proper function. However, a growing body of evidence suggests that uncontrolled cardiac inflammation—due to mechanisms we do not fully understand—can lead to adverse cardiac remodeling and facilitates the disease progression to its sequelae, dilated cardiomyopathy, congestive heart failure, or even sudden death. Discoveries over the last years have made it clear that an important regulator of the inflammatory pathways may be found in the cardiac extracellular matrix (ECM), known as the matrix metalloproteinase (MMP) system. It is now becoming clear that degradation and turnover of the cardiac ECM is neither the sole nor the main function of these proteinases. In fact, a growing body of evidence suggests that a complex bidirectional cross talk exists between the MMP system and a wide variety of cytokines, chemokines, growth factors, and other bioactive molecules that regulates diverse and sometimes opposing aspects of the inflammatory response in the injured heart. Furthermore, the use of single MMP-mutant mice seems to indicate MMP inhibition as potentially cardioprotective after myocardial infarction while detrimental during the pathogenesis of coxsackievirus B3-induced viral myocarditis. These findings

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enhance our knowledge in the MMP field and in that of inflammatory cardiomyopathy and will without a doubt allow for novel and highly specific therapeutic interventions in the future.

Keywords Matrix metalloproteinases • Extracellular matrix • Inflammation • Cardiac remodeling • Myocardial infarction • Viral myocarditis

11.1 Cardiac Injury, Inflammation, and Repair

Whether myocardial injury is induced by myocardial infarction (MI) or viral infection of the heart (e.g., coxsackievirus B3 (CVB3)-induced viral myocarditis), a tightly regulated orchestra of inflammatory mechanisms is provoked [1]. Cardiac inflammation functions to limit tissue damage and clears the wound from dead or infected cells and matrix debris while activating reparative pathways essential for proper scar formation, and this in an attempt to restore cardiac homeostasis and to preserve its proper function [1–3]. Interestingly, although both pathologies have their own unique features when it comes to etiology, pathogenesis, and disease progression, they both reflect inflammatory mechanisms that are partially shared.

11.1.1 Initiation of the Inflammatory Response

In contrast to cells undergoing apoptosis, necrotic cell death resulting from cardiac ischemia or viral infection leads to disruption of cell membranes and the release of cytoplasmatic and nuclear components that contain damage-associated molecular patterns (DAMPs) [2, 4]. Together with ECM fragments and pathogen-associated molecular patterns (PAMPs), DAMPs have the ability to activate the innate immune system through (1) toll-like receptor-mediated pathways, (2) the complement cascade, and (3) reactive oxygen species (ROS) [2, 4]. These three molecular "alarm" pathways converge on activation of the nuclear factor (NF)-Kβ system that drives the production of pro-inflammatory cytokines, chemokines, and growth factors by cardiac resident cells.

11.1.2 Pro-inflammatory Phase: "A Partnership Between the Innate and Adaptive Immune System"

At this point, an intense inflammatory chain reaction is initiated (Fig. 11.1). Pro-inflammatory cytokines and chemokines, including interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, and interferon (INF)-γ, fuel the inflammatory process by

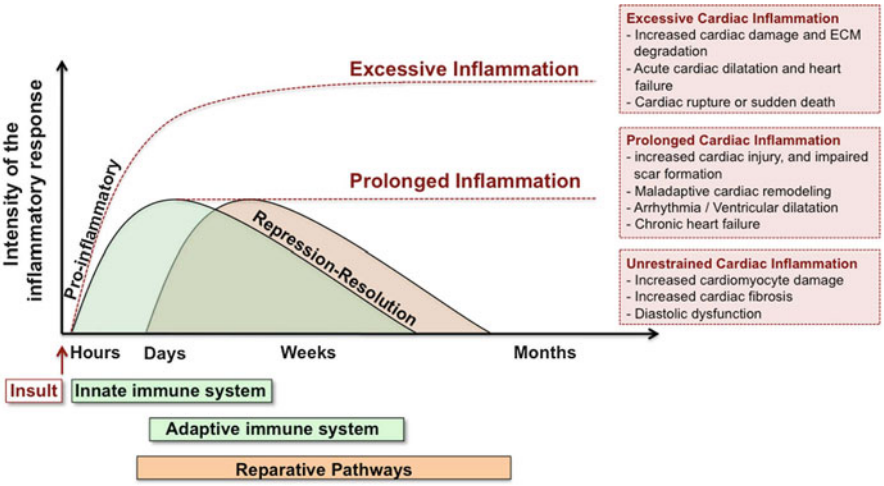

Fig. 11.1 *Cardiac inflammation: "when friend turns foe."* A complex inflammatory reaction is provoked in response to cardiac injury. In normal conditions, the pro-inflammatory phase is followed by an active phenomenon of inflammatory repression and resolution that involves both innate and adaptive immune system. In the meantime, reparative pathways are activated in order to replace the dead tissue by a fibrotic scar to restore cardiac homeostasis and preserve proper function of the injured heart. Unfortunately, in a subset of patients, cardiac inflammation becomes excessive, prolonged, or unrestrained and facilitates the disease progression to its sequelae

activating resident monocytes and macrophages [2, 5, 6]. In addition, they also upregulate cell adhesion molecules (e.g., vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1), monocyte- and neutrophil-attracting chemokines (CC, e.g., monocyte chemotactic protein-1 (MCP-1)/CCL2 and CXC, e.g., IL-8/CXCL8, respectively), and chemokine receptors (CXCRs), thereby generating directional cues for leukocyte subpopulations to egress from the vasculature into the injured or infected myocardium [2, 3].

Neutrophils and monocytes are recruited very early into the injured myocardium [2]. Once recruited and exposed to macrophage colony-stimulating factor, TNF-α, INF-γ, and others, monocytes differentiate into pro-inflammatory M1 phagocytotic macrophages [7, 8]. Together with neutrophils, they release matrix-degrading MMPs, which allow them to migrate to the site of injury where they jointly remove necrotic cardiomyocytes and secrete large amounts of pro-inflammatory mediators to further amplify the pro-inflammatory signaling cascade [2, 3, 9]. At the same time, MMPs released by neutrophils, macrophages, fibroblasts, and/or endothelial cells cleave a multitude of inflammatory mediators, consequently amplifying or dampening the inflammatory response. Besides neutrophils and monocytes, natural killer (NK) cells also infiltrate the injured heart. Although the significance of NK cells after MI is only beginning to be recognized [10], they play an important role in the pathogenesis of viral myocarditis by directly recognizing and killing virus-infected cells [6].

Subsequently, immune cells from the adaptive immune system also accumulate in the damaged myocardium (Fig. 11.1). During viral myocarditis, B-lymphocytes, $CD4^+$ T-helper, and $CD8^+$ cytotoxic T-lymphocytes are recruited to maximize the elimination of the virus-infected cells and to limit viral replication [3, 6]. The contribution of adaptive immunity in the context of MI, on the other hand, has been widely disregarded because it contradicts the classic notion that adaptive immunity is not stimulated by self-antigens. However, recent studies revealed the presence of conventional $CD4^+$ and $CD8^+$ T-lymphocytes as well as regulatory T cells in infarcted hearts [11], indicating that antigens that are normally not accessible (e.g., troponin I) or neoantigens created during ischemic injury are released from the myocardium and presented to T cells. Nevertheless, further investigations are mandatory to elucidate the full extent of the adaptive immune system post-MI.

The immune response is now at its peak. While this is beneficial for viral clearance and removal of dead cells and matrix debris, it can also contribute to irreversible myocardial damage due to the nonproliferating nature of cardiomyocytes (Fig. 11.1).

11.1.3 Suppression and Resolution of the Inflammatory Response

Once the initial inflammatory stimulus has been eliminated, a set of "brakes" is initiated to suppress and resolve the inflammatory infiltrate and promote the reparative pathways (Fig. 11.1). First, after their short life-span, apoptotic neutrophils themselves release mediators to abrogate further neutrophil recruitment and promote their resolution. A well-studied example, annexin A1, dampens neutrophil accumulation by several mechanisms, including downregulation of neutrophil transendothelial migration, increasing neutrophil apoptosis, and promoting macrophage-mediated phagocytosis of apoptotic neutrophils [2, 5]. Secondly, the clearance of apoptotic neutrophils by phagocytes launches an anti-inflammatory, pro-resolving transcriptional program with release of IL-10, IL-13, and transforming growth factor-β (TGF-β) [2, 5]. IL-10, for example, inhibits IL-1, IL-6, IL-8, and TNF-α production and has been reported to modulate the phenotypic switch from pro-inflammatory M1 to anti-inflammatory M2 macrophages [7, 8]. Furthermore, IL-10 may play a significant role in ECM remodeling by promoting TIMP-1 synthesis that contributes to the stabilization of the cardiac ECM [2]. Together with immunosuppressive $CD4^+CD25^+Foxp3^+$ regulatory T-lymphocytes, M2 macrophages further amplify the array of suppressive and pro-resolving cytokines, chemokines, and growth factors such as IL-10, decoy type II IL-1 receptor, IL-1 receptor antagonist (IL-1Ra), and TGF-β that dampens the immune response and promotes the replacement of dead tissue by a fibrotic scar [2, 5, 11]. TGF-β also modulates (myo)fibroblast phenotype and function, inhibits MMP production, induces TIMPs, and stimulates collagen synthesis [2]. Next, (myo)fibroblasts migrate into the empty spaces and produce large amounts

of ECM proteins, and an extensive microvascular network is established. Finally, (myo)fibroblasts and vascular cells undergo apoptosis, and a mature highly organized collagen-based scar is formed that provides the necessary mechanical support to the injured heart [3, 9].

11.1.4 Cardiac Inflammation: "When Friend Turns Foe!"

Unfortunately, in a subset of patients, the inflammatory response becomes excessive and chronic or expands into the noninjured myocardium and can facilitate the disease progression to its sequelae (Fig. 11.1) [2, 3, 6, 9]. An array of studies using various approaches to manipulate either the innate or adoptive immune response after MI and during viral myocarditis has revealed that (1) excessive inflammation due to enhanced expression of pro-inflammatory mediators or the absence of proper repression may activate proapoptotic pathways and augment matrix degradation causing cardiac rupture or sudden death; (2) prolongation of the inflammatory reaction—due to defective repression or resolution—may exaggerate cardiac injury, promote a disorganized collagen scar, and thereby alter the mechanical properties of the heart, resulting in exaggerated maladaptive cardiac remodeling which is associated with higher incidence of arrhythmia, ventricular dilatation, chronic heart failure, and mortality; and, finally, (3) defective containment of the inflammatory reaction may lead to extension of the inflammatory infiltrate into the healthy myocardium, thereby enhancing (myo)fibroblast-mediated collagen deposition and fibrosis, myocardial stiffness, and diastolic dysfunction. Moreover, in the setting of viral myocarditis, it is believed that not the direct infection of myocytes but rather the subsequent immune response plays a major role in the myocardial damage and ensuing morbidity [6].

Taken together, there is little doubt that uncontrolled cardiac inflammation is deleterious and codetermines the patients' clinical outcome. Unfortunately, the complex nature of myocardial inflammation has hindered the development of innovative inflammatory-based therapeutic strategies and underlines our need to better understand the regulatory mechanisms of the inflammatory response in cardiac diseases.

11.2 Matrix Metalloproteinases and Their Tissue Inhibitors: "The Basics"

Although originally discovered in 1962 as collagen-degrading enzymes responsible for tadpole morphogenesis, the MMP system is becoming widely accepted as a tightly regulated proteolytic system that modulates a wide variety of molecular and cellular processes during development, homeostasis, and disease [12–14].

11.2.1 The MMP Family

The MMPs constitute a superfamily of structurally related and highly conserved zinc-dependent endopeptidases (Table 11.1 and Fig. 11.2a) [13, 14]. To date, at least 25 MMPs have been identified. MMPs are either secreted or membrane bound, thereby confining their catalytic activity to membrane proteins in the secretory pathway or pericellular space. The MMP family is traditionally divided according to their sequence homology, ECM substrate specificity, and cellular localization in several subclasses: (1) the collagenases, (2) the gelatinases, (3) the matrilysins, (4) the stromelysins, and (5) the membrane-type MMPs [13, 14]. The first four groups of MMPs are secreted as latent zymogens, termed pro-MMPs. In marked contrast, the fifth group represents a unique subcategory that consists of all membrane-bound MMPs, the MT-MMPs, which are non-substrate related. A final subcategory comprises of all MMPs that do not fall in any of the categories above.

11.2.2 Regulation of MMP Activity

Similar to all secreted proteinases, the catalytic activity of MMPs is tightly regulated at four points: gene expression, compartmentalization, pro-MMP activation, and inhibition [18]. Typically, MMP expression is low in healthy tissues yet increases in all repair and remodeling processes, including those of the heart [3, 9].

MMP transcription is regulated by specific signals that are temporally limited and spatially confined. In the heart, their transcription can be regulated by pro-inflammatory cytokines (e.g., IL and TNF-α), growth factors (e.g., TGF-β), and other bioactive molecules (angiotensin II and endothelin), as well as ROS and mechanical stimuli [13, 19]. Moreover, all cell types found in the myocardium, either in basal conditions (myocytes, fibroblasts, endothelial cells) or in response to an inflammatory stimulus (neutrophils and macrophages), express one or more types of MMP species (Table 11.1) [5].

Upon secretion, MMPs are initially kept in a catalytically inactive state. In fact, a large reservoir of recruitable pro-MMPs exists within the myocardium—compartmentalization. MMPs can either be embedded in the cardiac ECM or bound to accessory molecules (e.g., MMP-9 to CD44) [18]. Subsequent activation can therefore result in a localized yet rapid surge of proteolytic activity. MMP activation requires proteolytic cleavage of the amino terminal pro-peptide domain by serine proteinases such as plasmin and urokinase plasminogen activator (uPA), thereby exposing their Zn^{2+}-binding site in the catalytic domain (a process known as the "cysteine-switch mechanism"; Fig. 11.2b) [13]. The formation of plasmin itself is under control of the plasminogen system (plasminogen activators (PA), tissue-type PA (t-PA), urokinase-type PA (uPA), and the PA inhibitor (PAI)) and thereby forms an important point of control of MMP activity (Fig. 11.3).

Table 11.1 Matrix metalloproteinases and their tissue inhibitors

		Substrates[a]			
	Cellular source	Collagen	Pro-MMPs	ECM related	Relevant to inflammation[a]
Collagenases					
MMP-1	CMC, Fb, ECs, and macrophages	I, II, III, VII, VIII, X	2, 9	Agg, Gel, PG	IL-1β; TNF-α; MCP-1, MCP-2, MCP-3, and MCP-4; SDF-1
MMP-8	Neutrophils, macrophages, and VSMC	I, II, III, V, VII, VIII, X		Agg, EL, FN, Gel, LN	CXCL5; CXCL8/IL-8; CXCL9
MMP-13	Fb	I, II, III, IV, VII, IX, X, XIV	9	Agg, FN, Gel	MCP-3; SDF-1; CXCL8/IL-8
Gelatinases					
MMP-2	CMC, Fb, mFb, ECs, VSMCs, macrophages, and lymphocytes	I, III, IV, V, VII, X, XI, XIV	1, 9, 13	Agg, EL, FN, Gel, LN, PG, VN	IL-1β; TNF-α; MCP-3; SDF-1; CX_3CL1; latent TGF-β1, TGF-β2, and TGF-β3
MMP-9	CMC, Fb, neutrophils, macrophages, lymphocytes, EC, and VSMC	IV, V, VII, X, XIV		Agg, EL, FN, Gel, PG, VN	IL-1β; TNF-α; IFN-β; MCP-1; SDF-1; CXCL1; CXCL4, CXCL8/IL-8; CXCL9; latent VEGF; latent TGF-β1, TGF-β2, and TGF-β3; TSP-1; IL-1R; IL-2R
Stromelysins					
MMP-3	CMC, Fb, macrophages, and VSMCs	III, IV, IX, X, XI	1, 7, 8, 9, 13	Agg, EL, FN, Gel, LN, PG, VN	IL-1β; TNF-α; MCP-1, MCP-2, MCP-3, and MCP-4; SDF-1; latent TGF-β1; OPN; E-cadherin
MMP-7	CMC and macrophages	IV, X	1, 2, 9	Agg, Casein, EL, FN, Gel, LN, PG, VN	Latent TNF-α; synd-1; OPN

(continued)

Table 11.1 (continued)

		Substrates[a]			
	Cellular source	Collagen	Pro-MMPs	ECM related	Relevant to inflammation[a]
Membrane-type MMPs					
MT1-MMP	CMC, Fb, mFb, ECs, and VSMCs	I, II, III	2, 13	Agg, EL, FN, Gel, LN	MCP-3; SDF-1; CXCL8/IL-8; synd-1, latent TGF-β
Other MMPs					
MMP-12	Macrophages	IV		Casein, EL, FN, Gel, LN, PG, VN	Latent TNF-α; MCP-1, MCP-2, MCP-3, and MCP-4
TIMPs	Cellular source	Substrates			
TIMP-1	CMC, Fb, macrophages, ECs, and VSMCs	Inhibits all MMPs, except MMP-2 and MT1MMP			
TIMP-2	CMC, Fb, macrophages, EC, and VSMCs	Inhibits all MMPs, except MMP-2; activates pro-MMP-2			
TIMP-3	CMC, Fb, and VSMCs	Inhibits MMP-1, MMP-2, MMP-3, and MMP-9 and inhibits TNF-α-converting enzyme			
TIMP-4	CMC, Fb, and VSMCs	Inhibits MMP-1, MMP-3, MMP-7, and MMP-9			

The data presented in this table are restricted to the MMPs and TIMPs relevant to this chapter and are collated from [12, 15–17]

MMP matrix metalloproteinase, *TIMP* tissue inhibitor of MMP, *ECM* extracellular matrix, *CMC* cardiomyocyte, *(m)Fb* (myo)fibroblast, *ECs* endothelial cells, *VSMCs* vascular smooth muscle cells, *Agg* aggrecan, *Gel* gelatin, *PG* proteoglycan link protein, *EL* elastin, *FN* fibronectin, *LN* laminin, *VN* vitronectin, *IL* interleukin, *IL-1R* IL-1 receptor, *IFN* interferon, *TNF* tumor necrosis factor, *MCP* monocyte chemotactic protein, *SDF-1* stromal cell-derived factor-1 (= CXCL12, CXC-chemokine ligand-12), *E-cadherin* epithelial cadherin, *VEGF* vascular endothelial growth factor, *TGF* transforming growth factor, *Synd* syndecan, *OPN* osteopontin, *TSP* thrombospondin

[a]The list of substrates has been limited to proteins that are cleaved by MMPs and relevant to the regulation of the inflammatory response

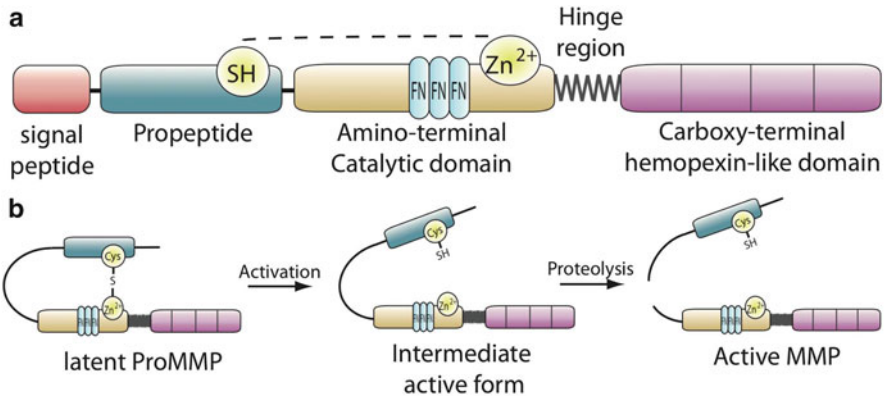

Fig. 11.2 *MMPs: "the basics."*(**a**) Domain structure of the mammalian MMP family. The general structure consists of (1) a signal peptide (SP), (2) a pro-domain, (3) a catalytic domain, and (4) a hemopexin-like domain which functions in substrate recognition. To be classified as an MMP, a protein needs to have at least the conserved pro-domain and catalytic domain. The catalytic domain typically contains a zinc ion (Zn^{2+}) in the active site (therefore the prefix "metallo") that interacts with a conserved cysteine residue (thiol, SH) in the pro-domain. Several MMPs, for example, MMP-2 and MMP-9, contain multiple fibronectin type II repeats, whereas the membrane-type MMPs (MT-MMPs) contain a transmembrane domain, a glycosylphosphatidylinositol anchor, or a signal anchor attached to the hemopexin-like domain. MT-MMPs also contain a furin-cleavage site at the C terminus of the pro-domain and are therefore activated intracellularly. (**b**) The cysteine-switch mechanism. The pro-peptide maintains an MMP inactive. When the interaction between the conserved cysteine residue in the pro-domain and the active site Zn^{2+} ion is disrupted, the active site becomes accessible, resulting in an intermediate active enzyme. Next, the pro-domain is removed by autocatalytic or other proteases, resulting in a fully active enzyme. Nevertheless, only the disruption of the Zn^{2+}–thiol interaction is an absolute requirement

Furthermore, several pro-MMPs (e.g., pro-MMP-9 and pro-MMP-2) are substrate for other MMPs (e.g., MMP-3 and MT1-MMP, respectively), leading to autocatalytic activation of MMPs and providing a mechanism for quantum bursts of proteolytic activity (Fig. 11.3). In contrast, MT-MMPs (e.g., MMP-14, also known as MT-MMP-1) contain a furin-cleavage site to allow intracellular activation before insertion in the cell membrane by members of the proprotein convertase family of proteases [18].

A final point of control is the delicate balance between MMPs and their endogenous inhibitors. The latter has been shown to be critical during cardiac homeostasis and in the development of heart failure [15]. Four endogenous tissue inhibitors of metalloproteinases (TIMP-1 to TIMP-4) exist that firmly regulate MMP activity (Fig. 11.3). TIMPs bind to the catalytic site of specific MMPs in a stoichiometric 1:1 molar ratio, thereby blocking the access to their substrates. In addition, TIMPs can also bind pro-MMPs at the aminoterminus and regulate MMP function prior to activation. Finally, TIMP binding does not exclusively lead to MMP inhibition. Indeed, the binding of TIMP-2 to MMP-2 is required for activation of the enzyme in a complex with MT1-MMP [15].

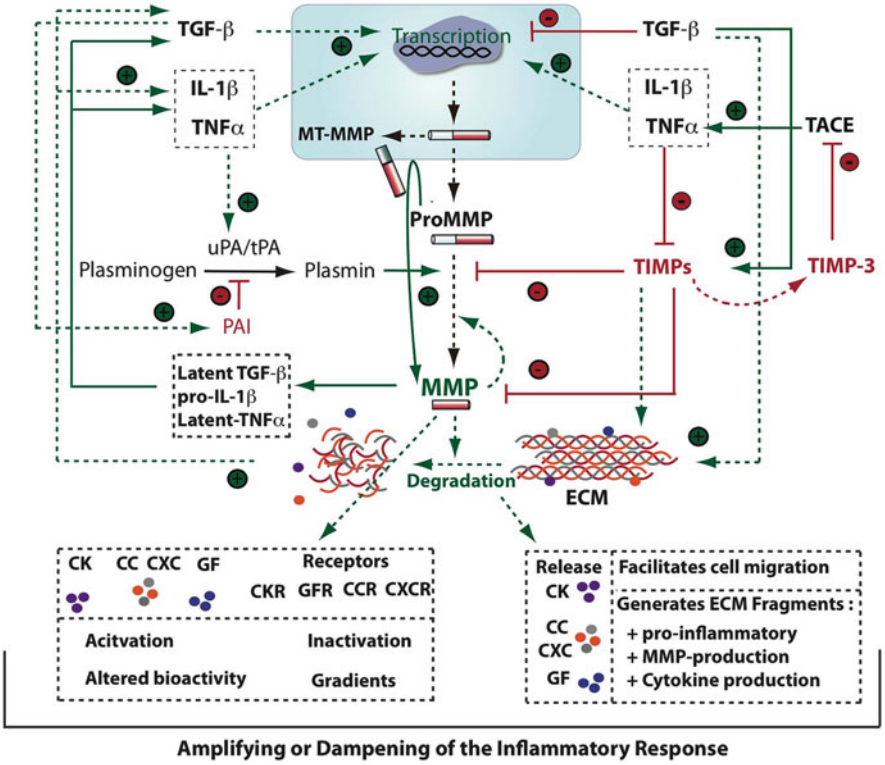

Fig. 11.3 *MMPs and inflammation: "a bidirectional cross talk."* A simplified scheme summarizing (1) MMP production, activation, and inhibition as well as (2) the complex interactions between the MMP system and inflammation as discussed in Sects. 11.2 and 11.3 of this chapter. *MMP* matrix metalloproteinases, *MT-MMP* membrane-type MMP, *TIMP* tissue inhibitor of MMP, *IL* interleukin, *TGF-β* transforming growth factor-β, *TNF-α* tumor necrosis factor-α, *uPA* urokinase plasminogen activator, *tPA* tissue PA, *PAI* PA inhibitor, *TACE* TNF-α-converting enzyme, *CK* cytokine, *CC and CXC* chemokine, *GF* growth factor, *R* receptor, + stimulatory, – inhibitory

11.2.3 MMPs in the Heart: "Much More than ECM-Degrading Enzymes"

As illustrated in Table 11.1, MMPs are collectively capable of degrading most structural components of the basement membrane and the cardiac ECM. In this aspect, increased MMP activity—in response to cardiac injury and stress—was rapidly associated with cardiac ECM remodeling and the development of progressive ventricular dilatation and dysfunction or even fatal cardiac rupture, for example, after MI [9, 15]. However, it is important to note that MMPs and TIMPs have far-reaching properties well beyond ECM turnover. Recent studies are continuously

identifying novel MMP substrates, implicating the MMP family in a wide variety of biological functions (Table 11.1). MMPs mobilize growth factors, cytokines, and chemokines, not only by liberating them from the ECM but also by cleavage and dissociation of cytokine- and growth factor-binding proteins [12]. In this capacity, MMPs can contribute to a multitude of localized signaling pathways and cellular responses that are varied (e.g., modulating cell proliferation, migration, apoptosis, angiogenesis, and inflammation) and complex in nature, evoking seemingly paradoxical effects (e.g., pro- or anti-inflammatory and pro- and anti-angiogenic responses) [14, 18]. In addition, intracellular roles are being discovered. For example, MMP-2 is involved in the proteolysis of sarcomeric and cytoskeletal proteins in cardiomyocytes under oxidative stress and may have additional biological actions in other subcellular locations including caveolae, nuclei, and mitochondria [20]. Finally, TIMPs also exhibit MMP-independent, cytokine-like properties that are beginning to be characterized (reviewed by Vanhoutte et al. [15]). Taken together, it is critical to recognize both ECM- and non-ECM-related aspects of MMPs and TIMPs when considering their role in the heart.

11.3 Inflammation and Matrix Metalloproteinases: "A Bidirectional Cross Talk"

Evidently, by physical degradation of the basement membrane and the cardiac interstitium, MMPs facilitate inflammatory cell migration to the site of injury [9]. However, a far more complex cross talk, including both positive and negative feedback and forward loops, has been observed between MMPs, inflammation, and ECM turnover and is summarized in Fig. 11.3.

First, although inflammatory mediators, including cytokines, chemokines, and growth factors (e.g., NF-Kβ, IL-1β, IL-6, TNF-α, MCP-1, and TGF-β), have distinct functions during the inflammatory process, they also regulate the expression of multiple MMPs and TIMPs [2, 19, 21] (Fig. 11.3). For example, IL-1β and TNF-α cause increased expression of MMP-2, MMP-9, and MMP-13 by cardiac fibroblasts [22]. Furthermore, they both have the capacity of inducing MMP activator uPA [21]. TGF-β, on the other hand, has a dual effect. TGF-β can suppress the transcription of MMP-1 and MMP-3, whereas it increases the expression of MMP-2 and MMP-9 [23, 24]. Conversely, inflammatory mediators can also control MMP activity by influencing the expression of endogenous protease inhibitors. IL-1 and TNF-α, for example, inhibit the expression of TIMPs, thereby tipping the net proteolytic balance towards increased MMP activity, whereas TGF-β can induce the synthesis of protease inhibitors, including PAI-1 and TIMPs (Fig. 11.3) [19].

Secondly, active MMPs can cleave a multitude of inflammatory mediators—including cytokines, chemokines, growth factors, and their receptors—and/or affect their bioavailability by releasing them from the ECM (extended overview in

Table 11.1). Examples of MMP substrates relevant to the inflammatory response include MCP-1, MCP-2, MCP-3, and MCP-4, CXCL12 (stromal cell-derived factor-1), fractalkine (CX_3CL1), pro-IL-1β, latent TNF-α, interferon-β, and latent TGF-β [12, 18]. Interestingly, cleavage of these molecules can either result in their activation, inactivation, or conversion to other molecules with altered bioactivity, consequently amplifying or dampening the inflammatory response. As such, MMP-2, MMP-3, and MMP-9 can proteolyze pro-IL-1β to activate pro-inflammatory cytokine IL-1β, while MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-12, MMP-14, and MMP-17 have TNF-α shedding activities [18, 25]. Similarly, MMPs can also establish chemokine gradients that enhance leukocyte trafficking to the site of injury [18, 25]. Conversely, MMPs are also able to suppress of the inflammatory response. MMP-1, MMP-3, and MMP-12, for example, have the ability to cleave MCP-1 to MCP-4 and CXCL1, thereby abrogating chemotaxis and putting a brake on leukocyte infiltration [26, 27], whereas MMP-2, MMP-3, MMP-9, and MT1-MMP can liberate latent TGF-β from the ECM, consequently increasing the bioavailability of the mature anti-inflammatory and pro-fibrotic TGF-β [18, 25]. Other MMP-mediated anti-inflammatory actions include proteolysis of IL-1 receptor (IL-1R), IL-2R, and IFN-β by MMP-9 rendering them inactive [25, 28]. Hence, MMPs form a negative feedback loop for controlling inflammatory cell recruitment and activation.

Next, through means of MMP-mediated proteolytic degradation, ECM fragments are generated that exert important immunomodulatory properties (Fig. 11.3), referred to as matrikines [29]. For example, various fragments of collagen type IV, elastins, and laminins have been shown to elicit neutrophil and/or macrophage chemotaxis. On the other hand, several fibronectin fragments influence the expression and secretion of MMPs (MMP-9 and MMP-12) and cytokines (IL-1 and IL-6) by monocytes/macrophages. Thus, besides their structural roles, ECM proteins can function as important actors in the cardiac inflammatory response [30].

Finally, studies indicate that pro-MMPs can associate with integrins or other cell surface receptors on leukocytes and promote their motility independent from their activation [31]. Furthermore, there are lines of evidence indicating TIMP-1, TIMP-2, and TIMP-3 might influence the inflammatory response through MMP-independent pathways [15]. As such, TIMP-3 was identified as one of the main inhibitors of TNF-α-converting enzyme (TACE), an enzyme primarily responsible for the activation of TNF-α (Fig. 11.3), thus adding yet another level of complexity to the MMP/TIMP system and their delicate regulatory role in the setting of inflammation.

Taken together, these studies expose the increasingly complex network of interactions between the MMP/TIMP system and inflammation. Moreover, they support the notion of MMPs and TIMPs as an integral part of the immune response. Nevertheless, it is important to note that many of these immunomodulatory functions are the result of in vitro studies and are yet to be validated in an in vivo setting.

11.4 MMPs, Inflammation, and Cardiac Remodeling: "Friend and Foe"

The discovery of the MMP family has been an enormous breakthrough in the field of ECM metabolism and cardiac remodeling [13]. Furthermore, keeping in mind the complex molecular cross talk between the MMP and inflammatory system as well as the importance of cardiac inflammation in the disease progression and clinical outcome of MI and viral myocarditis, it is not surprising that the MMP system has evolved to being a major focus of research in the pathophysiology of both diseases. As summarized in Table 11.2, genetic approaches have clearly demonstrated that any deviation from the delicate balance between MMPs and TIMPs can result in diverse and sometimes opposing consequences on the pathogenesis of MI and viral myocarditis. In fact, whereas genetic deletion of individual MMPs seems to be cardioprotective after MI, the deficiency of exactly the same family members produces catastrophic consequences during the pathogenesis of viral myocarditis (Table 11.2).

11.4.1 Spatiotemporal Expression Profile of MMPs/TIMPs in Response to Cardiac Injury

During MI or viral myocarditis, the homeostatic balance between MMPs and TIMPs present in the myocardium is largely disrupted. Both human and animal studies have revealed increased levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13, MMP-14, and TIMP-1 and TIMP-2, while TIMP-3 and TIMP-4 levels decrease after an ischemic insult to the heart [9, 16]. Similarly, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, TIMP-1, and TIMP-2 are upregulated in a mouse model of CVB3-induced viral myocarditis, while others, such as MMP-11 and MMP-7, remain unchanged or such as TIMP-4 decreased [3, 17]. Importantly, in both diseases, MMPs and TIMPs follow a unique spatiotemporal localization, thus pointing towards highly specific effects on molecular and cellular responses during the acute and chronic phases in response to cardiac injury. Nevertheless, to date, the potential roles of many MMPs have not yet been evaluated, underscoring the continuing need to unravel the full complement of MMPs and TIMPs in response to MI or viral infection of the heart.

11.4.2 MMPs/TIMPs and Myocardial Infarction

Heymans and colleagues were the first to indicate that acute—yet transient—protease inhibition may represent a viable therapeutic target to prevent cardiac rupture after MI, whereas prolonged inhibition leads to cardiac failure [35]. Using

Table 11.2 The role of individual MMPs and TIMPs in myocardial infarction and viral myocarditis

MMPs	Mouse model	Cardiac phenotype	Proposed mechanism	References
Collagenases				
MMP-8	MMP-8$^{-/-}$, CVB3	Decreased T-lymphocyte migration	Lack of collagenase activity necessary for lymphocyte migration	[32]
Gelatinases				
MMP-2	MMP-2$^{-/-}$, MI	Decreased leukocyte infiltration and collagen degradation, slower wound healing, decreased LV dilatation and cardiac rupture	Lack of laminin- and fibronectin-peptide fragments mediated macrophage migration	[33, 34]
	MMP-2$^{-/-}$, CVB3	Increased susceptibility to viral myocarditis	Lack of CCL7 (MCP-3) degradation	[17]
MMP-9	MMP-9$^{-/-}$, MI	Decreased leukocyte infiltration and collagen deposition, increased angiogenesis, decreased LV dilatation and cardiac rupture	Impaired ECM degradation	[35, 36]
	MMP-9$^{-/-}$, CVB3	Increased viral load, CD3^{+} T-lymphocytes, myocardial damage, and fibrosis, yet preserved cardiac output	Lack of IFN-β/IFN-γ inactivation by MMP-9	[32]
Stromelysins				
MMP-3	MMP-3$^{-/-}$, MI	No differences in cardiac rupture, leukocyte infiltration, and collagen deposition	NA	[35]
MMP-7	MMP-7$^{-/-}$, MI	Increased macrophage infiltration, decreased arrhythmia, and improved survival	Impaired connexin-43 processing	[37]
Membrane-type MMPs				
MT1-MMP	MT1-MMP$^{+/-}$, MI	Decreased collagenolytic potential of cardiac fibroblasts, decreased dilatation and dysfunction, and increased long-term survival	Attenuated collagen type I catabolism	[38]
Other MMPs				
MMP-12	MMP-12$^{-/-}$, MI	No differences in cardiac rupture, leukocyte infiltration, and collagen deposition	NA	[35]
	MMP-12$^{-/-}$, CVB3	High mortality early post infection	Lack of innate antiviral immune response	[39]

TIMPs	Mouse model	Cardiac phenotype	Proposed mechanism	References
TIMP-1	TIMP-1$^{-/-}$, MI	Increased hypertrophic response, reduced fibrillar collagen content and adverse LV remodeling	Increased MMP-13 activity and decreased collagen deposition	[40, 41]
	AdTIMP-1, MI	Decreased leukocyte infiltration, wound healing, and angiogenesis; decreased cardiac rupture	Increased MMP inhibition	[35]
	TIMP-1$^{-/-}$, CVB3	Increased survival and decreased myocardial apoptosis	Impaired retention of immune cells at sites of infection and increased MMP-mediated cell death	[42]
	AdTIMP-1, CVB3	Reduced cardiac inflammation, necrosis, fibrosis, LV dilatation, and dysfunction	No cleavage of MMP zymogen	[43]
TIMP-2	TIMP-2$^{-/-}$, MI	Increased inflammation and IL-6 and MCP-1 expression; augmented MMP-8, MMP-9, MMP-13, and MT1-MMP activity; decreased collagen density; exaggerated infarct expansion and LV remodeling	Loss of TIMP-2-mediated MT1-MMP inhibition	[44]
	AdTIMP-2, MI	Decreased MMP-2 and MMP-9 activity, leukocyte infiltration, and LV dilatation and improved long-term survival	Decreased MMP-mediated leukocyte infiltration	[45]
TIMP-3	TIMP-3$^{-/-}$, M	Increased neutrophil infiltration and MMP-2 and MMP-9 activity; suppressed TGF-β1 expression and collagen synthesis; increased LV dilatation and dysfunction; and higher incidence of cardiac rupture	Increased EGF/EGFR signaling downregulates TGF-β1 expression and collagen synthesis	[46, 47]
TIMP-4	TIMP-4$^{-/-}$, MI	ND	ND	NA
	TIMP-4$^{-/-}$, CVB3	ND	ND	NA

MMP matrix metalloproteinase, *TIMP* tissue inhibitor of MMP, *AdTIMP* adenoviral-mediated TIMP overexpression, *MI* mouse myocardial infarction, *CVB3* human coxsackievirus B3 mouse model of viral myocarditis, *LV* left ventricle, *CCL* CC-chemokine ligand, *MCP* monocyte chemotactic protein, *IFN* interferon, *IL* interleukin, *TGF* transforming growth factor, *EGF/EGFR* epidermal growth factor/EGF receptor, *ND* not done, *NA* not applicable

various genetic approaches to reduce protease activity, including uPA-deficient mice and adenoviral-mediated overexpression of protease inhibitors TIMP-1 or PAI-1, the authors revealed significantly reduced leukocyte infiltration and protection against acute cardiac rupture in a mouse model of myocardial infarction. Furthermore, lack of uPA suppressed the expression of IL-6 and MPO, indicating that PA/MMPs influence the cytokine response and oxidative stress, thereby potentially promoting cardiomyocyte injury in the infarcted heart [35]. However, these acute cardioprotective effects were followed by delayed wound healing, decreased angiogenesis, and contractile dysfunction in uPA-deficient hearts, indicating that a timely tuned balance between inflammation and proteases is essential for proper wound healing and functional recovery of the infarcted heart [35]. Similarly, Creemers et al. [48] revealed that after MI, the absence of MMP-activating plasminogen depressed MMP-2 and MMP-9 activity and macrophage infiltration and completely abolished wound healing, thereby promoting altered ventricular remodeling and contractile dysfunction. PAI-1-null mice, on the other hand, had increased leukocyte infiltration and died of ventricular wall rupture within 7 days post-MI [49].

Subsequently, several genetic studies have tried to expand our knowledge on the individual roles of MMPs and TIMPs post-MI (Table 11.2). Lack of MMP-2 or MMP-9 in mice almost completely protected against cardiac rupture post-MI [33, 35]. Both phenotypes were marked by reduced inflammatory influx—macrophages and total leukocytes, respectively—and a slower removal of necrotic debris from the infarcted left ventricle, whereas others demonstrated attenuated left ventricular dilatation and dysfunction in both MMP-2- and MMP-9-deficient mice, without affecting collagen scar formation 14 days post-MI [34, 36]. In contrast, similar cardiac rupture rates were observed in MMP-7-null and WT mice after MI [37]. The absence of MMP-7 did, however, improve long-term post-MI survival by taking MMP-7-mediated cleavage of gap junction protein connexin-43 out of the equation, consequently leading to improved electrical conduction patterns in the infarcted heart. Interestingly, this phenotype was associated with an increased macrophage density in MMP-7-null hearts post-MI. In contrast, membrane-anchored MT1-MMP-null mice display multiple organ defects and die within weeks of birth. Given this morbid status, Koenig et al. [38] were limited to the use of mice heterozygous for an MT1-MMP-null allele (MT1MMP$^{+/-}$ mice) to investigate the role of MT1MMP post-MI. MT1MMP$^{+/-}$ mice display reduced numbers of macrophages, a significant post-MI survival benefit, and retain myocardial structure and function, without affecting MMP-2 and MMP-9 activity post-MI. Instead, the beneficial effects observed in MT1MMP$^{+/-}$ hearts were attributed to a decrease in MT1-MMP-mediated interstitial collagen type I catabolism. Finally, no differences were noted in the incidence of cardiac rupture, leukocyte infiltration, and collagen deposition within the first 14 days post-MI in MMP-3 and MMP-12 knockout mice, indicating either functional redundancy, compensation by other MMPs, or that these MMPs might only be important in the later stages after MI [35].

On the other side of the spectrum, gene deletion of several TIMPs convincingly showed that further activation of MMPs results in a higher risk of acute cardiac

rupture or heart failure post-MI (Table 11.2). Following MI, TIMP-1-null mice had elevated activity levels of MMP-13, lower fibrillar collagen content, and more severe LV dilatation and dysfunction, however, without significantly changing rupture rates as compared to WT controls [40, 41]. Kandalam et al. [44, 50] revealed that TIMP-2 deficiency leads to exacerbated LV dilatation and dysfunction post-MI, associated with increased neutrophil and macrophage infiltration, expression of pro-inflammatory mediators IL-6 and MCP-1, and decreased fibrillar collagen content. Although MMP-8, MMP-9, and MMP-13 activity levels were increased in absence of TIMP-2, the authors ascribed the protective role of TIMP-2 to the inhibition of MT1-MMP-mediated degradation of fibrillar collagen in the infarcted heart. Concordantly, adenoviral-mediated TIMP-2 overexpression reduced MMP-2 and MMP-9 gelatinase activity, reduced leukocyte infiltration, preserved cardiac structure and function, and improved long-term survival post-MI [45]. Finally, two independent studies reported a high incidence of post-MI cardiac rupture and poor long-term survival after MI in TIMP-3-null mice. The latter was associated with higher density of neutrophil infiltration as well as overt activity of MMP-2 and MMP-9 in the infarcted myocardium [46, 47]. Furthermore, Hammoud et al. [46] indicated that lack of TIMP-3 resulted in increased EGF/EGFR signaling which downregulates TGF-β and collagen synthesis.

Taken together, these studies collectively indicate a cardioprotective effect of MMP inhibition in the setting of MI. With exception of MMP-7, many of the authors tried to pinpoint the mechanism solely to the effects of the MMP system on ECM metabolism or to the generation of ECM-peptide fragments that elicit chemotactic properties (e.g., MMP-2; Table 11.2) [33]. However, given our current knowledge, one must wonder whether ECM degradation is really the primary target of MMP inhibition post-MI and to what extent MMPs and TIMPs directly influence the pro-inflammatory and/or pro-resolving pathways as well as the cellular components of the inflammatory response.

11.4.3 MMPs/TIMPs and Viral Myocarditis

Similar as to what was previously observed after MI, Heymans and colleagues revealed that broad inhibition of MMP activity was protective in a mouse model of CVB3-induced viral myocarditis. More specifically, targeted deletion of uPA or adenoviral-mediated overexpression of TIMP-1 and PAI-1 reduced cardiac inflammation ($CD4^+$ and $CD8^+$ T-lymphocytes and neutrophils), necrosis, and fibrosis while preserving ventricular function without affecting viral load (Table 11.2) [43]. This potential therapeutic effect of MMP inhibition was further substantiated when Pausinger et al. [51] showed that lowering MMP activity in CVB3-infected mice through the administration of carvedilol significantly reduced cardiac inflammation and ventricular dysfunction.

Although the number of studies is still limited, mice deficient for a single MMP have yielded very contrasting findings in a mouse model of CVB3-induced

viral myocarditis, as to what previously was observed after MI, thus highlighting the disease-specific context of MMP/TIMP biology (Table 11.2). Furthermore, these recent studies start to incorporate the notion of non-ECM-related targets of the MMP system during cardiac disease, inflammation, and remodeling. Whereas no differences in viral load and functional parameters were noted between MMP-8-null mice and their WT counterparts, ablation of MMP-9 increased the severity of CVB3-induced viral myocarditis in mice [32]. More specifically, lack of MMP-9 resulted in increased levels of INF-β/INF-γ and increased $CD3^+$ lymphocytic infiltration, leading to a dramatic increase in immune cell-mediated damage of the myocardium, uncontrolled viral replication, and faster transition to heart failure after CVB3 infection. The authors ascribe the protective role of MMP-9 to the inactivation of pro-inflammatory cytokines IFN-β/IFN-γ and hence the dampening of the immune response [32]. In addition, preliminary studies also suggest a protective role for MMP-2 and MMP-12 in CVB3-induced myocarditis. Upon CVB3 infection, MMP-2-null hearts displayed increased levels of pro-inflammatory mediators MCP-3, IL-1β, TNF-α, and fractalkine—all known substrates of MMP-2—leading to an exaggerated inflammatory response and immune-mediated cardiac damage [17]. MMP-12-null mice, on the other hand, produced the most dramatic response to CVB3, as high rates of mortality were observed 3 days post infection. However, more in-depth investigations are mandatory to elucidate the exact mechanistic role of MMP-12 during innate antiviral immunity [39].

Concordant with the detrimental effects observed in MMP-null mice, TIMP-1 deficiency leads to improved survival rates when challenged with CVB3 (Table 11.2) [42]. This was not attributed to changes in viral replication or T-cell response, but this rather indicated that local TIMP-1 expression is mandatory to retain and concentrate the immune response at sites of CVB3 infection, resulting in myocarditis and tissue injury [42].

11.5 Conclusions and Future Directions

It has long been presumed that the functional contribution of MMPs in the development of cardiac disease, remodeling, and failure was limited to their capacity to degrade and turnover the structural components of the ECM. Unfortunately, this functional misconception led clinical studies to evaluate MMP inhibitors, for example, in patients with MI, that failed to meet their endpoints and casted a shadow on MMPs as valid therapeutic targets [52]. In addition, these studies underscore our lack of knowledge within the MMP field and the complexity of translating basic studies into clinical therapeutics in general.

However, accumulating evidence from a wide variety of studies is changing this functional dogma. The growing number of new substrates discovered for MMPs and TIMPs increases their significance in regulating many localized signaling pathways and cellular responses. In this respect, a complex bidirectional cross talk

between the MMP system and inflammation is emerging, where location, timing, stimulation, or inhibition will be the key to prevent adverse cardiac remodeling and heart failure in response to cardiac stress and disease. Particularly striking is that the analysis of single-MMP-mutant mice seems to identify MMP inhibition as a potential tool to dampen cardiac inflammation and therefore as cardioprotective after MI, whereas the same mutants exaggerate inflammation and proved to be detrimental during CVB3-induced viral myocarditis. However, further efforts to delete the remaining mouse MMPs and TIMPs and the continuing analyses of existing mutants will be essential to see how all MMPs are deployed within the wide range of cardiovascular diseases. Another major challenge will be to carefully map the complete spatiotemporal and cell-specific expression profiles of the many inflammatory mediators, MMPs, and their inhibitors in a cardiac disease-specific manner to confirm known biological mechanisms and to uncover novel functions. Finally, we will need to identify the full portfolio of MMP- and TIMP-specific substrates using approaches and systems relevant to cardiac health and disease while keeping an open mind towards both intra- and extracellular functions of MMPs as well as the emerging MMP-independent functional properties of TIMPs. This will, for example, provide a detailed view into the complex interactions of the MMP system with the pro-inflammatory and pro-resolving immune cascades as well as the cell-specific reparative pathways that are activated during cardiac repair, and vice versa.

Taken together, further in-depth studies should lay the foundations for the development of new therapeutic strategies that specifically inhibit cardiac disease-related MMPs and/or their effectors that cause deleterious effects while sparing or even enhancing MMP functions that are protective.

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Chapter 12
Role of Chymase in Matrix and Myocardial Remodeling Due to Mitral Regurgitation: Implications for Therapy

Spencer J. Melby, Carlos M. Ferrario, Chih-Cheng Wei, and Louis J. Dell'Italia

Abstract The pure volume overload of mitral regurgitation (MR) has many unique features including matrix metalloproteinase (MMP) activation, increased bradykinin, extracellular matrix loss, disruption of the focal adhesion complex, and cardiomyocyte myofibrillar loss—all of which either directly or indirectly are beneficially affected by inhibition of chymase. Cardiomyocyte myofibrillar loss and cytoskeletal disruption may be related to intracellular oxidative stress and/or increased chymase production within the cardiomyocyte. Increased adrenergic drive is also an important underlying pathophysiologic feature, which, like chymase activation, is present both early and late in course of MR. There is now both dog and human data

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demonstrating the benefit of β_1-receptor blockade in isolated MR. However, neither chymase inhibition nor β_1-receptor blockade alone attenuates left ventricular (LV) dilatation. These data raise the intriguing question whether the combination of a chymase inhibitor and β_1-receptor blocker would have a synergistic effect in preventing LV remodeling, especially if started early in the course of isolated MR.

Keywords Mitral regurgitation • Volume overload • Chymase • Matrix metalloproteinase • Focal adhesion kinase

12.1 Introduction

Mitral regurgitation (MR) creates a unique hemodynamic stress by inducing a low-pressure form of volume overload caused by ejection into the low-pressure left atrium. Mechanisms of left ventricular (LV) dysfunction in isolated MR are not well understood. Vasodilator therapy in other forms of LV dysfunction reduces LV wall stress and improves LV function; however, studies in isolated MR show no beneficial effect on LV remodeling using standard medical therapy with renin–angiotensin system (RAS) blockade [1, 2]. As opposed to other forms of heart failure in which RAS blockade has been highly successful, chronic isolated/pure volume overload is associated with loss of interstitial collagen surrounding cardiomyocytes and upregulation of the cardiac kallikrein–kinin system, which further promotes collagen loss as well as inflammatory cell infiltration that is not improved by RAS blockade [3–6].

The study of LV remodeling in isolated MR was initially fueled by questions related to (1) difficulty in determining optimal timing for surgical intervention because of the common incidence of heart failure and LV dyofunction post-valve repair/replacement and (2) the failure of conventional medical therapy to attenuate LV remodeling and to improve LV function. These important clinical questions provided the impetus to better understand the myocardial mechanisms of LV remodeling of an isolated volume overload which are totally distinct from pressure overload.

12.2 Studies in Isolated Volume Overload

To better understand why standard therapy with RAS blockade was ineffective, we performed gene arrays in both the dog with isolated MR and in the rat with the pure LV volume overload produced by aortocaval fistula (ACF) [7–9]. These animals developed LV and cardiomyocyte eccentric remodeling with a loss of interstitial collagen throughout a 4-month time course [6–9]. In a dog model with isolated MR, the loss of collagen weave that connects individual cardiomyocytes was also associated with a significant downregulation of matrix genes and profibrotic growth factors, in particular transforming growth factor-beta (TGF-β), in addition to

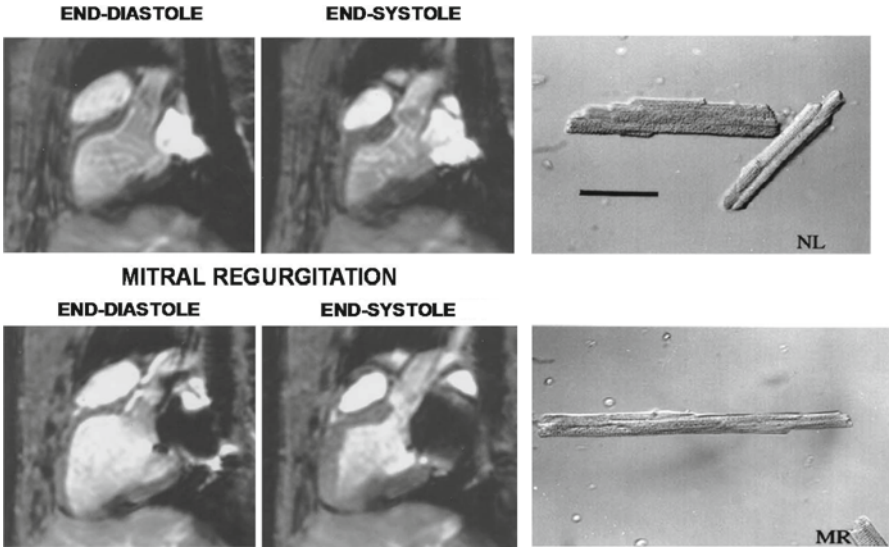

Fig. 12.1 Magnetic resonance images of a dog at baseline and 4 months after induction of MR with representative isolated cardiomyocytes. The LV demonstrates an increase in LV end-diastolic volume with wall thinning resulting in a decrease in the LV mass-to-volume ratio. There is also a corresponding increase in the length-to-width ratio of cardiomyocytes after 4 months of MR

upregulation of matrix metalloproteinase (MMP) genes and the antifibrotic kallikrein–kinin system [6–9]. The MR heart has a less robust hypertrophy response (despite a significant increase in LV angiotensin II levels) than the volume overload of aortic regurgitation where as opposed to ejection into the low-pressure left atrium in isolated MR, the excess volume overload of aortic regurgitation is ejected into the high-pressure aorta. However, the hemodynamic response cannot completely explain the LV remodeling of isolated MR because there is a decrease in protein synthesis that has been attributed to the toxic effects of excessive adrenergic drive throughout the course of MR in the dog [10]. The loss of collagen and noncollagen extracellular matrix combined with a weak hypertrophy response produces LV wall thinning and a decrease in the LVEDV mass/volume ratio that is characteristic of isolated MR in the dog model and in humans (Fig. 12.1) [11]. Taken together, the loss of collagen combined with a decrease in profibrotic growth factors is a poor therapeutic match for the antifibrotic and antihypertrophic effects of RAS blockade or vasodilators in general.

The loss of surface cellular proteins that connects collagen weave to the cell surface results in the dephosphorylation of focal adhesion kinase (FAK) in the dog with isolated MR [12]. An intact focal adhesion complex is essential for cell growth, survival, and myofibril assembly. As opposed to the volume-overloaded MR heart, increased FAK phosphorylation has been documented in animal models of pressure overload [13]. Interestingly, mice with selective inactivation of cardiomyocyte FAK demonstrate increased myofibrillar degeneration, elongation and thinning of

cardiomyocytes, and eccentric LV remodeling and heart failure after 6 months [14]. The disruption of FAK and cellular integrins is also associated with disruption, disorganization, and decreased respiratory capacity of subsarcolemmal mitochondria early in the course of volume overload in the rat with ACF, which is prevented by MMP inhibition in the acute 24 h ACF [15].

Another key factor in pure volume overload is the role of increased adrenergic drive, which is central to both early- and late-stage MR [16–18]. However, prolonged excessive adrenergic stimulation has a cytotoxic effect on cardiomyocytes [19], resulting in myofibrillar loss and cardiomyocyte elongation and thinning that is reflected in increased LV end-systolic dimension or volume and wall stress [11]. In the dog and human with isolated MR, β_1-receptor blockade results in significant improvements in myofibrillar loss, calcium homeostasis, and cardiomyocyte and LV function; however, there is no improvement in extracellular matrix loss, LV dilatation, or cardiomyocyte elongation [20–22]. The discordance between LV remodeling and improved LV systolic function with β_1-receptor blockade is most likely related to the failure of β_1-receptor blockade to improve the loss of extracellular matrix components, which are essential to cardiac geometry. Thus, the hallmark features of early and persistent adrenergic drive and extracellular matrix loss of a pure volume overload elude a completely effective medical therapy that both attenuates LV dilatation and improves LV function.

The pure stretch of volume overload also demonstrates a molecular and cellular inflammatory response that contributes to MMP activation and matrix degradation. However, as with studies in human heart failure, prolonged inflammatory cytokine or inflammatory cell blockade in pure volume overload has not met with success [23, 24]. Although mast cell stabilizer therapy has reported success in the rat model, in the dog with isolated MR, mast cell stabilizer results in worsening LV and cardiomyocyte function that is attributed to the inherent calcium entry-blocking effects of this class of drugs [24]. Thus, no clear target of the extracellular matrix degradation (either direct or indirect) has been found, especially in view of the results in other models which demonstrated that prolonged MMP inhibition resulted in excessive collagen deposition (fibrosis) [25] and that blockade of the bradykinin type 2 receptor resulted in a marked increase in blood pressure [5]. It is of interest that, as opposed to the beneficial effects of kallikrein delivery in pressure overload, blockade of kallikrein results in matrix preservation and improved LV function in the chronic volume overload of ACF in the rat [6]. However, at this point in time, there are no existing drugs that target kallikrein blockade.

12.3 Chymase in Cardiovascular Disease and in Isolated Mitral Regurgitation

The interest in chymase in isolated MR emanates from the significant increase in LV mast cell infiltration and degranulation in the dog with isolated MR [26, 27], resulting in release of chymase, a proteolytic enzyme which is increased at both early and

late stages of MR [26]. Chymase belongs to a family of chymotrypsin-like serine proteases stored in secretory granules of mast cells, which was first described in the human heart in 1990 [28, 29]. Based on amino acid sequence homology, chymase is divided into two groups: α and β [30]. Only α-chymase has been identified in human, dog, and hamster, while multiple isoforms of α- and β-chymase are present in rodents. It is thought that chymase is tonically released from mast cells and released into the interstitium of the heart. In the mast cell-deficient mouse, there is no chymase [31, 32]. In addition, chymase mRNA is at a very low level in the human and mouse heart, supporting the notion that mast cell release is the predominant source of chymase protein in the heart.

In addition to an Ang II-forming capacity from Ang I that is 20-fold higher than ACE [28, 29], chymase directly activates MMPs [33, 34], degrades fibronectin [35], and activates kallikrein [36]. The addition of chymase to isolated smooth muscle cells [37] or neonatal cardiomyocytes [38] results in cell death due to chymase degradation of cell surface connections to extracellular matrix and disruption of the focal adhesion complex. In addition, chymase activates kallikrein [38] and transforming growth factor (TGF)-β and interleukin (IL)-1β [39]. A newly described propeptide cleaved from angiotensinogen, Ang-(1–12), may be an alternate substrate for the formation of biologically active angiotensins [40, 41]. Ang-(1–12) is increased in cardiac myocytes of adult spontaneously hypertensive rats (SHR) [42]. In the anephric rat, cardiac Ang-(1–12), Ang I, and Ang II increase, while plasma levels decrease [43], suggesting a role of ANG-(1–12) as an intermediate substrate for cardiac Ang II formation. Although the enzymatic mechanism responsible for cardiac Ang-(1–12) formation from angiotensinogen remains an open question, a recent study suggested a chymase-mediated mechanism in the ischemia–reperfusion rat heart [44]. Recently, Ferrario and coworkers have reported that this chymase-mediated Ang II formation from Ang-(1–12) represents the predominant mechanism of Ang II formation in the left atria of humans having surgery for correction of chronic atrial fibrillation [45] and in the human LV (data submitted for publication). Thus, the chymase/Ang-(1–12) axis may represent a totally renin-independent mechanism of Ang II formation in the heart (Fig. 12.2).

Chymase has multiple mechanisms to mediate LV myocardial remodeling by its activation in the cardiac interstitium and after release along with other constituents of activated mast cells. Blockade of chymase has implications beyond the effects on Ang II formation because chymase activates MMPs and directly degrades fibronectin, which promotes tissue remodeling and apoptosis by disrupting matricellular connections. The positive effects of ACE inhibition on progression of cardiovascular disease and heart failure have obscured the fact that chymase rather than ACE may be the major Ang II-forming enzyme in humans. Studies by Dell'Italia and collaborators demonstrated that cardiac Ang II production and/or degradation in the interstitial and intravascular spaces is compartmentalized and mediated by different enzymatic mechanisms—by ACE in the intravascular space and by chymase in the interstitium [32, 46]. In addition, the presence of bradykinin $(BK)_2$ receptors on mast cells may contribute to the mechanism of "ACE escape" via an ACE inhibitor-mediated chymase release from mast cells into the interstitium (Fig. 12.3) [32].

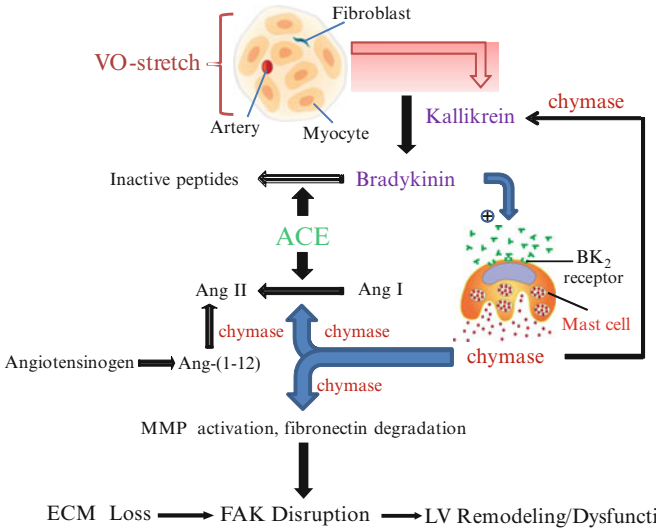

Fig. 12.2 Chymase-related mechanisms in pure volume overload. Volume overload causes an increase in cardiac kallikrein expression and mast cell degranulation and recruitment. Chymase release also has a myriad of effects on factors that promote the inflammatory response and tissue remodeling including kallikrein activation, MMP activation, and fibronectin degradation, resulting in disruption of focal adhesion kinase (FAK) complex. Chronic inhibition of ACE increases BK_2-dependent chymase release from mast cells, which counteracts the direct effect of ACE inhibition on Ang II formation through the chymase pathway of Ang II formation via Ang I and Ang-(1–12)

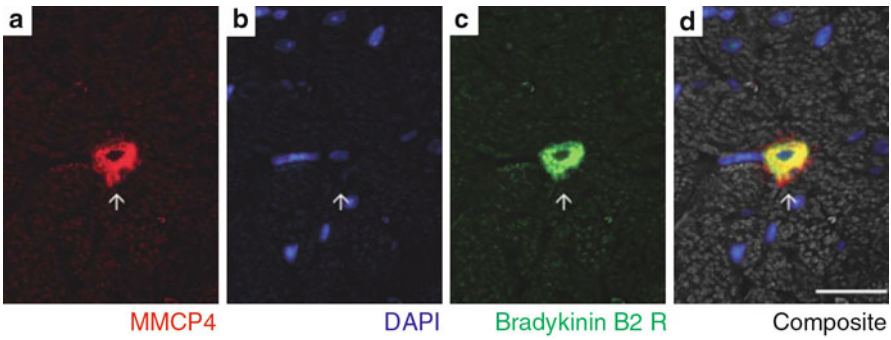

Fig. 12.3 Bradykinin BK2 receptors in LV MMCP4+ MCs. Photomicrographs of a LV section from an 8-week-old wild-type C57B6 mouse stained for (**a**) mast cell protease 4 (MCP4) to identify mast cells, (**b**) DAPI to detect nuclei, (**c**) BK_2 receptor, and (**d**) composite image. *Arrows* show the location of the MMCP4 + MC. Scale bar: 20 μm (Reproduced with permission *J Clin Invest*)

The multiple actions of chymase on tissue remodeling, in particular MMP activation, have confirmed a role for cardiac chymase in the adverse LV remodeling following myocardial infarction [32,47,48], atherosclerosis [49–52], and in the pathophysiology of abdominal aortic aneurysm in the human [53].

There is now mounting evidence that chymase may provide an important intracellular mechanism for Ang II formation. Cardiac myocytes, fibroblasts, renal mesangial, and vascular smooth muscle cells have been shown to synthesize intracellular Ang II under hyperglycemic conditions [54–57], and chymase was upregulated in coronary and renal arteries in patients with diabetes [58]. Rapid field electrical stimulation of HL-1 atrial cells induces a sustained augmentation of intracellular Ca^{2+} that is associated with increases in ACE and chymase activity and angiotensinogen expression [59].

Chymase inhibition has been reported to improve hemodynamics and prevent fibrosis in animal models of heart failure due to pacing tachycardia, myocardial infarction, and hypoxia/reoxygenation [60–63]. A more recent study in the pig demonstrated that acute intravenous administration of a chymase inhibitor significantly decreased infarct size after 1 h of ischemia and 2 h of reperfusion. The protective role of chymase inhibition was related to its attenuation of MMP-9 activation and a decrease in inflammatory cytokines [64]. Exposure of mice to intermittent hypoxia increased LV chymase activity, Ang II, oxidative stress, and IL-6, TNF-α, and TGF-β activity—all of which were significantly decreased by treatment with a chymase inhibitor [65]. Treatment also resulted in a decrease in LV fibrosis and hypertrophy as well as a decrease in RV systolic pressure. In addition, the combination of ACE inhibitor and chymase inhibitor decreased infarct size and LV dilatation and improved LV function compared to ACE inhibitor alone in hamsters 1 month after coronary occlusion (Fig. 12.4) [32]. These results suggest that chymase inhibitors, which target MMPs [33, 34] and fibronectin [36], prevented cell death through attenuation of the loss of cell–ECM connections in the pathophysiology of LV remodeling [36, 37]. This suggests that chymase inhibitors might be a useful addition to ACE inhibition or AT_1-receptor blockade in the treatment of heart failure.

12.4 Effects of Chymase Inhibition in the MR Dog

Chymase activity is increased early and throughout the course of MR in the dog. In dogs with 4 months of isolated MR, chymase inhibition normalizes the significant decrease in fibronectin and FAK phosphorylation and prevents cardiomyocyte myofibrillar loss (Figs. 12.5 and 12.6) [66]. One of the major myofibrillar proteins in the heart is the large sarcomeric protein titin, which exists as two isoforms—N2BA and N2B [67–71]. Total titin and its stiffer isoform N2B are increased in the LV epicardium and parallel the changes in fibronectin and FAK phosphorylation in MR dogs treated with chymase inhibitor. Chymase inhibition also improves depressed isolated cardiomyocyte function without improving intracellular calcium transients in dogs with MR. Although the increase in cardiomyocyte function did not translate to an improved LV ejection fraction, magnetic resonance imaging with tissue tagging demonstrated an increase in LV torsion angle in chymase treated compared to untreated MR dogs [66].

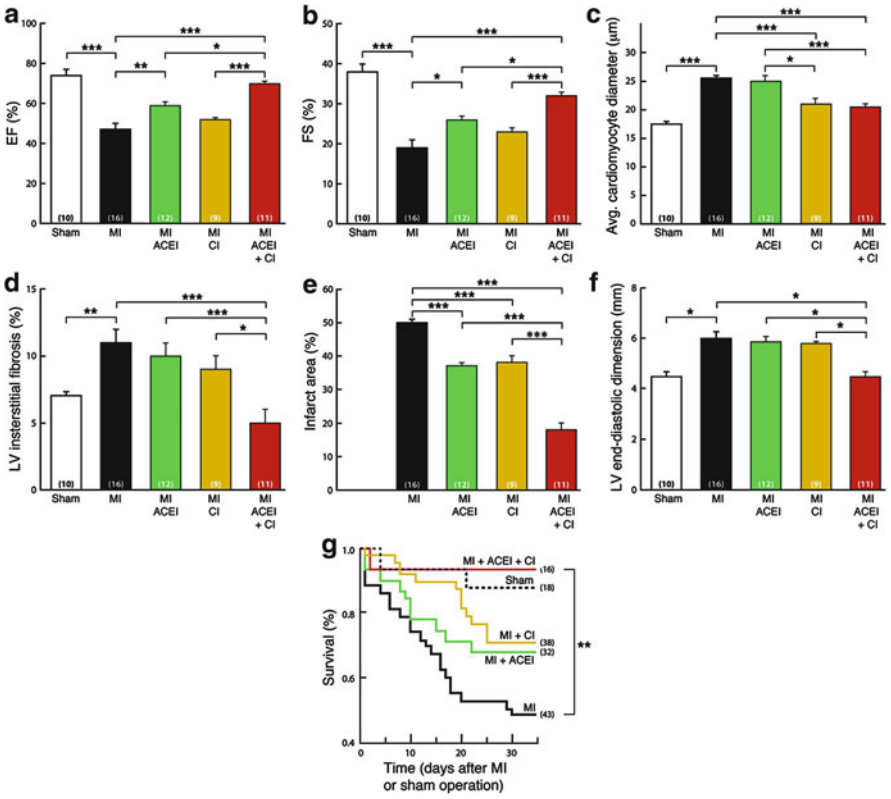

Fig. 12.4 Effect of chymase inhibition on LV remodeling and function and mortality after coronary occlusion in the hamster. The combination of ACE inhibitor and chymase inhibitor produces the greatest improvement in LV remodeling, systolic function, and mortality. Graphs demonstrating LV ejection fraction (EF) (**a**), fractional shortening (FS) (**b**), average cardiomyocyte diameter (**c**), LV interstitial fibrosis (**d**), infarct area (**e**), LV end-diastolic dimension (**f**), and survival (**g**) are all significantly improved by ACE inhibitor + chymase inhibitor in hamsters 1 month after total left anterior descending artery occlusion. Drugs were started within 24 h after total occlusion. MI myocardial infarction; *ACEI* ACE inhibitor; *CI* chymase inhibitor (Reproduced with permission *J Clin Invest*)

Torsion is the wringing motion of the LV along its long axis during systole, induced by contracting myofibers, which are aligned 180° from endocardium to epicardium. Studies in the intact animal demonstrate that acute increases in preload and contractility are associated with increased LV torsion [72, 73]. Preservation of the fibronectin–FAK complex that tracks titin N2B expression from endocardium to epicardium in the chymase inhibitor-treated MR dogs may indeed mediate the higher torsion angle. Further, FAK is located at the Z disk at the insertion of titin in the cardiomyocyte, and there is evidence that the N2B region of titin interacts with integrins and FAK [71]. The improvement in cardiomyocyte shortening and LV torsion with chymase inhibition may be related to the maintenance of a functional

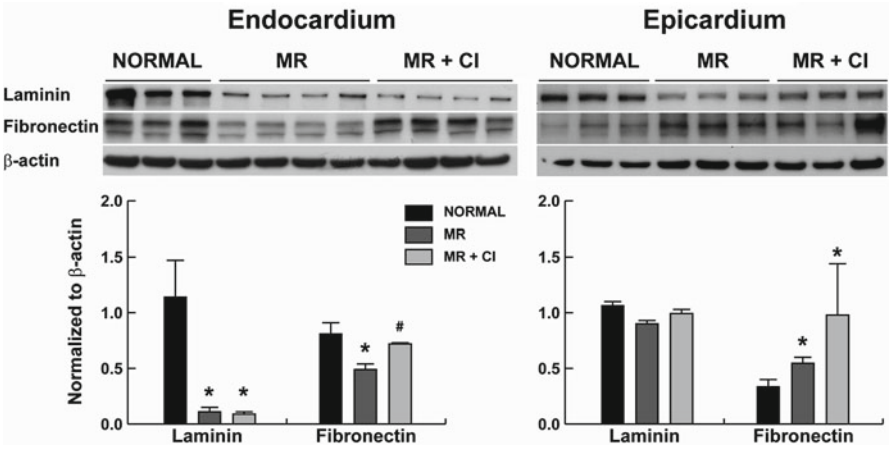

Fig. 12.5 Effect of chymase inhibition on noncollagen extracellular matrix (ECM) proteins in dog model of MR. Representative Western blots of laminin and fibronectin protein in normal ($n=7$), MR ($n=7$), and MR+CI ($n=8$) LV endocardium and epicardium. Chymase inhibition attenuated the loss of laminin and fibronectin in dogs with MR. Densitometric analysis of laminin and fibronectin protein expression is normalized to β-actin. Values mean ± SEM. $^{*}P<0.05$ vs. Normal; $^{\#}P<0.05$ vs. MR. *CI* chymase inhibitor (Reproduced with permission *Circulation*)

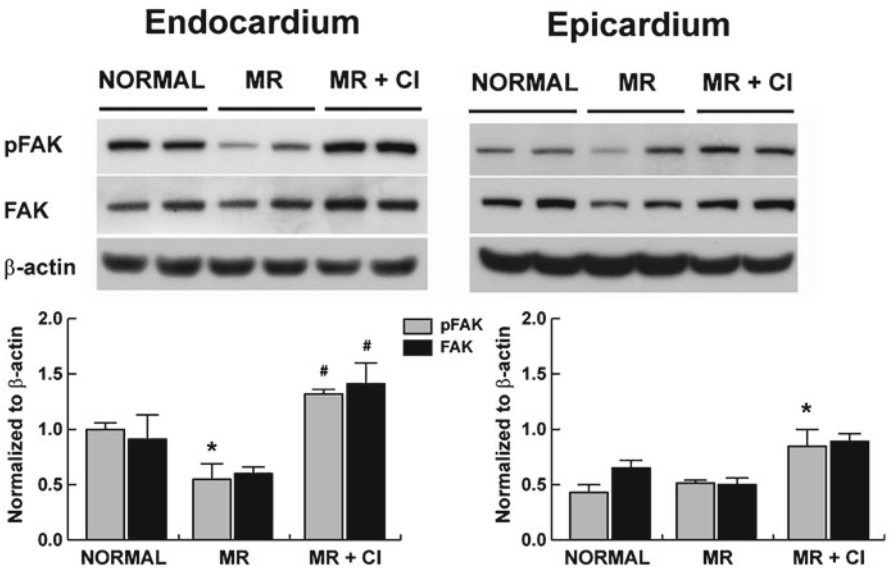

Fig. 12.6 Effect of chymase inhibition on focal adhesion kinase (FAK) phosphorylation in a dog model of MR. Representative Western blot of phospho-FAK (tyrosine 397) and total FAK (band size 125 kD) in normal ($n=7$), MR ($n=7$), and MR+CI ($n=8$) LV endocardium and epicardium. Chymase inhibition attenuated the decrease of phosphorylation of FAK. Densitometric analysis of phospho-FAK and total FAK protein expression is normalized to β-actin. Values mean ± SEM. $^{*}P<0.05$ vs. Normal; $^{\#}P<0.05$ vs. MR (Reproduced with permission *Circulation*)

fibronectin–FAK interaction that promotes an increase in titin-dependent cardiomyocyte shortening in MR. Future studies will elucidate the mechanism that causes a titin isoform shift in the myocardium and whether this is dependent on preservation of an intact fibronectin–FAK interaction with chymase inhibition.

Another important feature that can contribute to the negative extracellular matrix homeostatic balance is that the isolated volume overload is associated with an increase in the cardiac kallikrein–kinin system in both the dog model of isolated MR [66] and in the rat model of aortocaval fistula [6]. Adult rat cardiomyocytes undergoing cyclical stretch increase kallikrein mRNA expression and bradykinin release which are inhibited by aprotinin, a kallikrein inhibitor, further supporting the connection of stretch and kallikrein upregulation. It is of interest that inhibition of chymase normalized a 16-fold increase of bradykinin in the MR dog [66], which is consistent with the reported activation of kallikrein by chymase in bronchial tissue [36]. Bradykinin reduced collagen I and fibronectin mRNA and protein expression by adult cardiac fibroblasts [74], while knockdown of kallikrein increased fibronectin expression in kidney mesangial cells [75]. Thus, chymase may mediate fibronectin loss by direct degradation and/or indirectly by inhibiting its synthesis through increased bradykinin formation in the MR heart.

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Chapter 13
Cardiac Remodeling Due to Aortic Regurgitation and Mitral Regurgitation

Blase A. Carabello

Abstract Mitral regurgitation and aortic regurgitation (MR, AR) both impose a volume overload on the left ventricle (LV). Despite this similarity, the two lesions and loads they present are quite different from one another. In MR, the extra volume pumped by the LV is ejected into the left atrium, while in AR the extra volume is delivered into the aorta where it increases pulse pressure and systolic pressure. Thus MR is a "pure" volume overload, while AR is a combined pressure and volume overload, and afterload excess plays a much greater role in AR. As a consequence, the ventricle of AR demonstrates both eccentric and concentric hypertrophy, while the MR ventricle is a thin-walled dilated chamber. These different pathologies are associated with different mechanisms of hypertrophy and different mechanisms that lead to LV dysfunction.

Thus, while once MR and AR were lumped together as volume-overload lesions, their differences are considerable so that each lesion should be approached as a uniquely separate entity with differing pathophysiologies and differing clinical outcomes.

Keywords Volume overload • Mitral regurgitation • Aortic regurgitation • Remodeling

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13.1 Introduction

All valvular heart diseases place a hemodynamic overload upon the left and/or right ventricle, and for each valve lesion, this overload is unique. Uniqueness extends to mitral and aortic regurgitation (MR, AR). While MR and AR were once lumped together as volume-overloading lesions, it is now clear that mitral regurgitation and aortic regurgitation are actually quite different from one another, presenting the left ventricle (LV) with different hemodynamic overloads, in turn resulting in different geometric adaptation and remodeling [1]. Decades ago, Grossman et al. postulated that hemodynamic stress (σ) was the progenitor of cardiac hypertrophy [2]. Laplacian stress (σ) = $P \times r/2$th where P = ventricular pressure, r = ventricular radius, and th = wall thickness. In their formulation, an increase in systolic stress, mediated by increased systolic pressure, caused the myocardium to lay down new sarcomeres in parallel, increasing myocyte thickness and thus increasing LV wall thickness. In this formulation, the increase in the pressure term in the numerator of the Laplace equation would be offset by an increase in the thickness term of the denominator, in turn normalizing systolic wall stress (afterload), an adaptation that is beneficial in maintaining normal LV ejection. Conversely in volume overload, increased diastolic stress mediated by increased LV filling pressure would signal sarcomeres to be laid down in series, causing each myocyte to elongate [3], increasing LV volume so that it could accommodate the volume and cardiac output needs of the patient. While many biologic systems modulate how stress results in remodeling and hypertrophy, the Grossman's hypothesis serves as a guide to how the ventricle responds to an overload.

It is generally held that the processes creating and maintaining LV remodeling and hypertrophy can be both adaptive and maladaptive. Since there is limited ability for myocytes to divide after birth, most cardiac growth occurs through the hypertrophic process. Clearly if the heart failed to enlarge, it could not generate the cardiac output needed to compensate for the volume demands of normal body growth let alone the ability to compensate for a volume overload. As such, hypertrophy and remodeling are not only adaptive but also necessary for life. On the other hand, increased LV wall thickness is associated with increased mortality, an obviously maladaptive consequence of concentric hypertrophy. Likewise, increased LV volume may decrease LV efficiency as the radius term in the Laplace equation increases in turn increasing afterload, limiting LV ejection, a maladaptive feature of the eccentric hypertrophic process [4].

This chapter will review the unique loads of mitral regurgitation and aortic regurgitation, contrasting the major differences that exist between the two entities. Going forward, it is important to contrast the terms hypertrophy and remodeling which are not synonymous. Ventricular hypertrophy indicates that the ventricle has gained weight, while remodeling indicates that there has been a change in ventricular geometry. While both usually occur together, they do not always do so. For instance, in a patient whose LV increases in volume but decreases in wall thickness, it is possible for there to be no increase in LV mass. In such a case, there would be eccentric remodeling without eccentric hypertrophy.

13.2 Mitral Regurgitation (A Pure Volume Overload)

13.2.1 Definitions

In discussing mitral regurgitation (MR), it is necessary to distinguish primary (organic) MR from secondary (functional) MR. In the former condition, one or more of the components of the mitral valve malfunction causing it to leak, imparting a volume overload on the LV [5]. Conversely in functional MR, the mitral valve itself is usually normal, but disease of the LV leads to ventricular dilatation, papillary muscle displacement, and annular dilatation in turn causing MR. In organic MR, valve disease leads to a hemodynamic overload, eventually leading to ventricular damage, heart failure, and death. In functional MR, there is already severe LV damage caused by myocardial infarction or cardiomyopathy in turn leading to MR as a secondary phenomenon.

13.2.2 Organic MR

13.2.2.1 Load and Remodeling

In most types of volume overload including aortic insufficiency (see below) anemia, hyperthyroidism, and heart block, the extra volume pumped by the LV to compensate the pathologic volume requirements of the disease is delivered into the aorta. Because pulse pressure is in part dependent upon stroke volume, increased stroke volume in most volume overloads widens pulse pressure, tending to increase systolic pressure. Thus most such volume overloads are in fact combined pressure and volume overloads. In this context, mitral regurgitation is nearly unique among LV volume overloads. In MR, the extra volume is pumped into the left atrium as opposed to the aorta. Thus aortic flow is normal or reduced, not increased. As a consequence, systolic pressure in patients with MR tends to be normal or even lower than normal, averaging 110 mm Hg in one study [4].

Because the development of remodeling and hypertrophy responds to hemodynamic loading, this characteristic loading in MR leads to characteristic LV geometry. The typical LV in the normotensive MR patient is thin walled and dilated [1] having the largest LV radius-to-thickness (r/h) ratio and the smallest ratio of mass-to-volume ration (m/v) of the left-sided overloading lesions. Thus, in MR, there is least amount of LV mass to pump the required increased stroke volume.

13.2.3 Consequences of Remodeling in MR

The thin-walled, enlarged LV of MR is conducive to LV filling as it allows the LV to accept the increased volume stored in the LA during systole at a normal filling pressure. In fact, MR is one of the few heart diseases in which diastolic function is actually supernormal, at least in compensated disease [6, 7].

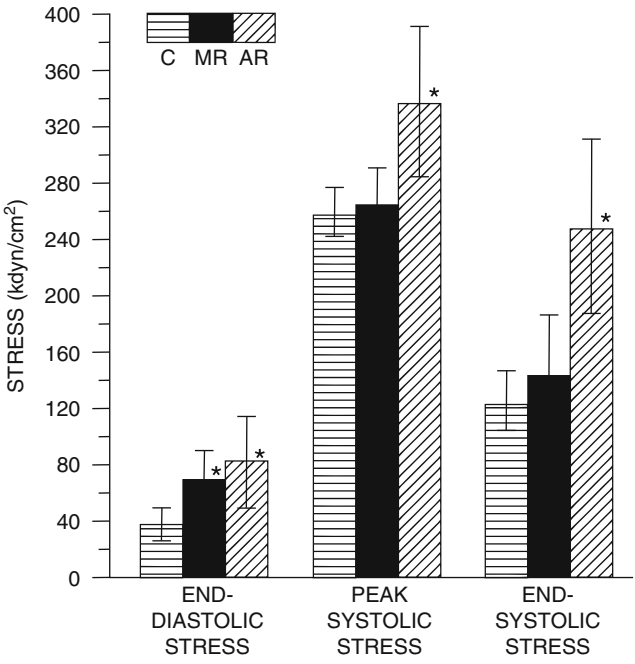
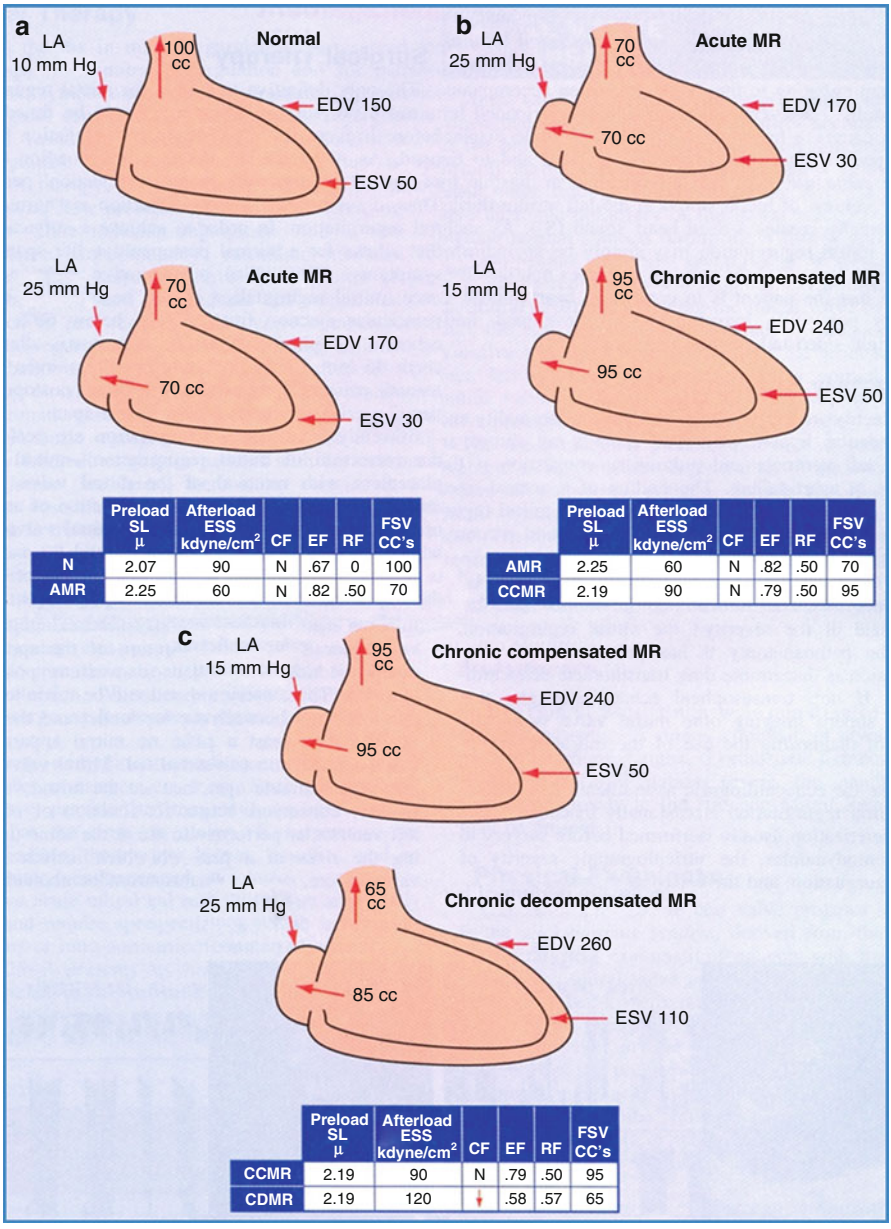


Fig. 13.1 End-diastolic and peak and end-systolic wall stress for normal subject (C) patients with mitral regurgitation (MR) and matched patients with aortic regurgitation (AR) is demonstrated. Preload represented by end-diastolic stress is elevated in both volume-overload lesions, but afterload (systolic stress) is elevated only in AR (taken from Wisenbaugh et al. [4])

While the typical MR geometry is beneficial to diastolic function, it is detrimental to systolic function. As noted above, systolic wall stress $(\sigma) = P \times r/2h$. Thus the r/h ratio forms two thirds of the Laplace equation, and r/h is increased in MR. While the leak of output into the LA during systole provides a second and low-impedance pathway for LV ejection and must in part unload the LV, the MR pattern of LV remodeling tends to increase systolic wall stress (afterload) (Fig. 13.1) [4]. Indeed, in decompensated MR, afterload is increased not decreased [8] as is often held erroneously about the pathophysiology of MR. The stages of remodeling in MR are shown in Fig. 13.2 [9]. In acute MR as might occur following rupture of a chorda tendinea, volume overload leads to increased sarcomere stretch; LV pumping ability is increased via the Frank–Starling mechanism, and

Fig. 13.2 (continued) compensated MR (CCMR). Eccentric hypertrophy and remodeling in CCMR have led to a large increase in EDV, permitting a large increase in total and forward stroke volume despite afterload returning to normal which increases ESV modestly. Increased SL still maintains a high EF. An enlarged LA now accommodates the regurgitant volume at a lower pressure. Bottom panel compares CCMR to chronic decompensated MR (CDMR). Contractile dysfunction and increased afterload impair LV emptying, increasing ESV while decreasing total and forward stroke volume. EF is also reduced but may remain within the normal range. Additional left ventricular dilatation my increase MR and RF, and LA pressure is again elevated (taken from Carabello [9])



	Preload SL μ	Afterload ESS kdyne/cm²	CF	EF	RF	FSV CC's
N	2.07	90	N	.67	0	100
AMR	2.25	60	N	.82	.50	70

	Preload SL μ	Afterload ESS kdyne/cm²	CF	EF	RF	FSV CC's
AMR	2.25	60	N	.82	.50	70
CCMR	2.19	90	N	.79	.50	95

	Preload SL μ	Afterload ESS kdyne/cm²	CF	EF	RF	FSV CC's
CCMR	2.19	90	N	.79	.50	95
CDMR	2.19	120	↓	.58	.57	65

Fig. 13.2 The stages of MR are depicted. Upper left panel compares normal physiology (N) with the pathophysiology of acute MR (AMR). Preload (sarcomere length, SL) is increased, while afterload (end-systolic stress, ESS) is decreased, and contractile function (CF) is normal. These changes increase end-diastolic volume (EDV) modestly and decrease end-systolic volume (ESV) so that ejection fraction (EF) increases. Total stroke volume increases to 140 cc, but because 50 % (regurgitant fraction, RF) of the stroke volume is regurgitated into the left atrium (LA), forward stroke volume (FSV) decreases to 70 cc. In the *upper right panel*, AMR is compared to chronic

end-diastolic volume increases modestly. The extra pathway for LV ejection into the left atrium (LA) acutely reduces afterload, reducing end-systolic volume. These changes act in concert to increase total stroke volume. However, since 50 % or more of the total stroke volume is ejected into the LA, forward stroke becomes less than normal, and cardiac output is reduced. Volume overload on the small LA increases LA pressure inducing pulmonary congestion. Thus the patient suffers heart failure even though ejection fraction is supernormal. If MR develops more gradually, the patient may enter a chronic compensated phase in which eccentric hypertrophy and remodeling allow increased total stroke volume. Supernormal diastolic compliance and enlargement of the LA permit operation at near normal filling pressure. Increased LV radius returns afterload to normal, while increased diastolic filling maintains increased preload and increased ejection fraction (EF). In this phase, the patient may be completely asymptomatic despite severe MR. However, eventually chronic volume overload leads to LV decompensation in which LV contractility is impaired. End-systolic volume and afterload increase reducing ejection fraction although EF may remain in the "normal" range. Contractile impairment is in part predicated upon loss of contractile elements and is also in part due to abnormal calcium handling [3, 10]. What causes these abnormalities is uncertain, but increased catecholamines probably play a role. Support for this concept comes from evidence that catecholamines are increased in MR patients with LV dysfunction and that beta adrenergic blockade reverses the abnormalities in contractility [11, 12]. Thus a likely sequence in the pathophysiology of MR is that remodeling, while initially compensatory, eventually causes increased afterload, forcing adrenergic overdrive to maintain cardiac output. Increased catecholamines in turn cause LV damage and reduced contractility compounding the heart failure syndrome.

It is unlikely that myocyte elongation alone can explain LV enlargement in MR. Indeed there must be some rearrangement of LV connective tissues. This aspect of remodeling is in part dependent upon matrix metalloproteinases which in turn are activated by LV stretch and also by chymase and mast cell activity [13, 14].

13.2.4 The Hypertrophic Process

The myocardial contractile proteins turn over approximately every 10 days, and LV mass is maintained by an equilibrium between protein synthesis rate (k_s) and protein degradation rate (k_d). For hypertrophy (increased muscle mass) to develop, k_s must exceed k_d, either because k_s increases or because k_d decreases. Because the myocardial proteins turn over relatively rapidly, uptake rate of a labeled amino acid as it becomes incorporated into the myocardium can be used to calculate k_s. Myosin heavy chain synthesis rate increased by 35 % within 6 h of imposition of a pressure overload (Fig. 13.3) [16]. However, no increase could be detected in k_s after imposition of the severe volume overload of MR. Nor could any increase be detected at 2 weeks, 4 weeks, or 3 months after severe MR was created in dogs, yet LV mass did

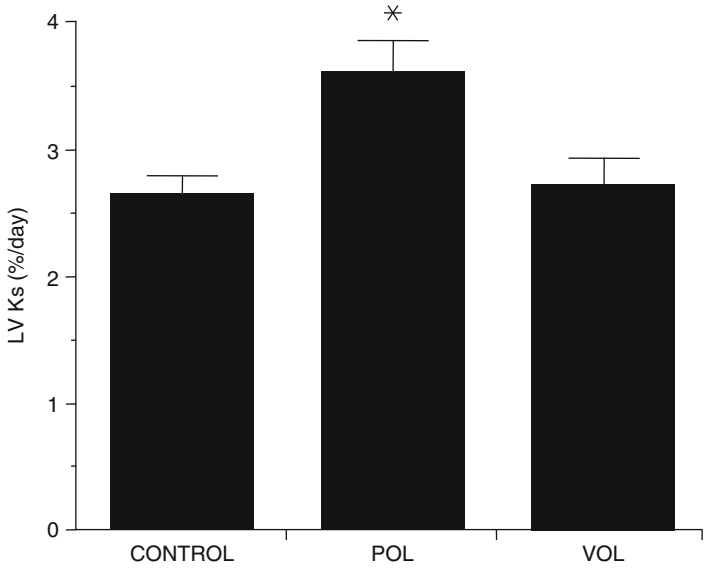


Fig. 13.3 Left ventricular (LV) myosin heavy chain synthesis rate (k_s) for control dogs, those with severe pressure overload (POL) and those with severe MR (volume overload, VOL), is demonstrated. Acute pressure overload but not acute volume overload causes a marked almost immediate (within 6 h) increase in k_s (taken from Imamura [15])

increase [17]. These data suggest that hypertrophy in MR accrues from a decrease in degradation rate [15] rather than an increase in protein synthesis rate, opposite to the mechanism of mass accrual in pressure overload hypertrophy.

13.2.5 Functional MR

In functional MR, the pathologic processes that have led to valve incompetence (myocardial infarction, ischemia, and primary cardiomyopathy) have already produced substantial eccentric LV modeling. It is highly likely that once functional MR develops, it accentuates this remodeling as shown in an ovine model of functional MR [18]. However, in any given patient, it is impossible to know how much the MR is driving the remodeling process versus how much is driven by the muscle disease independent of the MR. This fact has made it very difficult to know in whom correction of MR is likely to be effective. It does seem that once extreme eccentric remodeling has developed, mitral surgery becomes ineffective [19].

In summary, organic MR places a nearly unique hemodynamic load on the LV that of a pure volume overload. The LV responds to this burden by developing eccentric remodeling and eccentric hypertrophy, generating a thin-walled, enlarged chamber. This pattern increases the volume pumping capabilities of the LV and also

enhances diastolic filling. However, this pattern of remodeling also eventually impairs systolic emptying. While MR is often viewed as a lesion that unloads the LV via the low-impedance ejection pathway into the LA, remodeling increases systolic wall stress (afterload) and thus actually impairs ejection, reducing forward output. These factors may activate the sympathetic nervous system leading to myocardial damage and dysfunction which can be reversed by beta blocker administration and/or mitral valve repair [20, 21]. Increased muscle mass in the MR LV probably accrues not from increased protein synthesis but rather from decreased protein degradation. What effect the presence of older contractile proteins might have on the natural history of MR is currently unknown.

In functional MR, the contribution of the MR to remodeling versus that of the muscle disease that caused the MR is under intense investigations that may help to determine what therapeutic strategies will be useful in treating that entity.

13.3 Aortic Regurgitation (A Mixed Pressure and Volume Overload)

13.3.1 Load and Remodeling

As noted above for many years, aortic regurgitation and mitral regurgitation were lumped together as volume-overloading lesions. Indeed, aortic regurgitation (AR) does require the LV to increase the amount of volume that it must pump in order to compensate for volume lost to regurgitation. However, unlike MR, in AR, the entire compensatory stroke volume is ejected into the aorta. While a large portion leaks back into the LV during diastole, during systole, the large stroke volume increases systolic pressure. Recalling the $\sigma = P \times R/2th$, the increased LV systolic pressure in conjunction with the large LV radius increases systolic wall stress (Fig. 13.1). While LV thickness is indeed greater in AR than it is in MR [1, 22] (as the LV responds to systolic pressure load), increased thickness often does not compensate the increased numerator of the Laplace equation. Indeed, wall stress in AR can be as high as is seen in the pressure overload of aortic stenosis and is consistently higher than the afterload of MR [1, 4, 23, 24]. Accordingly, LV remodeling in AR differs from that of MR. There is combined eccentric and concentric LV hypertrophy, making the typical LV mass in AR the largest of human valvular heart disease.

13.3.2 Load and LV Function

Whereas afterload excess in MR only occurs in late-stage disease and is partially offset by the low-impedance LA ejection pathway, afterload excess plays

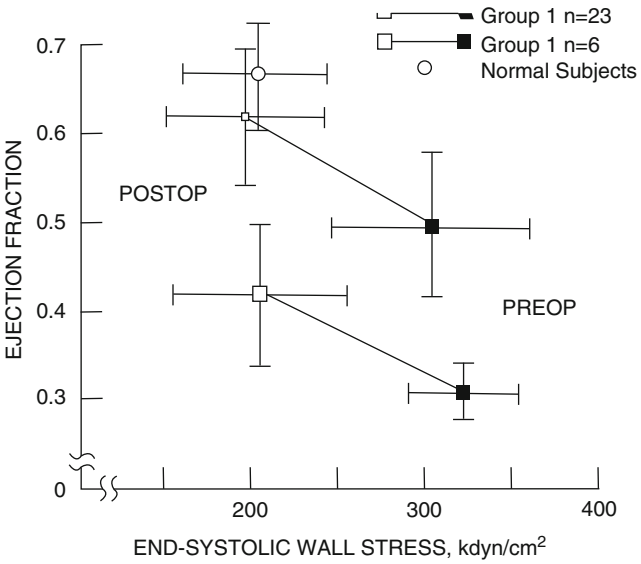


Fig. 13.4 Afterload (end-systolic wall stress) and ejection fraction are shown for normal subjects and for aortic regurgitation patients before (preop) and after (post-op) aortic valve replacement. Reduced post-op afterload both in patients with mild left ventricular dysfunction (group 1) and those with severe dysfunction (group 2) improves post-op ejection fraction (taken from Taniguchi [25])

a much more prominent role in the systolic dysfunction of AR. As can be seen in Fig. 13.4 [25], preoperative afterload excess impairs ejection fraction substantially, while aortic valve replacement leads to afterload reduction and improved postoperative EF. However, AR reduces EF not only by increasing afterload but also because persistent overload eventually impairs contractility. Borer et al. examined the contributions of afterload and contractile reserve to changes in EF during exercise [26]. If EF decreased out of proportion to exercise-induced increases in load (indicating impaired contractile reserve), prognosis was reduced. The mechanisms of LV dysfunction in AR seem different from those in MR. In both the experimental animal and in man, contractile elements of the myocardium are displaced by collagen (man) and/or fibronectin (rabbit) [27, 28], whereas in MR, collagen and other elements of the interstitium are usually normal. Also unlike MR, the hypertrophic process in AR is initiated by increased protein synthesis as is seen in pressure overload but maintained by a decrease in protein degradation as seen in the pure volume overload of MR [29]. Still other differences in AR versus MR that stem from remodeling relate to coronary blood flow reserve. Reserve is reduced in the thicker AR left ventricle [30] while normal in the thinner MR ventricle [31].

Table 13.1 Comparison of mitral (MR) and aortic (AR) regurgitation

	MR	AR
Preload	++++	++++
Afterload	+	+++
ECC hypertrophy	+	++
ECC remodeling	+++	++++
CONC hypertrophy	0	++
MECH SYS DYS	AE, Ca++	F, CBF
Diastolic PROP	++	–

AE adrenergic excess, *Ca++* abnormality in calcium handling, *CBF* coronary blood flow abnormality, *DYS* dysfunction, *ECC* eccentric, *F* fibrosis, *MECH* mechanism, *SYS* systole

13.4 Summary

Aortic regurgitation and mitral regurgitation hold in common the creation of a volume overload on the left ventricle. However, apart from this similarity, many differences exist between the character of these two overloads and the ventricular remodeling and hypertrophy that they produce (Table 13.1). MR is a "pure" volume overload leading to eccentric remodeling and a thin-walled dilated LV with modest hypertrophy and supernormal diastolic properties. "Paradoxically," this pattern of LV geometry tends to increase afterload despite the low-impedance pathway for LV ejection into the LA. On the other hand, AR causes a combined pressure and volume overload on the LV, leading to the heaviest chamber in valvular heart disease. Afterload excess plays a prominent role in the mechanism of LV dysfunction in AR, and geometry and architecture are far different from those found in patients with MR.

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Chapter 14
Reducing Oxidative Stress and Manipulating Molecular Signaling Events Using Resveratrol as a Therapy for Pathological Cardiac Hypertrophy

Shereen M. Hamza, Miranda M. Sung, and Jason R.B. Dyck

Abstract Left ventricular hypertrophy is a significant independent risk factor for sudden cardiac death, dysrhythmia, heart failure, ventricular ischemia, and coronary heart disease. However, the pathophysiological mechanisms which lead to the development of cardiac hypertrophy and eventually heart failure are not completely understood. Although the specific steps that lead to hypertrophy are not altogether clear, it appears that oxidative stress plays a significant role. As such, the possibility of a treatment approach involving antioxidant therapies such as the naturally occurring polyphenol resveratrol is a potentially fruitful strategy that can be used to target several signaling and pathogenic events in the etiology of cardiac hypertrophy and may be useful alone or in combination with other drugs. Over the past 15 years, a great deal of evidence supporting the amelioration of hypertrophy and improvement of cardiac function by resveratrol has been documented. However, this is largely in cell-based and animal models with limited evidence from human studies. Clinical trials utilizing resveratrol as a supplementary therapy in heart failure patients are just underway, and much is left to be understood regarding the distinct cellular mechanism of action of this compound in physiological and pathological states. Thus modulation of oxidative stress pathways and cardiac function by resveratrol remains an exciting and undoubtedly rewarding area of research with important clinical implications.

Keywords Oxidative stress • Resveratrol • Hypertrophy • Cardiac metabolism • AMPK • MAPK • NOS • Nitric oxide • Pressure overload

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14.1 Introduction

Increased hemodynamic load on the left ventricle initiates a cascade of events that leads to ventricular remodeling, cardiac hypertrophy, and eventually heart failure [1]. This ventricular remodeling is a myocardial response to various stresses and involves gradual and progressive changes in chamber architecture, cardiomyocyte phenotype, as well as non-myocytic components [2, 3]. Clinical conditions that produce the pressure-overload stimulus include essential hypertension, aortic stenosis, aortic coarctation, and drug-induced hypertension. The pattern of ventricular remodeling and hypertrophy is dependent upon the type of cardiac stress. In the case of pressure overload, the left ventricle responds with concentric hypertrophy in contrast to the eccentric hypertrophy which occurs in response to volume overload. The process of hypertrophy is initially considered to be an adaptive response because it serves to normalize wall stress and helps to mechanically compensate heart function. However, with progression of the stimulus, the initial compensatory hypertrophy can lead to maladaptive responses that can eventually lead to heart failure. Although the exact point at which hypertrophy becomes maladaptive is difficult to identify, there may be a clear delineation of compensated and decompensated phases of hypertrophy [4–6]. Alternatively, it has also been proposed that the initial hypertrophic process involves a combination of both adaptive and maladaptive cardiac processes and that initiation of any pathological hypertrophy will eventually compromise heart function [7].

Left ventricular hypertrophy is recognized as a major independent risk factor for cardiovascular morbidity and mortality from sudden cardiac death, dysrhythmia, heart failure, ventricular ischemia, and coronary heart disease [6, 8–11]. A growing body of evidence suggests that oxidative stress, which occurs when the generation of reactive oxygen species (ROS) exceeds the cell's intrinsic antioxidant defenses, is an important contributor to the development and progression of cardiac hypertrophy, in part by activating multiple intracellular signaling pathways [12]. Therefore, ameliorating oxidative stress has been proposed as a potential strategy for preventing and/or treating pathological cardiac hypertrophy and heart failure [13, 14]. Our current understanding of the pathophysiology of cardiac hypertrophy as well as potential new therapies targeting oxidative stress will be reviewed herein.

14.2 Physiological Process of Ventricular Hypertrophy

It is generally accepted that mechanical stress begins the cascade of events that leads to ventricular remodeling. However, it is important to note that in vivo, there is rarely, if ever, an isolated increase in pressure independent of any neurohormonal activation. As a result, pressure overload involves mechanical as well as neurohormonal activation that then modulates the ventricular response to stress.

Left ventricular hypertrophy begins with normal ventricle and myocyte function; however, with prolonged hypertrophy this situation eventually decompensates into a hypertrophied ventricle with corresponding myocyte dysfunction. These changes influence both structural and mechanical properties of the myocardial tissue and coronary circulation [1].

At the cellular level, concentric left ventricular hypertrophy is identified by an increase in cardiomyocyte cell size, parallel addition of new sarcomeres, and enhanced protein synthesis arising from alterations in gene transcription and translation [15]. In addition to changes to the cardiomyocyte, left ventricular hypertrophy is also characterized by proliferation of fibroblasts, increased generation of extracellular matrix proteins, and fibrosis [8, 16, 17]. This increased fibroblast growth in combination with the deposition of collagen and fibrosis leads to myocardial stiffening, which on its own is an important contributor to cardiac dysfunction [18, 19]. In the adult mammalian heart, α-myosin heavy chain (MHC) is the predominant isoform expressed and possesses a high ATPase activity and faster rate of contractility than the fetal β-MHC isoform. During hypertrophy, α-MHC expression is reduced, and β-MHC expression is increased which is characteristic of the re-induction of the fetal gene program [20]. Indeed, the transition from α- to β-MHC isoforms that occurs during pathological cardiac hypertrophy may play a role in the development of cardiac dysfunction by reducing myocardial contractility [19].

Generally there are three broad steps regarding the molecular mechanism of ventricular hypertrophy. Initially, heart remodeling is activated by biomechanical stress induced by increased pressure. The left ventricle is stretched during diastole and overloaded during systole which initiates both local myocardial and systemic neurohormonal responses. The mechanical stretch is sensed by myocyte membrane and sarcomere-coupled stretch receptors [21], whereas neurohormonal signals [i.e., endothelin-1 (ET-1), angiotensin-II (Ang-II), catecholamines] are sensed by G protein-coupled receptors that lead to activation of $G\alpha_{q/11}$ proteins [22]. In the second stage of the hypertrophic process, these mechanical and neurohormonal stimuli activate intracellular signaling pathways which eventually transmit a signal to the myocyte nucleus [7, 21]. Finally these signaling pathways activate transcription factors to induce expression of particular genes in the cardiac myocyte which ultimately causes phenotypic changes in the myocytes as well as altered gene transcription that includes re-expression of the fetal gene profile [i.e., atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP)] and new cellular growth [21, 23]. Activation of specific pathways leads to beneficial and adaptive remodeling, while activation of other pathways eventually has maladaptive and harmful results [21, 24]. Ultimately the imbalance between maladaptive and beneficial signaling results in the development of pathological hypertrophy and the progression to heart failure. Since multiple molecular signaling pathways have been shown to be involved in the development of pathological hypertrophy, these pathways are becoming potential targets for cardiovascular treatments [25].

14.3 Transition from Compensated Hypertrophy to Heart Failure

As mentioned, the initial stimulus for hypertrophy may also stimulate maladaptive events in the cardiac tissue [2, 18, 26]. Several of the initial changes occurring at the level of the cardiomyocyte may be detrimental in left ventricular hypertrophy. For instance, the aforementioned re-expression of the fetal gene profile also induces a change in energy metabolism that results in enhanced glycolysis and a reduction in overall oxidative metabolism [27]. Rates of glycolysis are accelerated in the hypertrophied heart likely as an initial adaptive mechanism to increase ATP production in the presence of impaired oxidative metabolism [28–31]. As a result of this metabolic reprogramming, there is a decrease in myocardial ATP production, leading to energy deprivation [23, 32]. In addition, when glucose oxidation is not correspondingly increased along with glycolysis, there is an accumulation of protons and ROS [28, 33, 34]. Moreover, the reduction in fatty acid utilization itself may lead to lipid accumulation within cardiomyocytes, which has been shown to result in contractile dysfunction [35]. In addition to metabolic changes, apoptosis of cardiomyocytes is augmented in hypertensive heart disease, which leads to a reduction in contractile mass and affects contractility [36]. Other events which occur during hypertrophy such as collagen deposition may promote ventricular stiffness and diastolic dysfunction and heart failure [37–39]. As a result of these and a myriad of other changes that occur [1], it is apparent that myocardial hypertrophy in the setting of pressure overload is ultimately a maladaptive process that should be prevented or reversed in order to prevent the development of impaired cardiac contractile function and heart failure.

14.4 Oxidative Stress in Cardiac Hypertrophy and Heart Failure

Although the fundamental mechanisms underlying the development and progression of cardiac hypertrophy and heart failure are not fully understood, evidence from experimental models of heart failure and clinical studies demonstrates increased oxidative stress in the failing myocardium and strongly supports the concept that it plays a major role in the development of cardiac hypertrophy and progression to heart failure [40–43]. Oxidative stress is defined as the excessive production of ROS relative to the body's natural antioxidant systems. ROS are a group of molecules including oxygen and its derivatives that are produced in all aerobic cells; this includes highly reactive free radicals such as superoxide anion ($^{\bullet}O_2^-$) and hydroxyl radical ($OH^{\bullet}$), as well as compounds such as hydrogen peroxide (H_2O_2) that can be converted to reactive radical species [13]. Nitric oxide (NO) is an important molecule that regulates normal cardiac function by activating soluble guanylyl cyclase and synthesis of intracellular cGMP to inhibit activation of hypertrophic signaling.

In the setting of oxidative stress, $^{\bullet}O_2^-$ can react with NO to form highly reactive species such as peroxynitrite, which has cytotoxic effects and further reduces NO bioavailability.

There are several enzymatic sources of ROS in mammalian cells including mitochondrial respiration, arachidonic acid pathway enzymes (lipoxygenase and cyclooxygenase), cytochrome p450s, xanthine oxidase, NADH/NADPH oxidases, nitric oxide synthase (NOS), peroxidases, and other hemoproteins [12]. The three key producers of ROS in the cardiovascular system are xanthine oxidase, NADH/NADPH oxidase, and NOS [12]. To combat ROS, the body has many specific and nonspecific antioxidant defense mechanisms that scavenge and degrade ROS to nontoxic molecules, including scavenging enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase as well as nonenzymatic antioxidants, such as vitamins E and C [44]. Under normal conditions, SOD converts $^{\bullet}O_2^-$ to H_2O_2, which is then further detoxified by catalase and glutathione peroxidase to H_2O [13]. Excessive production of ROS causes cell dysfunction, protein and lipid peroxidation, and DNA damage—all of which lead to cell damage and death [12]. In addition, ROS appear to participate in several steps involved in the development and progression of myocardial remodeling and failure, including modification of proteins critical for proper contractile function [45], activation of hypertrophy signaling kinases or transcription factors [46] and apoptosis [47], as well as stimulation of cardiac fibroblast proliferation and activation of matrix metalloproteinases leading to extracellular matrix remodeling [48–50].

14.4.1 Cardiac Sources of Reactive Oxygen Species

Increased oxidative stress in the failing heart may be reflective of an increase in the production of ROS from many sources and also a deficiency in antioxidant capacity. The mechanisms responsible for increased oxidative stress in cardiac hypertrophy are unknown, and little is known about the mechanism by which stimuli such as mechanical strain, neurohormones, or cytokines lead to increased ROS. However, cardiac sources of ROS include cardiac myocytes, endothelial cells, and neutrophils. Within cardiac myocytes, ROS can be produced by mitochondria due to leakage of electrons from the respiratory chain which results in the conversion of oxygen to $^{\bullet}O_2^-$ [51]. Mitochondria from the failing heart produce more superoxide than normal mitochondria, suggesting that mitochondrial electron transport could be a predominant source of $^{\bullet}O_2^-$ production [52]. In addition, these mitochondria were associated with decreased complex enzyme activity, making mitochondria an important source of ROS in failing hearts and indicating a link between mitochondrial dysfunction and oxidative stress [52].

Regarding ROS production via oxidative enzymes, NADPH oxidase activity was found to be increased by stimuli relevant to the development of heart failure (i.e., mechanical stretch, Ang II, ET-1, tumor necrosis factor-α) [53]. This increased cardiac NADPH oxidase activity has also been observed in human heart failure [54];

increased xanthine oxidase expression and activity have been similarly noted [55]. In addition to these sources of ROS, uncoupled NOS has also been shown to produce ROS in a variety of pathological conditions [56, 57]. Under normal conditions, endothelial NOS (eNOS) also known as NOS3 catalyzes the formation of NO from L-arginine and oxygen. Uncoupling of NOS due to absence of the necessary cofactor tetrahydrobiopterin (BH4), for example, produces $^{\bullet}O_2^-$ which is known to contribute to the progression of atherosclerosis and hypertension [58, 59]. eNOS is also uncoupled and figures importantly in pathological remodeling and progression to heart failure in response to chronic pressure overload in mice [60].

In addition to being a potential source of ROS during cardiac hypertrophy, mitochondria themselves can also be damaged by ROS. As ROS damage mitochondrial components, the $^{\bullet}O_2^-$ produced within the mitochondria is unable to pass through the membranes, causing still more damage to this organelle, thus compromising mitochondrial function [61]. Increased ROS generation in failing hearts was associated with mitochondrial damage and dysfunction with increased lipid peroxidation in the mitochondria and reduced oxidative capacity [62]. This establishes a vicious cycle in which mitochondrial functional decline promotes further ROS generation, more mitochondrial damage, and cellular injury.

14.5 Hypertrophic Signaling Pathways

A number of complex signaling cascades have been identified that regulate the hypertrophic process in the heart. There appear to be distinct signaling cascades that distinguish physiological versus pathological hypertrophy (see [63] for review of physiological hypertrophy). At a molecular level, pathological cardiac hypertrophy is mediated by several molecular growth signals and induction of fetal gene expression programs (for proteins involved in cardiac contractility and calcium handling) [22, 63]. Some of the better characterized signaling pathways involved in the development of pathological cardiac hypertrophy will be discussed in the section below, with a focus on those pathways where ROS have been shown to be important in activation of these signaling pathways. Indeed, ROS are known to regulate the activity of a variety of signaling kinases and transcription factors to mediate the cardiac hypertrophic response, including calcineurin–NFAT, mitogen-activated protein kinases (MAPKs), and AMP-activated protein kinases (AMPK) among others [63].

14.5.1 Calcineurin–NFAT Signaling

Calcineurin is a ubiquitously expressed calcium (Ca^{2+})–calmodulin-regulated serine/threonine phosphatase that consists of a catalytic A and regulatory B subunit. In response to sustained increases in intracellular Ca^{2+} levels, Ca^{2+}/calmodulin complexes bind to the B subunit, inducing a conformational change allowing calcineurin

to dephosphorylate downstream effector proteins, most notably the members of the nuclear factor of activated T-cells (NFAT) family of transcription factors [64]. NFAT transcription factors are normally sequestered in the cytoplasm; however, upon calcineurin-mediated dephosphorylation of NFAT, these proteins rapidly translocate into the nucleus and activate gene expression [65, 66]. Calcineurin activation is observed in hypertrophied hearts following pressure overload induced by constriction of the abdominal aorta [67]. Moreover, partial inhibition of calcineurin by nonspecific immunosuppressive drugs cyclosporin A (CsA) and FK506 has been shown to prevent hypertrophy in some studies [67, 68].

Using a more targeted genetic approach, several transgenic mice have been generated and characterized in order to better define the role of calcineurin in the development and progression of cardiac hypertrophy. Mice expressing a constitutively active form of calcineurin in the heart are sufficient to induce cardiac hypertrophy (two- to threefold increase in heart size) and the expression cardiac fetal hypertrophic genes (i.e., BNP, β-MHC), a phenotype which then rapidly progresses to dilated cardiomyopathy, interstitial fibrosis, and heart failure [66]. Furthermore, targeted inhibition of calcineurin by cardiac overexpression of the inhibitory domains of Cain/Cabin-1 and A-kinase anchoring protein (AKAP) 79, which are endogenous calcineurin inhibitors, reduced cardiac hypertrophy in response to both isoproterenol infusion and pressure overload [69].

Calcineurin activity is negatively regulated by myocyte-enriched calcineurin-interacting protein (MCIP1), which is highly expressed in striated muscle and capable of binding and inhibiting the activity of calcineurin [70]. The cardiac hypertrophic response induced by aortic banding was significantly blunted in transgenic mice overexpressing the calcineurin inhibitory domain from MCIP1 [71]. Mice expressing either a cardiac-specific dominant-negative mutant of calcineurin [72] or a deletion of calcineurin Aβ [73] both displayed significantly less cardiac hypertrophy following aortic banding. Lastly, similar protection from pressure-overload-induced hypertrophy is observed in mice with targeted disruption of NFATc3, a downstream transcription factor effector of calcineurin-mediated hypertrophy [74]. Collectively these different animal models provide strong evidence that the calcineurin–NFAT pathway plays a fundamental role in regulating sustained hypertrophic growth of the heart. Moreover, it is thought that activation of the calcineurin–NFAT pathway is specific to pathological hypertrophy, such as that produced by hypertension or aortic constriction, and does not contribute to the development of physiological cardiac hypertrophy (i.e., exercise), which does not lead to deleterious consequences, thus creating a distinction between signaling pathways responsible for the two different types of cardiac hypertrophy [64].

Alterations in calcium homeostasis, as a result of changes in expression and/or activity of Ca^{2+} handling proteins, are a common phenomenon observed in the setting of cardiac hypertrophy and heart failure [75]. Since cardiac contractile function is highly dependent upon intracellular Ca^{2+} concentration, modifications in Ca^{2+} handling proteins can have a profound effect on cardiac contractility and function [76]. Oxidative stress plays a major role in the indirect regulation of intracellular Ca^{2+} homeostasis and in general is thought to lead to a rapid increase in cytosolic Ca^{2+} levels.

Both the sarcoplasmic reticulum ATPase (SERCA2), an ATP-dependent Ca^{2+} pump that transports Ca^{2+} from the cytoplasm into the endoplasmic/sarcoplasmic reticulum, and the ryanodine receptor (RyR) SR Ca^{2+} release channels are redox sensitive [45]. ROS have been shown to reduce expression and/or activity of the SERCA2A [77] and increase the activation of the RyR [78]. Indeed, SERCA protein expression and activity are found to be decreased in pressure-overload-induced cardiac hypertrophy [79]. As well, oxidants have been shown to stimulate reverse mode of the sarcolemmal sodium–calcium exchanger (NCX) to facilitate Ca^{2+} entry into the cell [45]. Therefore, together these mechanisms may lead to an overall increase in intracellular Ca^{2+} levels that then may result in activation of the calcineurin–NFAT signaling pathway to trigger hypertrophy of the myocardium [80].

Since the cardiac myocyte is constantly beating with cyclic Ca^{2+} transients mediating excitation–contraction coupling, it is likely that Ca^{2+}-dependent activation of the calcineurin–NFAT pathway is more complex than in other cell types where sustained increases in total intracellular Ca^{2+} are sufficient to trigger activation of this pathway [81]. More recently, it has been proposed the existence of specialized cellular microdomains where Ca^{2+} concentration is locally regulated and sensed by Ca^{2+}-dependent proteins localized within these microdomains [81]. Transient receptor potential (TRP) channels, which are G protein-coupled receptor (GPCR)-operated Ca^{2+} channels that are located on the sarcolemma and membrane of the SR/ER, are now emerging as potential key regulators of Ca^{2+} entry during hypertrophy [82]. Indeed, TRP channel expression and activity are upregulated in pathological hypertrophy and heart failure [83–85]. Transgenic mice overexpressing various forms of TRP channels (i.e., TRPC3 and TRPC6) display marked ventricular hypertrophy at baseline and are more sensitive to pressure-overload-induced hypertrophy, which is associated with increased calcineurin–NFAT activity [85, 86]. Moreover, targeted disruption of the calcineurin Aβ gene blocked the exaggerated hypertrophic response in TRPC3 transgenic mice when subjected to TAC [86], supporting a link between TRPC and activation of the calcineurin–NFAT pathway. ROS generated during pressure-overload hypertrophy may also contribute to activation of TRP channels in this pathological setting [87]. Collectively these data suggest that TRP channels may play an important role in mediating pathological cardiac hypertrophy, likely by increasing local Ca^{2+} concentrations in microdomains resulting in activation of the calcineurin–NFAT signaling pathway and subsequent gene expression.

14.5.2 Mitogen-Activated Protein Kinases (MAPKs)

In general, mitogen-activated protein kinases (MAPKs) are divided into three classes based on the terminal kinase in the pathway: the extracellular signal-regulated kinases (ERKs), the *c-Jun* amino-terminal kinase (JNKs), and the p38 MAPKs [88–90]. There is evidence for activation of all three families of MAPKs in cultured cardiac myocytes in response to hypertrophic stimuli (i.e., mechanical stress, GPCR agonists, Ca^{2+}) [91–93], in the experimental pressure-overloaded heart

[94, 95], and in the human failing heart [96]. Although these MAPKs have been largely considered to be pro-hypertrophic, the precise role of each of these MAPKs in the pathophysiology of cardiac hypertrophy and whether they are necessary mediators and/or modulators of the hypertrophic process remains to be clearly identified. However, early during the development of cardiac hypertrophy, it has been thought that ROS may contribute to activation of each these MAPK signaling pathways [97]. As these kinases are considered pro-hypertrophic, antioxidant therapies that target these signaling pathways may also prove to be advantageous in the treatment of pathological cardiac hypertrophy and the progression to heart failure.

14.5.3 Extracellular Signal-Regulated Kinases (ERKs)

ERK1/2 are ubiquitously expressed protein kinases that are activated in the setting of pathological cardiac hypertrophy and heart failure [63, 90, 98, 99]. In cultured cardiac myocytes, ERK1/2 are activated in response to pro-hypertrophic agonist stimulation, oxidative stress, or mechanical loading [91, 97, 100, 101]. Furthermore, activation of ERK 1/2 is required for increased protein synthesis in isolated cardiac myocytes in response to hypertrophic stimuli, such as ET-1 and α-adrenergic agonists [102]. Consistent with these in vitro findings, expression of a dominant-negative mutant of Raf-1, an upstream kinase of Erk1/2, inhibited the activation of ERK1/2 and blunted development of pressure-overload-induced cardiac hypertrophy [103]. Interestingly, mice with expression of cardiac-specific constitutively active MEK1, an immediate upstream MAPK kinase of ERK1/2, led to the development concentric physiological hypertrophy associated with enhanced cardiac systolic function without decompensation over time [104]. Furthermore, neither a global deletion of ERK1 or ERK1 −/− and ERK2 +/− mice led to a reduction in cardiac hypertrophy in mice subjected to TAC [105], suggesting that ERK1/2 may be sufficient, but not critical for mediating pathological pressure-overload-induced cardiac hypertrophy.

14.5.4 c-Jun Amino-Terminal Kinase (JNKs)

The JNK family consists of at least ten isoforms derived from three mammalian genes: JNK1, JNK2, and JNK3 [106]. JNK is rapidly activated in vitro in response to multiple cellular stresses, including mechanical stress, oxidative stress, and proinflammatory cytokines [106]. Activation of JNK is mediated via phosphorylation by MAPK kinase 4 (MEK4) and MEK7, which in turn are activated by MAPK kinase kinase (MEKK1) [107, 108]. Although several in vitro studies suggest that JNKs may contribute to the regulation of pathological hypertrophy [109, 110], data from in vivo studies have proven inconclusive. Studies in transgenic mouse models with blunted JNK activation have provided conflicting results with some showing

that cardiac hypertrophy in response to pressure overload is either attenuated [111] or enhanced [112]. Moreover, TAC in mice with selective deletions of JNK1, JNK2, or JNK3 show that the cardiac hypertrophy developed to a similar extent as in wild-type mice [113], suggesting that either JNK isoforms perform similar functions and are redundant or that JNK activation may not be required for cardiac growth. At present, the exact role of JNK in the development of pathological cardiac hypertrophy remains unclear.

14.5.5 p38 MAPKs

The p38 MAPK family is an important regulator of a diverse array of biological functions including cell growth, cell proliferation, metabolism, and cell death [108, 114]. Of the four p38 isoforms, it appears that only p38α and p38β are expressed in the heart. Similar to JNK, p38 is a stress-activated protein kinase that is activated by a multitude of external stimuli including cytokines, oxidative stress, osmotic stress, and growth factors among others (see [63] for review). In vitro studies have largely supported a key role for p38 in promoting cardiac growth and hypertrophy with small molecular inhibitors of p38 and dominant-negative p38 adenovirus inhibiting hypertrophic growth [115]. Similar to the MAPKs described above, myocardial p38 activity is found to be increased by pressure overload [95] and ET-1/phenylephrine stimulation [116, 117]. Despite these findings, targeted activation of p38 in the heart by transgenic overexpression of upstream kinases MKK3 or MKK6 did not produce a significant degree of cardiac hypertrophy in the basal state. However, these mice did have increased interstitial fibrosis and ventricular wall thinning and died prematurely at 7–9 weeks as a consequence of heart failure [118], suggesting that p38 may be important in mediating late-stage ventricular remodeling as opposed to hypertrophy. Furthermore, studies in cardiac-specific p38 dominant-negative transgenic mice (DN-p38) showed that loss of p38 activity either had no effect on the development of cardiac hypertrophy [119] or enhanced the hypertrophic response [120] to pressure overload by aortic banding. In support of the latter finding, p38 MAPK has been shown to inhibit NFAT-transcriptional activity and nuclear translocation by directly phosphorylating NFAT [120, 121], suggesting that p38 may in fact be a negative regulator of hypertrophy. As several studies performed by different groups even in the same transgenic mice have given contradictory results, the precise role of p38 in the development and progression of cardiac hypertrophy and heart failure remains unclear.

14.5.6 AMP-Activated Protein Kinase (AMPK)

AMPK is a key metabolic sensing serine/threonine kinase that is activated by cellular and metabolic stresses that deplete ATP (i.e., ischemia). AMPK responds to increases in the AMP/ATP ratio by activating energy-producing metabolic pathways

and inhibiting energy-consuming pathways [122]. The exact role of AMPK in cardiac hypertrophy has not yet been clearly defined. Protein translation and synthesis are known to be necessary mediators of increased myocardial cell size, and pharmacological activation of AMPK (i.e., AICAR, resveratrol) has been shown to inhibit protein synthesis associated with cardiac hypertrophy via numerous molecular pathways (see [122, 123] for review). Thus, while AMPK may act as a negative regulator of cardiac hypertrophy [124–127], it is not certain if inactivation of AMPK is a necessary step of the hypertrophic process or if it simply creates a permissive environment for cardiac growth. Supporting this idea, recent evidence has shown that impaired AMPK activity makes the heart more susceptible to pro-hypertrophic stimuli, such as hemodynamic overload [128–130]. In the SHR, increased oxidative stress [specifically the lipid peroxidation by-product, 4-hydroxy-2-nonenal (HNE)] leads to a reduction in AMPK activity via inhibition of its upstream activating kinase LKB1 [128], and this was associated with the development of cardiac hypertrophy. Consistent with studies in isolated myocytes, impaired AMPK activity was associated with activation of the mTOR/p70S6 kinase pathway that regulates protein synthesis in the heart [128]. Furthermore, cardiomyocyte-specific deletion of LKB1 results in left ventricular hypertrophy [131], suggesting that modification of LKB1 activity may contribute to the hypertrophic process. Although some studies have shown that the activation of AMPK is associated with the development of pressure-overload-induced cardiac hypertrophy [132], this is likely due to the heart becoming energetically compromised in the later stages of the disease and requiring AMPK to restore depleted ATP levels. Therefore, AMPK may play dual roles in the cardiac hypertrophic process whereby early in the disease, inactivation of AMPK may be necessary for cardiac growth, whereas activation of AMPK occurs during the later stages in order to maintain adequate ATP supply to the heart (see [122] for review).

14.5.7 JAK–STAT Pathway

Janus kinase (JAK) proteins are a family of cytosolic tyrosine kinases associated with the intracellular domain of membrane-bound receptors, which act to transduce signals from extracellular ligands such as cytokines, growth factors, and hormones to the nucleus to elicit cellular responses [133]. The JAK family of proteins rapidly transduces signals by recruitment of signal transducers and activators of transcription pathway (STAT) transcription factors. The JAK–STAT pathway plays a critical role in cardiac hypertrophy and the transition from hypertrophy to failure as well as mediates signal transduction from gp130 cytokine receptor [134] and GPCR [135] to the nucleus. Gp130 is a promiscuous receptor for several cytokines including interleukin 6/11 and transduces its signal mainly through induction of STAT3. Specifically, STAT3 is translocated to the nucleus in response to gp130 activation, which results in the induction of genes involved in hypertrophy [136]. Overexpression of STAT3 is sufficient to induce cardiomyocyte hypertrophy under both in vitro [137] and in vivo [138] settings. Transgenic mice with a deletion of gp130 in the

myocardium have reduced STAT3 activity and display normal cardiac structure and function at baseline; however, these mice fail to develop compensatory hypertrophy following acute pressure overload and develop heart failure [139]. Therefore, the JAK–STAT pathway may be a novel therapeutic target for developing agents that prevent the development and/or progression of pathological cardiac hypertrophy.

The signaling pathways involved in cardiac hypertrophy are numerous and complex. The generation and characterization of transgenic rodent models have allowed investigators to better delineate the potential molecular mechanisms responsible for mediating distinct forms of cardiac growth. However, future research is still needed to clearly identify the pathways that are most critical for the development of pathological cardiac hypertrophy, and in doing so this may provide new targets for drug discovery in the management and treatment of cardiac hypertrophy and heart failure.

14.6 Antioxidants: Implications for Therapy

Since oxidative stress has been strongly implicated in the pathophysiology of cardiac hypertrophy induced by pressure overload, antioxidant therapies have received much attention as a potential therapeutic strategy to prevent and/or regress cardiac hypertrophy and/or prevent the transition to heart failure. To date, this therapeutic approach using antioxidants such as vitamin E and *n*-acetylcysteine, while having had some success in experimental animal models, has not successfully translated to the clinic for use in humans [140]. However, the natural polyphenol resveratrol that is found in a number of dietary food sources is emerging as a potential novel new therapy that may be a treatment option for those with cardiovascular disease, in particular cardiac hypertrophy.

14.6.1 Vitamin E

The antioxidant vitamin E is a naturally occurring lipid-soluble vitamin found in a variety of food sources, including nuts and sunflower seeds, and is a nonenzymatic part of the cell's intrinsic antioxidant system to counter the accumulation of ROS [141]. Indeed, symptoms in humans with a vitamin E deficiency further support that vitamin E plays an important role in protecting membranes and the nervous system from oxidative stress [142]. Of the various forms of vitamin E, α-tocopherol is the most abundant and biologically active form of vitamin E, with the acetate and synthetic forms of α-tocopherol being the primary constituents of vitamin E supplements [141, 143]. Vitamin E is a potent peroxyl radical scavenger that breaks radical-propagated chain reactions and due to its lipid solubility is present in cell membranes and plays an integral role in protecting cell membranes and plasma lipoproteins from lipid peroxidation [142, 143]. Indeed, peroxyl radicals (ROO^{-}) react at a rate 1,000 times faster with vitamin E than with polyunsaturated fatty acids [144].

In experimental models, treatment with vitamin E has been shown to reduce oxidative stress and prevent the development of cardiac hypertrophy and heart fail-

ure [145]. In the guinea pig model of pressure overload, chronic vitamin E treatment improved cardiac function and blunted the progression of heart failure over 20 weeks. Interestingly, however, this occurred in the absence of improvements in hypertrophy [145]. In contrast, clinical studies in humans have produced conflicting results regarding the cardiovascular benefit of vitamin E supplementation with some studies showing a significant reduction in cardiovascular risk [146–151], as well as a number of studies having failed to show a benefit to vitamin E supplementation [152–155]. Therefore, simply supplementing the antioxidant defenses of the heart using vitamin E may be insufficient to prevent cardiac hypertrophy and cardiovascular disease.

14.6.2 N-Acetylcysteine

N-acetylcysteine is a widely used thiol-containing antioxidant that is a pharmacological precursor of L-cysteine [156]. Reduced thiols are molecules with a sulfhydryl group whose biological properties include scavenging of oxygen free radicals, acting as cofactors for enzymatic reactions and potentiating the half-life and activity of NO by forming NO adducts (*S*-nitrosothiols) which are more stable than NO itself [157, 158]. Oral treatment with *N*-acetylcysteine (500 mg/kg/day) administered in the drinking water for 7 days led to a marked reduction in left ventricular weight induced by 2 weeks of abdominal aortic constriction in mice [159], which is consistent with a major role for ROS in the generation of pressure-overload-induced left ventricular hypertrophy in vivo. Interestingly, *N*-acetylcysteine administration (1.5 g/kg/day) to adult spontaneously hypertensive rats with established hypertension is both unable to reduce blood pressure and cardiac hypertrophy [160, 161]. Due to a lack of large-scale clinical trials, it is not clear whether *N*-acetylcysteine is effective in reducing cardiac hypertrophy in humans. However, *N*-acetylcysteine may be beneficial following myocardial infarction to limit infarct size [162, 163]. As well, *N*-acetylcysteine treatment has been shown to improve endothelial function and reduce systolic blood pressure in hypertensive diabetic patients via a reduction in oxidative stress and increased NO bioavailability [164]. This suggests that *N*-acetylcysteine can reduce oxidative stress in vivo and may be a useful antioxidant strategy to supplement current therapies in a variety of cardiovascular conditions.

14.7 Resveratrol as an Antioxidant

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenol that is found in a variety of berries, grapes, peanuts, and medicinal plants. Scientific interest in resveratrol has increased over the past 15 years since it has been shown to be a calorie-restriction mimetic and increase overall health in mammals [165]. Of relevance, resveratrol also appears to prevent the development of pressure-overload-induced cardiac

hypertrophy [128, 166, 167] as well as show benefit in a variety of other cardiovascular diseases [168]. Indeed, resveratrol has many properties which are considered the basis of its cardiovascular effects and protection. Some of these include antioxidant, $^{\bullet}O_2^-$ scavenging, ischemic preconditioning, and angiogenic actions [169]. The exact mechanism of action of resveratrol in vivo has not been unequivocally determined; however, many cell components and processes are thought to be involved such as surface receptors, signaling pathways, metabolic pathways, nuclear receptors, and gene transcription and translation [170]. The antioxidant and $^{\bullet}O_2^-$ scavenging properties of resveratrol are of interest as they directly relate to the potential therapeutic benefits of this compound to reduce oxidative stress in the setting of cardiac hypertrophy. Some experiments suggest that resveratrol can directly scavenge $^{\bullet}O_2^-$ in potassium superoxide and xanthine oxidase-based antioxidant systems [171], with added evidence of direct inhibition of xanthine oxidase. Resveratrol has also been shown to inhibit NADPH oxidase and subsequently reduce $^{\bullet}O_2^-$ production as a mechanism for vasorelaxation in isolated rat aorta [172]. Additional evidence supports direct scavenging of ROS by resveratrol [173, 174] as well as increased expression of antioxidant enzymes such as SOD, catalase, and glutathione peroxidase [174–176]. Thus a key protective mechanism of resveratrol appears to be an augmentation of endogenous antioxidant systems in addition to direct inhibition/scavenging of ROS to counter the characteristic increase in oxidative stress during the development of cardiac hypertrophy.

In vivo experiments suggest that resveratrol is indeed a potent antioxidant via upregulation of NOS and increased NO production or by modulation of thioredoxin and heme oxygenase systems [177]. In fact, NO may be an important component in resveratrol-induced changes in intracellular redox state because NO has a greater affinity for $^{\bullet}O_2^-$ radicals compared to superoxide dismutase and thus can rapidly lower $^{\bullet}O_2^-$ concentrations [178]. In support of this concept, platelet eNOS was activated at physiologically achievable doses of resveratrol and blunted the proinflammatory pathway linked to p38 MAPK to inhibit production of ROS [179]. The proposed mechanism for increased NO production by resveratrol is thought to involve activation of the sirtuin SIRT1 protein deacetylase [180] which in turn has been shown to deacetylate eNOS at lysine residues to stimulate NO production [181, 182]. Related to this, resveratrol also activates AMPK [165, 183], which can then directly phosphorylate eNOS to stimulate NO production [184, 185]. As NO possesses vasodilatory, anti-inflammatory, anti-hypertrophic properties [186], increasing NO production may be an important pathway by which resveratrol ameliorates pathological cardiac hypertrophy.

As mentioned, mitochondria contribute importantly to the increased oxidative stress in cardiac hypertrophy. Interestingly, resveratrol has also been shown to attenuate mitochondrial oxidative damage [187], which could be another potential mechanism by which this compound is cardioprotective. Resveratrol-induced overexpression of SIRT1 attenuates mitochondrial oxidative damage in endothelial cells [188, 189], suggesting that prevention of mitochondrial oxidative damage alone could be an indirect mechanism for reduction of ROS by resveratrol. Furthermore, resveratrol

also reduces mitochondrial $^{\bullet}O_2^-$ production in many cell types, including human coronary arterial endothelial cells which have been attributed to direct stimulation of mitochondrial antioxidant systems [189].

14.8 Modulation of Cell Signaling by Resveratrol

Although a currently accepted mode of action of resveratrol is as an antioxidant, this polyphenol also triggers other mechanistic pathways, which include modulation of cell signaling, apoptosis, and gene expression [190]. For example, resveratrol modulates phorbol-ester-induced signal transduction pathways, which leads to elevated COX2 expression [191] as well as other signals such as NFkB, MAP kinases, transcription factor activating protein-1 (AP-1), and ERK [192]. In addition, resveratrol interferes with many intracellular signaling pathways that regulate cell survival and apoptosis [193].

With specific relevance to cardiac pathophysiology, it is known that resveratrol reduces phenylephrine-induced hypertrophy of rat cardiac myocytes through the activation of AMPK in an AMPK kinase, LKB1-dependent manner [194]. Resveratrol acts as a negative regulator of cardiac hypertrophy via inhibition of the mTOR–p70S6 kinase protein synthesis pathway [195, 196]. Furthermore, resveratrol has been shown to inhibit calcineurin activity, NFAT translocation to the nucleus, and NFAT-dependent transcription in phenylephrine-induced hypertrophy of isolated rat cardiac myocytes [194]. As the calcineurin–NFAT signaling pathway is a major contributor to the development of pathological hypertrophy, resveratrol's ability to suppress activation of this pathway may be an important mechanism by which resveratrol mediates its anti-hypertrophic effects.

As discussed, it is widely accepted that oxidative stress due to excessive production of ROS is a prominent factor in triggering the events that result in cardiomyocyte death. Cardiac SIRT1 is the mammalian ortholog of the silent information regulator 2 (Sir 2) family and is upregulated in response to oxidative stress [197]. Resveratrol has also been shown to be an important activator of SIRT1 [198], and in neonatal rat ventricular cardiomyocytes with simulated ischemia–reperfusion injury, resveratrol-induced SIRT1 activation and overexpression protected cardiomyocytes from oxidative injury, mitochondrial dysfunction, and cell death [198]. Resveratrol-induced SIRT1 overexpression in turn affects the MAPK pathway by reducing p38 and JNK phosphorylation [198]. In a porcine coronary artery preparation, resveratrol treatment countered ET-1 enhancement of MAPK activity by directly inhibiting MAPK activity and similarly reducing JNK-1 and p38 phosphorylation, as well as phosphorylation of ERK 1/2 was also reduced [199]. In support of this finding, resveratrol pretreatment protected against oxidative stress (H_2O_2)-induced cell proliferation and ERK 1/2 activation in human coronary smooth muscle cells [200]. In addition, resveratrol strongly inhibited Ang-II-induced hypertrophy and produced dose-dependent reductions in Akt 1 protein

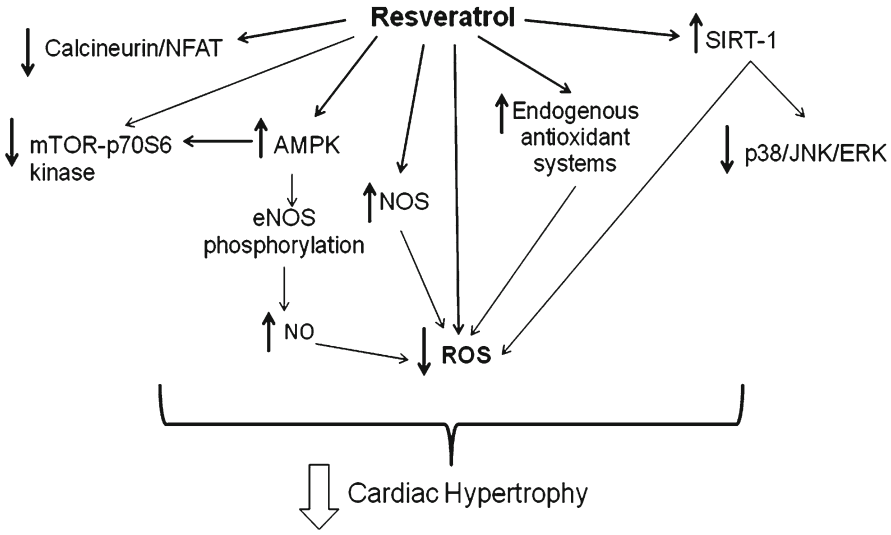

Fig. 14.1 Schematic representation of multiple cell signaling events modulated by resveratrol during the development of cardiac hypertrophy. Resveratrol affects several signaling events, many of which lead to a reduction in ROS and ultimately ameliorate cardiac hypertrophy. *NFAT* nuclear factor of activated T-cells, *mTOR* mammalian target of rapamycin, *AMPK* AMP-activated protein kinase, *eNOS* endothelial nitric oxide synthase, *NOS* nitric oxide synthase, *NO* nitric oxide, *ROS* reactive oxygen species, *SIRT1* sirtuin (silent mating type information regulation 2 homolog) 1, *p38*: p38 mitogen-activated kinase, *JNK*: c-Jun N-terminal kinases, *ERK* extracellular signal-regulated kinase

kinase and ERK 1/2 phosphorylation (two mediators thought to be essentially involved in Ang-II-mediated hypertrophy) [201].

Although activation of the p38 MAPK pathway is thought to contribute to the development of cardiac hypertrophy, recent but limited studies support a beneficial role for resveratrol-mediated activation of this pathway [202]. In H9c2 embryonic rat heart-derived cells treated with H_2O_2 to induce oxidative stress, resveratrol protected cells from oxidative damage, increased autophagy, and reduced peroxide-induced apoptosis [202]. All of these protective effects of resveratrol were prevented by prior treatment with a p38 MAPK inhibitor [202]. Overall resveratrol protected these cells from oxidative stress by upregulating autophagy via the p38 MAPK pathway [202]. Altogether these studies emphasize that the cellular actions of resveratrol are varied and dependent on the particular tissue or system under study (Fig. 14.1).

14.9 Therapeutic Applications for Resveratrol in Cardiac Hypertrophy

To complement direct antioxidant effects which could attenuate oxidative stress and the progression of hypertrophy, resveratrol also modulates other aspects of the pathophysiology of cardiac hypertrophy. As increased arterial pressure is often an initiating factor for cardiac remodeling, the ability of resveratrol to prevent increases in blood pressure in vivo is protective against left ventricular hypertrophy [203]. In several rat models of hypertension, resveratrol prevented left ventricular hypertrophy due to reduced systolic blood pressure [204–206]. Resveratrol also appears to have direct effects on cardiac myocyte growth since it can prevent cardiac hypertrophy in the absence of changes in blood pressure in spontaneously hypertensive rats or rats subjected to abdominal aortic banding as a model of pressure overload [128, 166, 167, 207]. This direct effect of resveratrol on cardiomyocytes is also supported by the modulation of signaling pathways that regulate growth and protein synthesis [194, 195]. Resveratrol also protects against other hypertrophy-induced events such as preventing the reduction in eNOS and iNOS [207] and presumably preventing a subsequent reduction in NO production. In addition, resveratrol inhibits critical steps in cardiac collagen deposition such as cardiac fibroblast proliferation which is thought to involve the cardiac NO–cGMP signaling pathway [196, 208]. Overall, evidence demonstrates that resveratrol protects cardiomyocytes from oxidative stress and death via ROS reduction, increased expression of antioxidant enzymes, and mitochondrial protection [209, 210], with ultimate reduction of fibrosis and preservation of cardiac function and survival in rodent models of heart failure [211].

14.10 Clinical Uses of Resveratrol

Although experimental models suggest that resveratrol may be an effective therapy for cardiac hypertrophy, to date there is a paucity of published human trials in this area. One study in healthy humans showed that a supplement which included 100 mg of resveratrol reduced the oxidative and inflammatory responses normally induced by a high-fat, high-carbohydrate meal [212]. A recent study focused on patients after myocardial infarction who were given a 10 mg/day resveratrol supplement for 3 months. In this study, resveratrol appeared to improve left ventricular diastolic function and endothelial function as measured by flow-mediated dilation of the brachial artery with no change in blood pressure [213]. However, despite the limited published evidence in humans, there are several clinical trials underway with this compound, and resveratrol may be a promising new therapeutic strategy for the treatment of cardiac hypertrophy and heart failure.

14.11 Conclusion

A thorough grasp of the pathophysiological mechanisms underlying the development of cardiac hypertrophy and the progression to heart failure is essential considering that left ventricular hypertrophy is a significant independent risk factor for sudden cardiac death, dysrhythmia, heart failure, ventricular ischemia, and coronary heart disease [214]. Although the specific steps that lead to hypertrophy are not completely clear, it appears that oxidative stress does play an important role in this condition. It is also promising that a treatment approach involving antioxidant therapies, including the natural polyphenol resveratrol, appears to target several signaling and pathogenic events in the etiology of this condition and may well prove to be effective in the prevention and treatment of cardiac hypertrophy alone or in combination with other drugs. Although we have a great deal of evidence supporting resveratrol treatment of hypertrophy, this is largely in cell-based and animal models with limited evidence from human studies—particularly focused on oxidative stress and cardiac function. Therefore, this is a critical and undoubtedly fruitful avenue of research in the future that may have significant clinical implications.

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Chapter 15
Angiogenesis, Arteriogenesis, and Mitochondrial Dysfunction

M.S. McMurtry

Abstract Angiogenesis and arteriogenesis are key processes involved in the response to occlusive arterial disease and the pathology of cancer. Angiogenesis remodels the circulatory bed by new capillary formation, while arteriogenesis remodels existing arterioles by increasing their diameter. The main molecular mechanism of angiogenesis is initiation by activation of hypoxia-inducible factor (HIF)-1, and arteriogenesis, initiated mainly by shear stress, is modulated by HIF-1 activation. Mitochondria can modulate HIF-1 activation by release of mitochondrial reactive oxygen species and alpha-ketoglutarate, a required cofactor for prolyl hydroxylation and destruction of HIF components, and thus, mitochondria can influence HIF-1-dependent angiogenesis and arteriogenesis. Mitochondria may serve as metabolic sensors that link metabolic derangements to appropriate neovascularization in health and in chronic ischemia and inappropriate neovascularization in the context of cancer. Mitochondrial remodeling or dysfunction may impair angiogenesis and contribute to the pathology of diseases, including diabetes and myocardial dysfunction. Drugs that alter mitochondrial function may alter HIF-1-dependent neovascularization and may represent novel therapies for chronic ischemic diseases and cancer.

Keywords Angiogenesis • Arteriogenesis • Neovascularization • Mitochondria

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15.1 Background: The Treatment Gap for Chronic Ischemic Diseases

Treatments for chronic refractory ischemia are needed for large numbers of patients that have failed conventional therapy. Cardiovascular diseases, including coronary disease (CAD), cerebrovascular, and lower extremity peripheral arterial disease (PAD), are leading causes of mortality and morbidity worldwide and in Canada [1–3]. Despite advances in medical therapy and revascularization, 10% of coronary artery disease patients have chronic refractory angina [4, 5], and 20–30% of critical limb ischemia patients need amputation [6]. Amputation rates for critical limb ischemia reach 1 per thousand of the general population [7–9], and the annual risk of amputation for those with PAD is approximately 1% [10]. New methods to revascularize ischemic tissues represent an important clinical need.

15.2 Neovascularization: A Therapeutic Target

Targeting innate mechanisms of vascular remodeling and repair, including angiogenesis and arteriogenesis, is an attractive but elusive therapeutic strategy. Three processes of new blood vessel development in humans have been described, including angiogenesis, arteriogenesis, and vasculogenesis [11–13]. Angiogenesis is the formation of new capillaries, but not large conduit vessels [12, 14]. Angiogenesis is initiated by hypoxia-induced stabilization of hypoxia-inducible factor 1 alpha (HIF-1α), causing nuclear translocation of HIF-1, a heterodimer of HIF-1α and HIF-1β, with subsequent transcription of angiogenic growth factors such as vascular endothelial growth factor (VEGF)-A (Fig. 15.1) [15]. Arteriogenesis is the maturation of preexisting arterioles into larger conduits and is the way arterial collaterals form [16]. The primary stimulus for arteriogenesis is thought to be shear stress-induced endothelial cell activation, causing proliferation and migration of endothelial and smooth muscle cells and monocytes, resulting in outward remodeling of the arteriole [17]. Vasculogenesis is the de novo blood vessel formation by circulating progenitor cells [18, 19], but the importance of this process in adults is unknown [14]. Despite promising preclinical work, trials to improve angiogenesis and arteriogenesis in human coronary and peripheral arterial disease utilizing protein or gene formulations of angiogenic growth factors such as VEFG-A, fibroblast growth factor (FGF) 2, FGF-4, and granulocyte–macrophage colony-stimulating factor have fallen short of demonstrating significant efficacy [10, 20–23]. Antiangiogenic treatments for cancer are also in their infancy [24, 25]. Despite challenges, targeting neovascularization remains attractive, and fundamental studies of mechanisms and treatments beyond single-factor replacement are required.

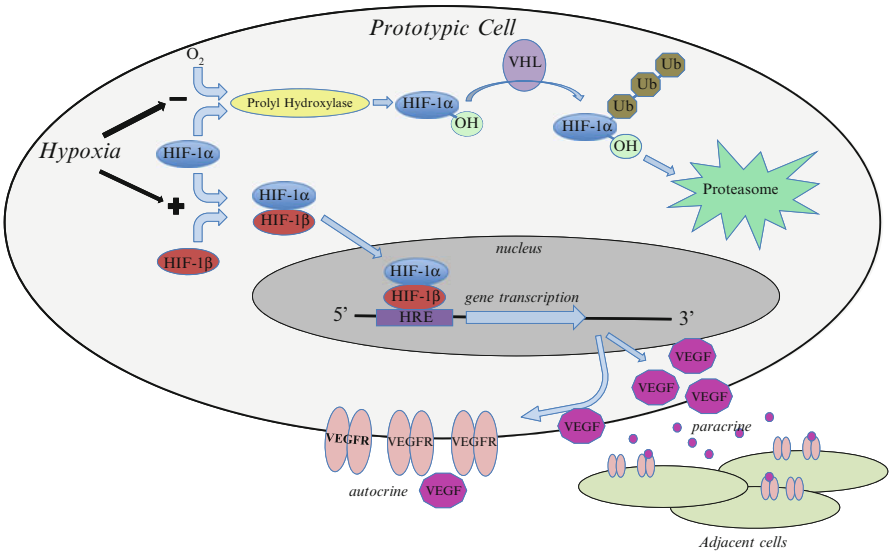

Fig. 15.1 The hypoxia-inducible factor (HIF)-1 system is activated by hypoxia, which promotes dimerization of HIF-1α and HIF-1β to form the active HIF-1. HIF-1 moves to the nucleus, activating transcription of proteins that induce angiogenesis, such as vascular endothelial growth factors (VEGFs)

15.3 Angiogenesis Is HIF-1 Dependent

Angiogenesis, or new capillary formation, is initiated by hypoxia-induced activation of HIF-1 [15]. Low local pO_2 inhibits the prolyl hydroxylation of HIF-1α, a reaction that requires both oxygen and α-ketoglutarate as substrates [26]. Decreased prolyl hydroxylation of HIF-1α prevents an interaction with the von Hippel–Lindau E3 ubiquitin ligase complex that targets HIF-1α for destruction [27–29]. Stabilized HIF-1α forms a heterodimer with HIF-1β to form HIF-1, which binds DNA and induces transcription of many angiogenic factors [30], including VEGFs [31], nitric oxide synthase [31], VEGF receptors (KDR/Flk and Flt) [32], angiopoietins [33], matrix metalloproteases [34], platelet-derived growth factor (PDGF) [35, 36], fibroblast growth factor (FGF) [36], and monocyte chemoattractant protein [37]. The VEGFs, including VEGF-A, -B, -C, and -D and placental growth factor (PlGF), bind to tyrosine kinase VEGF receptors including VEGF receptor 1 (VEGFR-1; Flt) and VEGFR-2 (KDR/Flk) on endothelial cells [38]. Bound ligands induce VEGF receptor dimerization and autophosphorylation, inducing signaling via the phosphatidylinositol 3-kinase/Akt pathway [39], the extracellular-regulated kinase pathway, and the mitogen-activated protein kinase pathway [40]. These pathways culminate in enhanced endothelial cell survival [39], endothelial cell proliferation [40], enhanced migration of endothelial cells, vascular smooth muscle cells

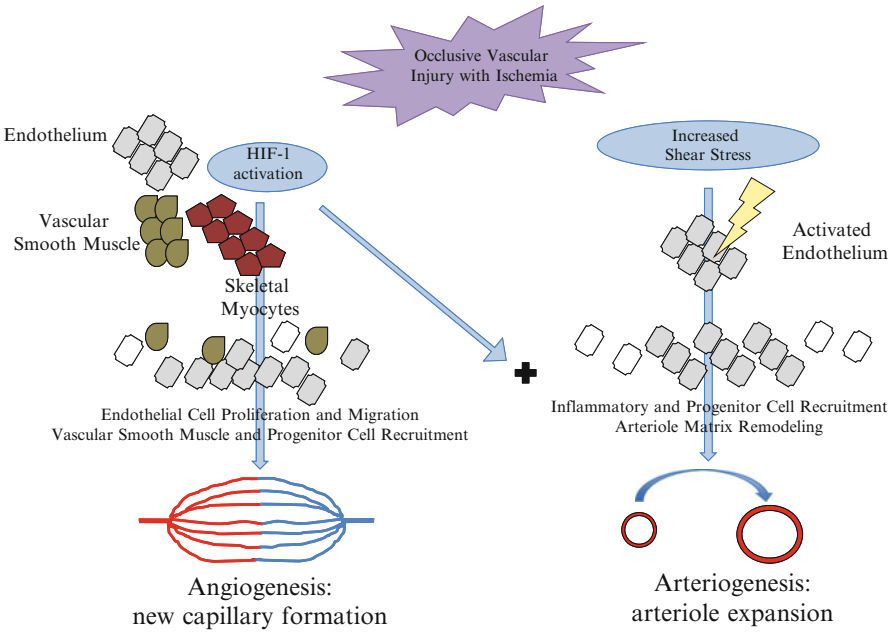

Fig. 15.2 Both angiogenesis, which is formation of new capillaries, and arteriogenesis, which is expansion of existing capillaries, are influenced by activation of HIF-1

and circulating inflammatory or progenitor cells [40], as well as enhanced angiogenesis in vivo (Fig. 15.2) [38, 41, 42]. Activation of HIF-1 can occur in endothelium [43], as well as vascular smooth muscle cells [44] and tissues, including cardiomyocytes [45] and skeletal myocytes [46, 47], fibroblasts [48], and bone marrow-derived cells [49]. HIF-1-mediated VEGF signaling and angiogenesis may be both autocrine [50, 51] and paracrine [51, 52], though the relative importance of autocrine versus paracrine signaling is incompletely understood [51]; Lee et al. suggest paracrine signaling predominates in developmental angiogenesis [52], but if this is true in pathologic models is not known. HIF-1 activation by hypoxia-induced inhibition of HIF-1α prolyl hydroxylation is the central trigger for angiogenesis.

15.4 Arteriogenesis Is Modulated by HIF-1 Activation

Arteriogenesis, or outward enlargement of preexisting arterioles, may also be partially HIF-1 dependent. Evidence suggests the primary stimulus for arteriogenesis is shear stress acting on endothelial cells [53–55], and not hypoxia [16]. Inflammatory markers and cells play a supporting role [56–62], as do growth factors such as basic fibroblast growth factor (FGF) and FGF receptors [63, 64], macrophage colony-stimulating factor [65], granulocyte–macrophage colony-stimulating factor

[66], and transforming growth factor beta [67]. At least three signaling mechanisms are activated by shear stress, including the RAS–ERK pathway, the Rho pathway, and nitric oxide [55]. Though HIF-1 is thought not to be a primary arteriogenic stimulus, variations in HIF-α genotype influence the development of human coronary collaterals in patients with coronary atherosclerosis [68]. HIF-1α expression in monocytes and lymphocytes in patients with coronary atherosclerosis also correlated with coronary collaterals in one study [69]. VEGF-A, a major effector of HIF-1, appears to enhance ischemic collateral formation in mice [70, 71] and rats [72], though the data in rabbits conflicts [73, 74]. In addition, genetically altered mice underexpressing or overexpressing VEGF-A demonstrate that native collateral density in the hind limb correlates with VEGF-A expression [75]. Blockade of the VEGF receptors decreases arteriogenesis [76], and VEGFR-2 is part of a complex that transduces endothelial cell shear stress, the key arteriogenic stimulus [77]. While experimental data supports that the major stimulus for arteriogenesis is shear stress [74], arteriogenesis also appears to be susceptible to modulation by changes in HIF-1 activation (Fig. 15.2).

15.5 Mitochondria Sense Hypoxia

Hypoxia reduces mitochondrial oxidative phosphorylation and changes Krebs cycle substrates and ROS production, allowing mitochondria to function as oxygen sensors. Mitochondria are the major ATP source in most cells [78]. Metabolites from glucose and fatty acids enter the Krebs cycle reducing NAD to NADH and FAD to $FADH_2$. As these reoxidize, they supply electrons to the electron transport chain (complexes I–IV). These electrons travel through the enzyme complexes to O_2, producing H_2O. During this process, protons translocate out of the inner mitochondrial membrane, generating a potential of −150 to −200 mV ($\Delta\Psi_m$) that reflects the level of the respiratory activity in the mitochondria [78]. ATP synthesis takes place at the ATP synthase (complex V), which contains a proton channel (F_0) and an ATP synthase (F_1). This enzyme is driven by the inward movement of protons through F_0 and phosphorylates ADP, producing ATP. It uses the stored energy of $\Delta\Psi_m$ to synthesize ATP, coupling respiration to ATP synthesis. Decreased $\Delta\Psi_m$, due to changes in substrate flux or the presence of uncoupling agents such as carbonyl cyanide-4(trifluoromethoxy)phenylhydrazone (FCCP), causes decreased ATP synthase activity [78]. Decreased electron transport chain complex activity would disrupt the normal electron flow and proton pumping, decreasing $\Delta\Psi_m$. Decreased $\Delta\Psi_m$ can also directly result from protonophores, such as FCCP or uncoupling proteins, generating heat [79]. As part of this process, ROS such as superoxide (O_2^-) are formed, which are rapidly dismutated by superoxide dismutase enzymes to hydrogen peroxide (H_2O_2). Vascular cells intricately balance energy supply and demand [80] and, with pO_2 reductions, shift from oxidative phosphorylation to glycolysis [81], resulting in changes of $\Delta\Psi_m$, ROS, and levels of Krebs cycle intermediates, such as α-ketoglutarate and succinate. In addition to other metabolic sensors like

AMP-activated protein kinase [82], mitochondria behave as sensors, with substrate flux, mitochondrial membrane potential, mitochondrial ROS, and Krebs cycle substrate levels shifting according surrounding metabolic state and pO_2 [78, 83, 84]. Endothelial and vascular smooth muscle cells are predominantly glycolytic, consistent with mitochondria being oxygen sensors [80, 81, 85]. Endothelial diversity [86, 87] is also consistent with EC mitochondria being fundamental in oxygen sensing, as different tissues respond to local conditions in different ways [81, 83]. Mitochondria are important sensors of intracellular hypoxia, the fundamental stimulus for HIF-1 activation [88].

15.6 Mitochondria Modulate HIF-1 Activation and Neovascularization

Changes in mitochondrial Krebs cycle substrates and ROS enhance HIF-1 activation [89], even in normoxia, linking mitochondria to HIF-1 and angiogenesis. Mitochondria interact with the HIF pathway in at least three ways. Mitochondria may act as a sink for oxygen [90] and in hypoxia may keep oxygen from prolyl hydroxylases. Mitochondrial ROS, frequently reported as increased by hypoxia [91–93], may also directly stabilize HIF-1α and therefore enhance activation of HIF-1 [92–97], as well as reinforce the glycolytic phenotype by enhancing PDK [98]. It should be noted, however, that controversy exists whether mitochondrial ROS increase [99] or decrease [100] in hypoxia, and discrepant findings might relate to differences between the systemic and pulmonary vasculature [83]. Furthermore, nitric oxide (NO) also modulates HIF-1, with prolonged NO exposure, decreasing HIF-1 activation by up to three different mechanisms, including direct inhibition of mitochondria by NO [101]. In addition, Krebs cycle substrates can directly interact with prolyl hydroxylation. The substrate α-ketoglutarate is required for prolyl hydroxylation of HIF-1α [26], and other Krebs cycle substrates, including succinate and fumarate, can directly inhibit prolyl hydroxylation [102], an effect that can be reversed with exogenous α-ketoglutarate [103, 104]. Mutations of succinate dehydrogenase lead to paragangliomas and pheochromocytomas, and mutations in fumarate hydratase lead to leiomyomas and renal cell carcinoma, all vascular tumors [105, 106]. It is debated in the literature whether mitochondrial ROS or Krebs cycle substrates are more important for HIF-1 activation, but both appear to play a role [89, 107–109]. These changes can occur when the metabolic balance shifts from oxidative phosphorylation to glycolysis, leading to activation of HIF-1 [110], even in normoxia (pseudohypoxia) [111]. Exogenous ROS can cause HIF-1 activation in normoxia [95, 97], and blocking prolyl hydroxylases, even in normoxia, activates HIF-1 [112]. Dysfunction of the Krebs cycle enzymes fumarate hydratase and succinate dehydrogenase causes accumulation of fumarate and succinate and activates HIF-1 in normoxia by inhibition of prolyl hydroxylases [106]. This normoxic HIF-1 activation can be reduced by replacement of α-ketoglutarate [103]. Since both mitochondrial ROS, in the form of hydrogen peroxide [81], and substrates such as α-ketoglutarate [103] are diffusible and can be modulated

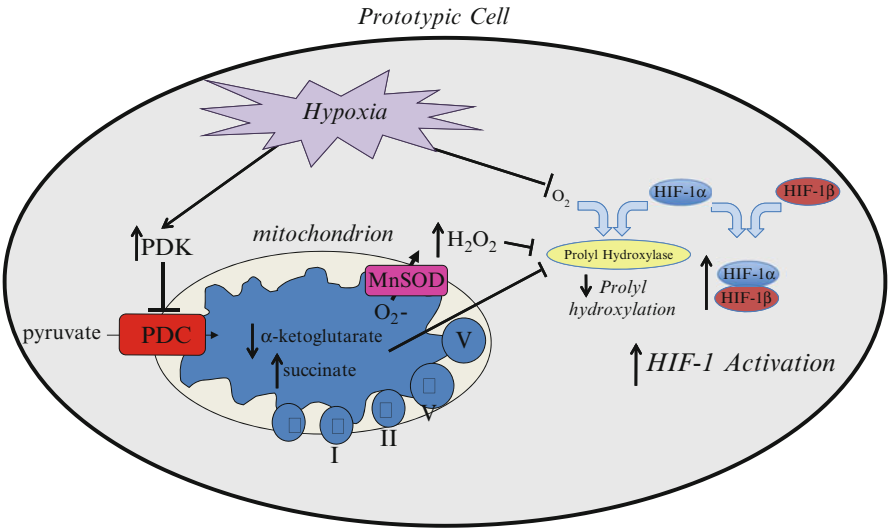

Fig. 15.3 Mitochondria respond to hypoxia by changes in release of the mitochondrial reactive oxygen species H_2O_2 and the prolyl hydroxylase cofactor α-ketoglutarate. These changes result in activation of HIF-1, supporting HIF-1-dependent neovascularization

independent of hypoxia, they represent possible mechanisms for both autocrine and paracrine activation of angiogenesis proximal to HIF-1 and VEGF, different from the usual mechanism of reduced pO_2 (Fig. 15.3).

15.7 Mitochondrial Dysfunction: An Effector of Impaired Neovascularization in Disease

The ability of mitochondria to modulate HIF-1-dependent processes, including neovascularization, has implications for the mechanisms of human diseases [89]. For example, an inhibitor of mitochondrial redox signaling, thioredoxin-interacting protein, is induced by glucose and may contribute to disordered angiogenesis in diabetes mellitus [113]. This is supported by evidence from thioredoxin-2 transgenic mice, which demonstrated enhanced arteriogenesis and angiogenesis in a murine hind limb ischemia model [114]. The glucose-derived oxoaldehyde glyoxal, present in hyperglycemia in the context of diabetes, also has been associated with mitochondrial dysfunction and impaired angiogenesis [115]. In aged rats, dysfunctional mitochondria, with lower expression of superoxide dismutase and other mitochondrial proteins, have been associated with impaired VEGF expression and angiogenesis in the rat kidney and may be a mechanism for exacerbation of renal injury [116]. Thyroid hormone (3,5,3′-Levo-triiodothyronine) replacement post-myocardial infarction has been demonstrated to improve left ventricular function in rats by improving both mitochondrial function and angiogenesis [117]. Conversely,

well-functioning mitochondria can promote pathology in the context of cancer by enhancing angiogenesis [118]. Mitochondrial dysfunction as an effector of pathology is just beginning to be explored.

15.8 Modulating Mitochondrial Function with Drugsto Alter Neovascularization

Hypoxia reinforces reductions in mitochondrial oxidative phosphorylation by inducing pyruvate dehydrogenase kinase. Oxidative phosphorylation is not only reduced by direct effects of hypoxia on the mitochondrial electron transport chain [84] but also by enhanced glycolysis at the expense of decreased substrate delivery to mitochondria. In many cell types, hypoxia activates HIF-1 and induces pyruvate dehydrogenase kinase (PDK) [119–121] and reduces expression of Krebs cycle enzymes [122]. PDK is a key gatekeeping enzyme that inhibits pyruvate dehydrogenase complex (PDH) [123], the major pathway for entry of pyruvate into mitochondria [124]. PDH is a multienzyme complex [125] that is redox sensitive [126] and inhibited by isoforms of PDK by phosphorylation of the E1a subunit [123]. As PDK is induced by hypoxia, there is less flux of substrate through into the mitochondria to the Krebs cycle and therefore lower oxidative phosphorylation [127]. There are four isoforms of PDK, and the expression is variable between organs [128]. Expression of PDK in endothelial cells is understudied, but it is known that PDK expression is increased in a HIF-1-dependent manner in ischemic skeletal muscle [129]. PDK isoforms are additionally regulated by nuclear receptors such as peroxisome proliferator-activated receptor alpha (PPARα) and gamma (PPARγ), liver X receptor, PPAR gamma coactivator alpha (PGC-1α), and insulin [130, 131]. Drugs are available that inhibit PDK (dichloroacetate) [132], indirectly increase PDK expression (WY-14,564; a PPARα agonist) [133], decrease expression of PDK (GW7845 [131], GW1929 [134]), or inhibit PDH (bromopyruvate and fluoropyruvate) [135]. In addition to hypoxia, other metabolic factors, including but not limited to fatty acids, can reduce oxidative phosphorylation, an effect previously described as the Randle cycle [136]. For example, the antianginal medication trimetazidine increases oxidative phosphorylation supposedly by inhibition of long-chain 3-ketoacyl-CoA thiolase, a mitochondrial enzyme [137]. Both cancer cells (the Warburg effect) and vascular cells are predominantly glycolytic, an observation that is incompletely explained [80, 81, 85]. Since the hypoxia-induced glycolytic shift mediated by increased PDK is associated with proliferation and apoptosis resistance in cancer, this phenotype might promote endothelial proliferation in angiogenesis [138, 139]. Drugs that activate PDK or inhibit PDH may promote angiogenesis and could be used as therapy for chronic ischemic diseases. Drugs that inhibit PDK or activate PDH may be useful as antiangiogenic therapies for cancer (Fig. 15.4). The concept that mitochondrial function can be pharmacologically manipulated is supported by reports of dichloroacetate suppressing cancer-associated angiogenesis as part of its therapeutic anticancer effect [140, 141].

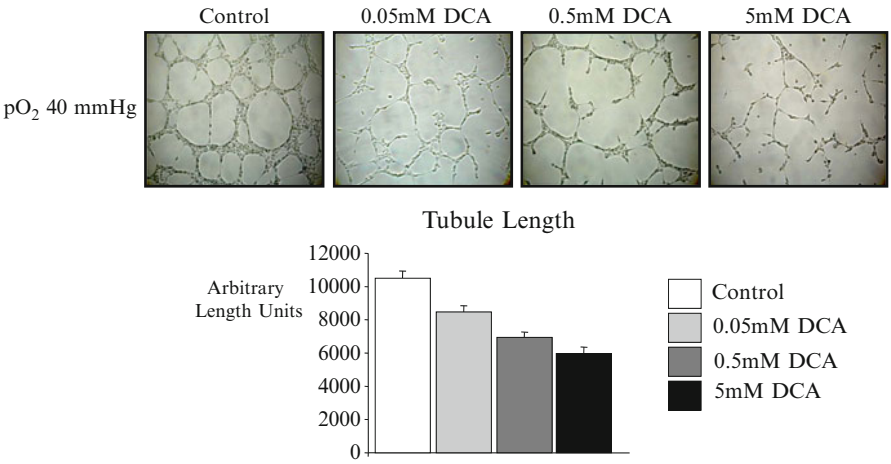

Fig. 15.4 Dichloroacetate (DCA), which inhibits pyruvate dehydrogenase kinase and promotes glucose oxidation, decreases angiogenesis in vitro in a dose-dependent manner. *Reproduced with permission from Michelakis et al.* [140]

15.9 Thiazolidinediones, Mitochondria, and Impaired Angiogenesis

Drugs that affect mitochondria can modulate HIF-1 activation and angiogenesis and may represent new avenues for therapies for diseases in which angiogenesis plays an important role. In contrast, there is a growing, yet somewhat conflicting, literature evaluating the effects of angiogenesis of agonists of peroxisome proliferator-activated receptors (PPARs) and angiogenesis [142–145]. In vitro PPARγ ligands inhibit angiogenesis on matrigel and on rat corneas [146], as well as human choroidal endothelial cells in vitro and in vivo [147]. Rosiglitazone reduces angiogenesis in human umbilical vein endothelial cells [148] and the human uterus [149], and both rosiglitazone and pioglitazone inhibit angiogenesis in the chick chorioallantoic membrane model [150]. While generally the literature suggests that PPARγ ligands, including thiazolidinediones (TZDs), reduce angiogenesis, rosiglitazone [151], troglitazone [152], and pioglitazone [153] have all been associated with increased angiogenesis in various reports. Clinical reports of TZD use also suggest that angiogenesis in vivo may be inhibited. Rosiglitazone has been associated with delayed onset of proliferative diabetic retinopathy [154], and the PROactive trial of pioglitazone in type 2 diabetics demonstrated an association between pioglitazone and increased rates of lower extremity revascularization [155]. Rosiglitazone and pioglitazone are associated with increased rates of heart failure and bone fractures, both of which could be plausibly related to impaired angiogenesis in myocardium and bone, respectively [156, 157]. There is interest in developing PPARγ agonists as antiangiogenic therapy for cancer [158]. Effects of TZDs may be mediated by both

PPARγ and non-PPARγ mechanisms. PPARγ appears to decrease PDK, while PPARα or PPARδ increases PDK [131]. PPARγ agonism decreases PDK expression in skeletal muscle [123, 131, 134] and other insulin-sensitive tissues, though one report demonstrated that rosiglitazone increased PDK-4 in fat [159]. TZD-mediated decreases in PDK may increase mitochondrial oxidative phosphorylation and via increases in α-ketoglutarate or decreases in mitochondrial ROS may decrease HIF-1 activation and angiogenesis. One experiment showed that GW1929, a selective PPARγ agonist, was associated with increased in vitro and in vivo angiogenesis, while rosiglitazone was not [160], suggesting that rosiglitazone's reduction on angiogenesis may be a non-PPARγ-mediated effect. TZDs directly inhibit complex I of the electron transport chain [161], and inhibition of complex I of the mitochondrial electron transport chain blocks HIF-1 activation [162]; therefore, TZDs may inhibit angiogenesis also by direct effects on the electron transport chain. Since the prevalence of peripheral arterial disease is as high as 1 in five diabetics [163, 164], TZDs are used in thousands of patients [165], and TZDs might increase in limb ischemia in human diabetics [155]; the association of TZDs and impaired angiogenesis strongly merits further study.

15.10 Perspective

Mitochondrial remodeling and changes in mitochondrial function, caused by hypoxia or a glycolytic shift in metabolism, enhance HIF-1 activation in via changes in α-ketoglutarate and mitochondrial ROS in endothelial cells and vascular smooth muscle cells, augmenting HIF-1-dependent autocrine and paracrine molecular pathways that result in increased angiogenesis. Though arteriogenesis is thought to be shear stress dependent, mitochondrial-dependent HIF-1 activation may improve arteriogenesis as well, both directly as well as indirectly by enhancing shear stress. Dysfunctional mitochondria that produce less α-ketoglutarate and mitochondrial ROS may be an effector for impaired neovascularization and vascular remodeling in disease. Mitochondria represent therapeutic targets to modulate neovascularization, either to enhance favorable vascular remodeling in chronic ischemic diseases or to decrease it in cancer.

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Part II
Molecular Mechanisms of Remodeling After Myocardial Injury and Infarction

Chapter 16
Subcellular Remodeling and Cardiac Dysfunction Due to Ischemia–Reperfusion Injury

Naranjan S. Dhalla, Vijayan Elimban, Larry Hryshko, and Darren H. Freed

Abstract Ischemic heart disease as a consequence of the blockade of coronary flow is associated with dramatic changes in cardiac function, metabolism, and ultrastructure. A wide variety of subcellular defects have been observed in ischemic and ischemia–reperfusion (I/R) hearts. There is evidence that various subcellular organelles become remodeled during the development of I/R injury and oxidative stress may be intimately involved in producing these abnormalities. In view of the direct participation of the sarcoplasmic reticulum (SR) and myofibrils in heart function, it appears that cardiac contraction and relaxation abnormalities in ischemic heart disease are due to remodeling of the SR and myofibrils, whereas remodeling of the sarcolemma membrane may determine the extent of intracellular Ca^{2+} overload, subsequent proteolysis, and irreversible injury to the heart. Furthermore, the acute effects of I/R injury on cardiac function are thought to be due to changes in the activities of subcellular organelles as a consequence of functional group modification, whereas the chronic effects of I/R yielding delayed recovery of cardiac function may be the consequence of changes in cardiac gene expression and subcellular remodeling. Although female hearts are less susceptible to I/R injury, in comparison to males, the basis for this gender difference in cardiac ischemic injury and protection needs to be defined. As females lose their resistance to different cardiovascular diseases after menopause, it appears that gender differences in cardiac susceptibility

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to I/R injury may be mediated through the participation of ovarian hormones. On the other hand, it is possible that the male sex hormone, testosterone, exacerbates I/R-induced cardiac dysfunction in adult males. Notably, in comparison to males, there is very little information in the literature on subcellular remodeling or on the mechanisms which regulate cardiac function during the development of I/R injury in female hearts.

Keywords Ischemia–reperfusion injury • Subcellular remodeling • Oxidative stress • Intracellular Ca^{2+} overload • Proteases • Gender differences

16.1 Introduction

Ischemic heart disease as a consequence of the blockade of coronary flow is associated with dramatic changes in cardiac function, metabolism, and ultrastructure [1, 2]; however, the exact cellular and molecular events leading to contractile dysfunction and derangement of cardiac structure are not fully understood. Although restitution of coronary flow to the ischemic heart is essential for the recovery of cardiac pump function, reperfusion after a certain period of ischemia has been shown to further aggravate the myocardial abnormalities [2–5]. As cardiac contractile defects due to I/R are almost invariably associated with situations such as angioplasty, thrombolytic therapy, cardiac surgery, and cardiac transplantation, studies on I/R injury are highly relevant for understanding the pathophysiology of an important clinical problem, namely, myocardial stunning. Both myocardial ischemia and I/R have been shown to generate different oxyradicals and oxidants such as H_2O_2, peroxynitrite, and HOCl, and these are suggested to be responsible for the occurrence of intracellular Ca^{2+} overload due to I/R injury [5–9]. Various active oxygen species such as superoxide radicals, hydroxyl radicals, and H_2O_2, which are formed during the development of I/R injury, produce electrical abnormalities [10–12], ultrastructural damage [13], intracellular Ca^{2+} overload [14], and cardiac dysfunction [15]. Both H_2O_2 and peroxynitrite have also been reported to activate some proteases and induce cardiac dysfunction [16–19]. Likewise, the intracellular Ca^{2+} overload may induce cardiac dysfunction and cell damage by activating different proteases and phospholipases [20, 21] and thus may modify the activities of various subcellular organelles such as sarcolemma (SL), sarcoplasmic reticulum (SR), myofibrils, and mitochondria. While both intracellular Ca^{2+} overload and oxidative stress have been shown to be involved in producing changes in cardiac gene expression as well as remodeling of subcellular organelles [20–22], oxidative stress seems to play a critical role in the genesis of intracellular Ca^{2+} overload and thus may induce cardiac dysfunction by remodeling of subcellular organelles during the development of I/R injury. A schematic representation of the events involving oxidative stress and intracellular Ca^{2+} overload due to I/R injury is given in Fig. 16.1. This view does not exclude the role of either lipid metabolites or oxidative stress/intracellular Ca^{2+} overload in apoptosis and necrosis commonly seen in ischemic and I/R hearts.

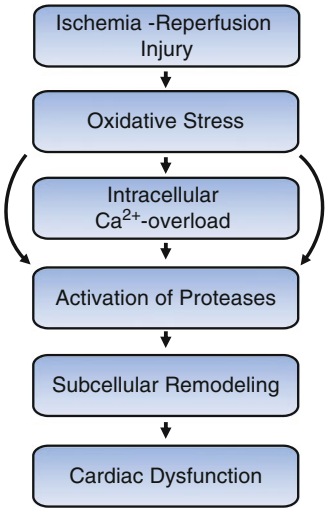

Fig. 16.1 Involvement of oxidative stress in inducing subcellular remodeling and cardiac dysfunction due to ischemia–reperfusion injury

16.2 Subcellular Remodeling and Molecular Abnormalities in I/R Hearts

Over the past 30 years, a wide variety of membrane defects have been observed in both ischemic and I/R hearts [5, 20–24]. It is now clear that the SR Ca^{2+} pump and associated regulatory mechanisms become defective due to changes in the molecular composition of the SR membrane as a consequence of I/R injury [25–35]. Several investigators have reported a reduction in the density of SR Ca^{2+}-release channels during I/R [36–39]. Various oxidants as well as hydroxyl radicals were also observed to depress the SR Ca^{2+}-pump activity [40, 41]. Although the efficiency of mitochondrial ATP production is impaired at the late stages of I/R injury, depression in both electron transport chain activity and Ca^{2+} transport in mitochondria also occurs at moderate degree of I/R injury [42, 43]. The biochemical activities of several SL membrane proteins including the Na^+–Ca^{2+} exchanger, Ca^{2+}-stimulated ATPase, Na^+–K^+ ATPase, and phosphoinositol turnover are markedly altered during myocardial I/R as well as during hypoxia–reoxygenation phases [44–55]. I/R, as well as reactive oxygen species and oxidants, have also been shown to reduce the sensitivity of myofilaments to Ca^{2+} by causing proteolysis of myofibrils [56–62]. These observations provide evidence that various subcellular organelles become remodeled or altered in I/R heart and that oxidative stress may be intimately involved in producing these abnormalities. In view of the direct participation of the SR and myofibrils in heart function, it appears that cardiac contraction and relaxation abnormalities in ischemic heart disease are due to remodeling of SR and myofibrils, whereas remodeling of the SL membrane may determine the extent of intracellular Ca^{2+} overload, subsequent proteolysis, and irreversible injury in the myocardium. It should also be noted that both I/R and oxidative stress have been shown to produce dramatic effects

on cardiac gene expression. We have demonstrated that mRNA levels for the SR Ca^{2+} pump, Ca^{2+} channels, phospholamban, and calsequestrin proteins were depressed in I/R hearts [25]. As H_2O_2 and I/R were observed to produce similar changes in SR gene expression, we suggested that these effects of I/R may be due to oxidative stress [25]. We have also observed that I/R produced differential changes in gene expression for SL Na^+–K^+ ATPase isoforms, and these alterations were simulated by perfusing the hearts with an oxyradical-generating system or H_2O_2 [63]. mRNA levels for the SL Na^+–Ca^+ exchanger were also depressed by I/R [55]. Although intracellular Ca^{2+} overload was demonstrated to occur in I/R hearts [64, 65], it is not known whether alterations in cardiac gene expression are affected by both intracellular Ca^{2+} overload and oxidative stress. Nonetheless, the acute effects of I/R injury on cardiac function are considered to be due to changes in the activities of subcellular organelles and proteins as a consequence of functional group modification, whereas the chronic effects of I/R including delayed recovery of cardiac function may be the consequence of changes in cardiac gene expression and subcellular remodeling.

16.3 Oxidative Stress and Development of Intracellular Ca^{2+} Overload in I/R Hearts

Some investigators have demonstrated the generation of oxygen free radicals in I/R hearts by employing electron paramagnetic resonance spectroscopy [66–68]. Oxidative stress has been shown to result in the development of intracellular Ca^{2+} overload due to I/R injury [69–73] because the activities of both SL Na^+–Ca^{2+} exchanger and SL Ca^{2+} pump were depressed following hypoxia or I/R as well as upon exposure of heart membranes to oxyradicals [74–78]. Oxyradicals were also reported to alter other SL activities such as Na^+–K^+ ATPase, Na^+–Ca^{2+} exchanger, phospholipid methyltransferase [46, 47, 78–82], Ca^{2+}/Mg^{2+} ecto-ATPase, superficial store of Ca^{2+} [83], and ATP receptors [84], which are considered to affect Ca^{2+} movements in the cell. The SL changes in different cation currents have also been observed upon exposure to oxyradicals and oxidants [85–87]. Several other defects such as changes in membrane permeability, loss of dystrophin, and alterations in phospholipases due to I/R injury have also been found in the SL membrane [88–92]. Oxidative stress has also been shown to produce marked alterations in myofibrils, mitochondria, and SR as well as induce autophagy during the development of I/R injury [62, 93, 94]. Thus, the increased formation of oxyradicals and oxidants in I/R hearts may induce a complex set of subcellular alterations with respect to their biochemical composition and functional activities related to Ca^{2+} movements, and these on balance may result in the development of intracellular Ca^{2+} overload and subcellular remodeling.

Oxidative stress seems to alter the subcellular activities by oxidizing different functional groups of subcellular organelles/proteins, and these changes seem to explain the development of intracellular Ca^{2+} overload and cardiac dysfunction due to I/R injury [3, 5, 21]. The effects of oxidative stress favoring the development of

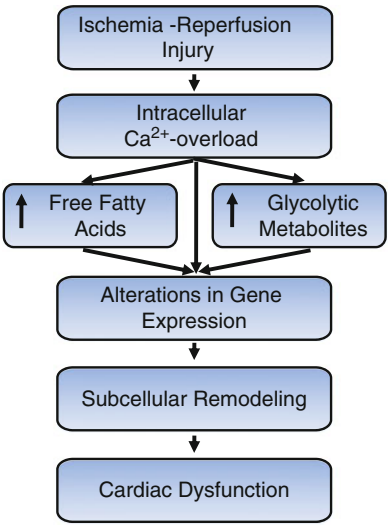

Fig. 16.2 Role of intracellular Ca^{2+} overload and changes in gene expression in inducing subcellular remodeling and cardiac dysfunction due to ischemia–reperfusion injury

intracellular Ca^{2+} overload are shown to be mediated through the activation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and/or stress-activated protein kinase [95–97], as well as translocation of PKC in ischemic heart [98, 99]. It is also noteworthy that besides the production of oxygen-derived free radicals, changes in nitric oxide (NO) metabolism have been observed in I/R hearts [67]. Although NO is known to regulate various events, its action becomes toxic by reaction with superoxide anion forming a potent oxidant, peroxynitrite, which has also been demonstrated to impair cardiac function [100, 101]. Thus, it appears that oxidative stress generated by different sources in the I/R heart plays an important role in the genesis of subcellular remodeling and cardiac dysfunction. A general scheme involving changes in gene expression for inducing subcellular remodeling due to I/R is shown in Fig. 16.2.

16.4 Activation of Proteases and Subcellular Remodeling in I/R Hearts

Activation of different types of proteases including calpain I and calpain II as well as metalloproteases (MMP-2 and MMP-9) has been suggested to be intimately involved in the pathophysiology of several forms of cardiac diseases [6–19, 102–106]. These proteases are activated by Ca^{2+}-dependent and Ca^{2+}-independent mechanisms and have been shown to cleave subcellular proteins and depress or alter their activities. While calpain I and calpain II are activated by Ca^{2+}, both MMP-2 and MMP-9 are activated by oxidative stress as well as by proteolysis. Pretreatment of heart with calpain inhibitors, MDL-28170 and A-70523, was observed to attenuate I/R-induced

cardiac stunning and infarct size [24, 106, 107]. The activation of calpain as well as defects in the SR Ca^{2+}-uptake and Ca^{2+}-release activities due to I/R injury was also attenuated by perfusing the heart with calpain inhibitors, leupeptin and E64d, or exercise training [103, 108, 109]. Preventing the activation of calpain by nitrosylation upon perfusing the heart with L-arginine was associated with improvements of SR function and cardiac performance in I/R hearts [51, 110]. Calpain-mediated depression in the activity of SL Na^+–K^+ ATPase and the loss of cytoskeleton protein α-fodrin due to I/R injury were prevented by a calpain inhibitor, MDL-7943, as well as ischemic preconditioning [111–114]. Likewise, the activation of MMP-2 [115, 116], changes in myosin light chain, and cardiac dysfunction due to I/R injury were prevented by doxycycline, an inhibitor of MMP-2 [116]. The influence of I/R injury on MMP-2 was observed to be mediated through the phosphoinositide 3 kinase (PI3K)/protein kinase B (Akt kinase) pathway [117]. These results provide evidence that activation of both calpain and MMP-2 due to I/R injury may depress cardiac performance due to subcellular defects; however, it remains to be examined if the observed changes in the activation of metalloproteases due to I/R injury are mediated directly through oxidative stress and/or indirectly through intracellular Ca^{2+} overload. It should be mentioned that both MMP-2 and MMP-9 are localized within cardiomyocytes [104, 105, 118] and their endogenous inhibitors, TIMP-2 and TIMP-1, are also found in these cells [104, 118]. Further, calpastatin serves as an endogenous inhibitor of both I and II isoforms of calpain in the heart [103]. Thus, the I/R-induced activation of MMP-2 and MMP-9 could also be due to a reduction in the TIMP-2 and TIMP-1 contents, whereas that of calpain I and II may be associated with the reduction of calpastatin content in cardiomyocytes. Nonetheless, the activation of different proteases under conditions of I/R injury would disrupt myocardial structure, remodel different subcellular organelles with respect to their protein content, and produce irreversible cardiac dysfunction.

16.5 I/R-Induced Subcellular Remodeling and Gender Difference

To date, the majority of work done to describe I/R-induced development of oxidative stress, occurrence of intracellular Ca^{2+} overload, activation of proteases, and defects in subcellular function has employed male animals, and little information is available for the female hearts. As female hearts are less susceptible to I/R injury, the basis for gender difference in cardiac ischemic injury and protection remains to defined [119, 120]. Several epidemiological studies have revealed sex differences with respect to the incidence of coronary artery disease, atherosclerosis, apoptosis, hypertension, and heart failure [120–134]. Various experimental investigations have also reported gender differences in the development of cardiac hypertrophy and heart failure due to myocardial infarction, pressure overload, and volume overload [135–146]. Gender difference in the properties of cardiac Na^+–K^+ ATPase due to hypertension [147], SR Ca^{2+} loading due to catecholamines [148], and Na^+–Ca^{2+} exchanger due to heart failure [149] has also been observed. As females lose their

resistance to different cardiovascular diseases after menopause, it appears that the intrinsic cardioprotection observed in females may be mediated through the participation of ovarian hormones [150]. Dramatic changes in Akt and NO synthase signaling as well as protein kinase A-mediated changes in Ca^{2+} handling involving SL Ca^{2+} channels, Na^{+}–Ca^{2+} exchanger, SR Ca^{2+} uptake, and Ca^{2+}-release channels have been observed upon ovariectomy [151–154]. Furthermore, estrogen, a major ovarian hormone, has been demonstrated to affect different Ca^{2+}-handling proteins, β-adrenoceptors, and Na^{+}–H^{+} exchanger as a consequence of its action on various kinase-mediated signal pathways [155–160]. However, a detailed study regarding the mechanisms of subcellular remodeling responsible for the resistance to ischemic insult in females, by employing ovariectomized animals with or without estrogen treatment, needs additional investigation before overarching conclusions can be made.

Several investigators have attempted to investigate the mechanisms of gender difference in cardioprotection against I/R injury in adult hearts. The postischemic recovery of cardiac function was greater and infarct size was smaller in female hearts in comparison to males, and these changes were attributed to differences in the Akt and PKC signal transduction [161, 162]. The loss of ischemic preconditioning effect on contractile function, infarct size, and enzyme leakage in I/R hearts was associated with impaired PKC phosphorylation [163]. Gender differences with respect to improved cardiac function in female I/R hearts were shown to be due to alterations in the regulation of $[Na^{+}]_i$ by a NO synthase-dependent mechanism [164–166]. Differences in the gender-dependent I/R-induced infarct size were associated with increased expression of SL K_{ATP} channels, and in fact, blockade of these channels was observed to abolish this difference [167, 168]. Conversely, gender differences with respect to resistance of female heart to I/R injury have also been attributed to difference in mitochondrial Ca^{2+} uptake [169] and tumor necrosis factor receptor signaling [170, 171]. By using ovariectomized animals, estrogen was found to attenuate I/R-induced changes in cardiac function and reduce infarct size as a consequence of changes in calpain and p38 MAP kinase activities [172–176]. Female mouse cardiomyocytes were protected against oxidative stress due to H_2O_2 as a consequence of Akt activation [177]. On the other hand, castration was found to decrease mRNA levels for L-type Ca^{2+} channels and Na^{+}–Ca^{2+} exchanger, and these alterations were reversed by testosterone [178–180]. Testosterone was also observed to modify I/R-induced changes in Akt signal transduction and apoptotic pathway [181–183]. Overall, there is a paucity of information on gender differences in subcellular remodeling as well as changes in mechanisms, which regulate cardiac function during I/R injury.

16.6 Conclusions

It is now known that the SR Ca^{2+} pump and associated regulatory mechanisms are defective due to changes in the molecular composition of SR membrane as a consequence of I/R injury. Various oxidants as well as hydroxyl radicals are observed to depress the SR Ca^{2+}-pump activity in I/R hearts. It should be noted that ischemic

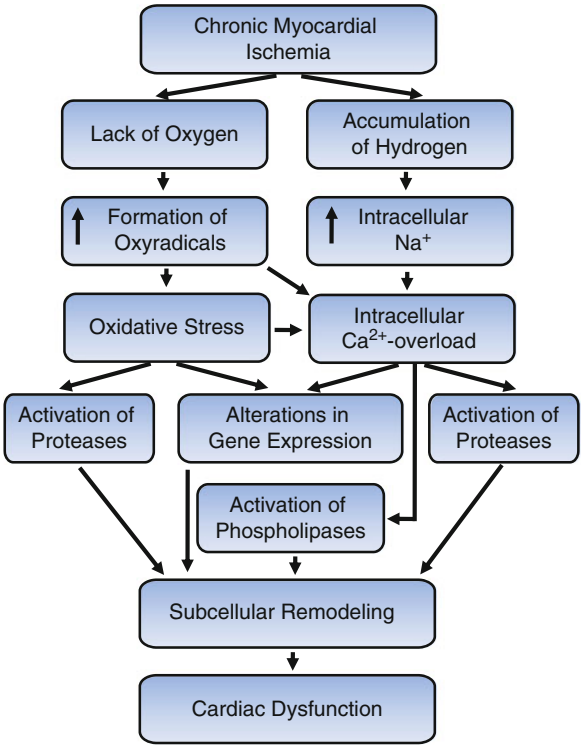

Fig. 16.3 Mechanisms of subcellular remodeling and cardiac dysfunction involving both oxidative stress and intracellular Ca^{2+} overload in ischemic heart disease

insult produces oxidative stress due to the generation of oxyradicals generating in addition to accumulating protons in cardiomyocytes. The magnitude of oxidative stress is amplified upon reperfusion of the ischemic myocardium, whereas protons are exchanged for Na^+ via the Na^+–H^+ exchanger. Furthermore, oxidative stress rapidly oxidizes the functional groups of Na^+–K^+ ATPase and augments the development of intracellular Na^+ overload. Intracellular Na^+ is exchanged with Ca^{2+} via the Na^+–Ca^{2+} exchanger and favors the occurrence of intracellular Ca^{2+} overload in the I/R heart. Thus, it is emphasized that alterations in the activities of Na^+-handling proteins (SL Na^+–K^+ ATPase and Na^+–Ca^{2+} exchanger) are critical for the net gain of Ca^{2+} within the cardiomyocytes. Oxidative stress and subsequent intracellular Ca^{2+} overload result in the activation of different proteases and induce dramatic changes in the composition of subcellular organelles/proteins in the I/R hearts. Accordingly, oxidative stress as well as changes in SL Na^+-handling proteins and protease activation plays an important role in inducing cardiac dysfunction due to I/R injury. Various events involved in subcellular remodeling during the development of cardiac dysfunction in ischemic heart disease are depicted in Fig. 16.3. It is also evident that females are more resistant to I/R-induced injury than males and the recovery of cardiac function upon reperfusing the male ischemic hearts is less

than that of the female. Although I/R-induced cardiac dysfunction in male hearts has been shown to be associated with the occurrence of oxidative stress, increase in development of intracellular Ca^{2+} overload, activation of proteases, cleavage of subcellular proteins, and alterations in subcellular activities, very little information regarding these changes in female hearts is available.

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Chapter 17
Role of MicroRNAs in Cardiac Hypertrophy and Postinfarction Remodeling

Jian Ding and Da-Zhi Wang

Abstract A family of small noncoding RNAs, termed microRNAs (miRNAs), plays critical roles in multiple physiological and pathological processes by negatively modulating gene expression at the posttranscriptional level. Cardiac hypertrophy and myocardial infarction, which are among the major causes of heart failure, have been found to be accompanied by dysregulated expression of miRNAs, suggesting that miRNA-mediated gene regulation is involved in cardiovascular pathogenesis. Loss- and gain-of-function studies by genetic or pharmacological manipulation of individual miRNAs further reveal that these small RNAs play critical regulatory roles in hypertrophy and postinfarction remodeling. Numerous miRNAs are cardiac protective, while some others are apparently detrimental during cardiac remodeling. Intriguingly, the functional consequences of some miRNAs in the heart appear to be cell type specific. Although the biological functions and molecular mechanisms of most miRNAs still remain elusive, their key regulatory roles in cardiac pathogenesis make them promising targets for therapeutic intervention. Here, we summarize the recent progress made in understanding the function and mechanisms of individual miRNAs in cardiac hypertrophy and postinfarction remodeling. The biological and clinical implications of miRNAs in the cardiovascular system are also discussed.

Keywords MicroRNA • Gene regulation • Cardiac hypertrophy • Myocardial infarction • Cardiac remodeling

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17.1 Introduction

miRNAs have emerged as pivotal regulatory factors of gene expression. This class of small RNAs inhibits the expression of downstream genes through translational repression or mRNA degradation by base-pairing with mRNA transcripts. The human genome may encode more than 1,500 miRNAs (http://www.mirbase.org), and most of them are conserved across species [1]. Additional miRNAs are continually being identified. Conversely, at least 60% of mammalian genes are directly regulated by miRNAs. An individual mRNA transcript usually contains binding sites for multiple miRNAs, while a single miRNA can also target numerous mRNA transcripts. These miRNAs and mRNAs form a complex regulatory network and modulate multiple physiological/pathological processes. Unlike transcription factors, which often act via a crude "on–off" mechanism, miRNAs function as "fine-tuners" rather than "master-switches" [2, 3]. However, growing evidence suggests that such "fine-tuning" regulation of gene expression by miRNAs can significantly influence pathogenesis in human diseases.

miRNA involvement in the regulation of cardiac remodeling has been implicated by numerous observations. First, cardiovascular diseases are often accompanied by dramatically altered miRNA expression [4–7]. Second, a cardiac-specific block of miRNA biogenesis in mice resulted in severe heart defects [8, 9]. Furthermore, a growing number of functional studies using genetic or pharmacological manipulation have demonstrated that many individual miRNAs significantly influence the pathogenesis of various cardiovascular diseases [10–12].

Cardiac hypertrophy and myocardial infarction are among the major causes of heart failure with a high risk of morbidity and mortality. Many regulatory networks, including multiple signal cascades, are involved in pathogenesis. Recent studies have uncovered that miRNAs play critical roles in cardiac hypertrophy and postinfarction remodeling. Due to their relative ease of pharmacological manipulation, miRNAs are considered an intriguing and promising target for therapeutic intervention. In this review, we summarize recent studies on the functions of numerous individual miRNAs in cardiac hypertrophy and postinfarction remodeling. The molecular mechanisms as well as the biological/clinical implications of miRNAs in the cardiovascular system are also discussed.

17.2 miRNAs in Cardiac Hypertrophy

Cardiac hypertrophy, primarily defined as an increase in the size of cardiomyocytes without a change in myocyte numbers, is further grouped in two different types: physiological hypertrophy and pathological hypertrophy. Pathologic cardiac hypertrophy is one of the major health problems in developed countries. It is usually accompanied with enhanced protein synthesis, an "adult to fetal" switch in cardiac gene expression (switch of adult α- to fetal β-myosin heavy chains in rodents), as well as reorganization of the cytoskeleton. It has long been speculated that the switch from α- to β-myosin heavy chain composition in myocytes may contribute

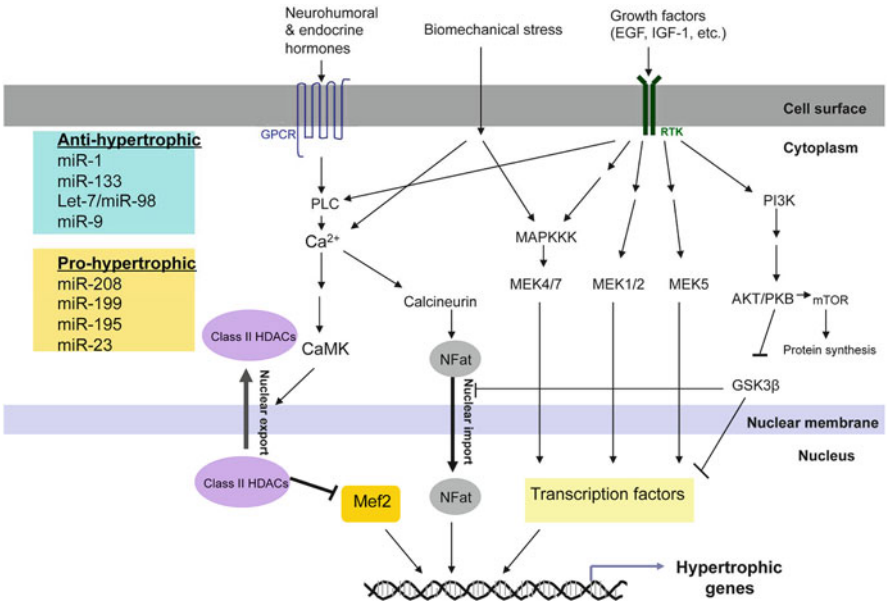

Fig. 17.1 Major cellular signal pathways that modulate cardiac hypertrophy. Signal cues, including biomechanical stress, neurohumoral, and endocrine hormones, can induce cellular hypertrophy responses by triggering a complex signal network involving MAPK, Ca^{2+}–calcineurin–NFAT, and PI3K-Akt pathways. miRNAs play critical roles in modulating cardiac hypertrophy by regulating the expression of multiple components in the signal network. Particularly, miR-1, miR-133, miR98, and miR-9 are apparently anti-hypertrophic, while miR-208, miR-199, miR-195, and miR-23 appear to be pro-hypertrophic miRNAs

to hypertrophic pathogenesis [13]. Alteration of the actin cytoskeleton not only affects the morphological integrity and mechanical resistance of the myocytes but also influences the transduction of stress signals, leading to changes in downstream gene expression and function [5]. Multiple cellular pathways and molecular events have been found to modulate cardiac hypertrophy. Signal cues, such as biomechanical stress, neurohumoral, and endocrine hormones (Endothelin-1, Insulin-like growth factor-1, etc.), could induce hypertrophic growth of cardiomyocytes by triggering numerous intracellular pathways, including the MAPK, Ca^{2+}–calcineurin–NFAT, and PI3K-Akt signal cascades [14–19]. Intriguingly, dysregulation of numerous miRNAs has been observed in cardiac hypertrophy in recent years, and direct evidence linking miRNAs to hypertrophy is emerging (Fig. 17.1).

17.2.1 Anti-hypertrophic miRNAs

Many miRNAs are shown anti-hypertrophic (Fig. 17.1) (Table 17.1). The bicistronic miRNA clusters miR-1-1/miR133a-2 and miR-1-2/miR-133a-1 are preferentially expressed in cardiac and skeletal muscle. miR-1-1 and miR-1-2 are probably the

Table 17.1 miRNAs in cardiac hypertrophy

	miRNA	Expression in cardiac hypertrophy	Targets (biological functions)
Anti-hypertrophic	miR-1	Downregulated	Calm1, Calm2, Mef2C, Gata4 (Ca^{2+} signaling)
			Twf1 (cytoskeleton regulation)
			IGF-1 (IGF-1 signaling)
	miR-133	Downregulated	RhoA, Cdc42 (cytoskeleton regulation)
			CnAβ, NFATc (Ca^{2+} signaling)
	let-7/miR-98	Downregulated	Cyclin D2 (MAPK signaling)
	miR-9	Downregulated	Myocardin (Ca^{2+} signaling)
Pro-hypertrophic	miR-208	miR-208a downregulated	Thrap1 (thyroid hormone signaling)
		miR-208b upregulated	Myostatin (inhibitor of Akt/PKB pathways)
	miR-195	Upregulated	
	miR-199	Upregulated	Dyrk1a (inhibitor of Ca^{2+} signaling)
	miR-23	Upregulated	Murf1, Foxo3A (negative regulator of cell growth)

most abundant miRNAs in the heart [20–22]. Downregulation of miR-1 levels in the heart has been consistently observed in different animal models of hypertrophy as well as in patients with hypertrophic myopathy [4, 23]. Inhibition of miR-1 activity with antagomirs appears to be sufficient to induce hypertrophic growth of rat ventricle cardiomyocytes in vitro, suggesting that downregulation of miR-1 may be causative in the pathogenesis of cardiac hypertrophy. Consistently, overexpression of miR-1 attenuates hypertrophy both in vitro and in vivo, further implying that this small noncoding RNA may be an important anti-hypertrophy regulator [5, 23]. miR-1 may exert its anti-hypertrophic function by repressing numerous pro-hypertrophic targets. A study by Ikeda et al. showed that miR-1 can repress the translation of the calmodulin-encoding mRNAs Calm1 and Calm2 by targeting their 3′ untranslated regions (3′ UTRs). miR-1 also has inhibitory effects on the expression of Mef2a and Gata4, key downstream transcription factors in calcium pathways. Together, these studies demonstrated that miR-1 could repress hypertrophic growth by negatively regulating calcium signaling [23]. miR-1 was also reported to target twinfilin-1 (Twf1), a cytoskeletal regulator. Overexpression of miR-1 represses the expression of endogenous Twf1, while attenuation of miR-1 results in an elevated level of Twf1 protein in cardiomyocytes. Thus, miR-1 could repress hypertrophy through regulation of the cardiac cytoskeleton [5]. Insulin-like growth factor-1 (IGF-1) is another pro-hypertrophic factor repressed by miR-1. It has been demonstrated that the expression levels of miR-1 and the IGF-1 protein

are inversely correlated in models of cardiac hypertrophy and heart failure. Intriguingly, IGF-1 signaling reciprocally regulates miR-1 expression. Thus, miR-1 and IGF-1 form a signal transduction feedback loop and together regulate hypertrophic growth [24].

Like miR-1, miR-133 has been found to be downregulated in hypertrophic cardiomyocytes. Overexpression of miR-133 in endothelin-1 (ET1) or phenylephrine (PE)-treated rat neonatal cardiomyocytes repressed hypertrophy, while loss of function of miR-133 by antagomir treatment was sufficient to induce hypertrophic growth of myocytes both in vitro and in vivo [25]. Studies have identified multiple targets of miR-133, many of which act as hypertrophic regulators. The RhoA GTPase and Cdc42, which function in modulating cytoskeletal and myofibrillar rearrangements during hypertrophy, have been shown to be targeted by miR-133 [25]. miR-133 also exhibits inhibitory effects on the expression of NFATc and CnA, two key players in calcium signal-mediated cardiac hypertrophy pathways [26].

Manipulation of miR-133 levels via adenovirus-mediated overexpression or antagomir-mediated knockdown altered the expression of its pro-hypertrophic targets and repressed or induced hypertrophic growth of cardiomyocytes, respectively. However, transgenic overexpression of miR-133 in cardiac muscle, though it inhibited myocardial fibrosis, did not affect the extent of hypertrophy in mice with transverse aortic constriction (TAC). Mice with a genetic deletion of miR-133a-1, miR-133a-2, or both did not exhibit a cardiac hypertrophy phenotype [27]. The discrepancy of the phenotypic outcomes may be attributed to the different approaches used to manipulate the level of miR-133. For instance, genetic deletion of miR-133 results in complete loss of 5p and 3p miR-133 species, both of which could have certain functions. In contrast, antagomirs only inhibit the function of one miRNA strand (5p or 3p). Moreover, conventional knockout or transgenic expression of miR-133 alters the gene expression program during cardiogenesis and, simultaneously or subsequently, could also initiate potential compensatory responses in the embryonic/fetal heart, which could be preserved and thus blunt miR-133's effects on hypertrophy in the adult heart. Alternatively, overexpression or antagomir inhibition of miR-133 could result in an "off-target" issue; therefore, caution should be used in interpreting the results.

Let-7/miR-98 are another set of miRNAs downregulated in cardiac hypertrophy that appears to be anti-hypertrophic. Adenovirus-mediated expression of miR-98 in cardiomyocytes resulted in reduced cell size and the repression of angiotensin II (ANG II)-induced hypertrophic growth. The anti-hypertrophic function of miR-98 may be partially attributed to its inhibitory effects on the expression of cyclin D2, a member of the G1-phase cyclin family, which is reportedly involved in cardiac hypertrophy. Intriguingly, expression of let-7/miR-98 is positively regulated by thioredoxin 1 (Trx1), which inhibits pathologic cardiac hypertrophy. Knockdown of miR-98 attenuated the inhibitory effects of Trx1 in Ang II-induced hypertrophic growth [28]. Thus, miR-98 appears to act downstream of Trx1 to mediate its anti-hypertrophic function.

Expression of miR-9 is downregulated in cardiomyocytes upon stimulation of hypertrophy with isoproterenol and aldosterone. On the other hand, administration

of miR-9 attenuated cardiac hypertrophy, suggesting that this miRNA functions as a key regulator in hypertrophy signaling. miR-9 appears to exert its function in hypertrophy by repressing myocardin, a key downstream mediator of NFATc3 in calcium-induced hypertrophic cascades [29]. In this manner, miR-9 appears to be anti-hypertrophic. However, the cardiac functions of this small RNA might be more complicated. As recently reported by Zhang et al. expression of miR-9 was induced by PDGF signaling in cardiomyocytes, while miR-9 reciprocally repressed the expression of PDGFR-β, a key component of the PDGF signaling cascade, which plays a pivotal role in stress-induced cardiac angiogenesis during hypertrophy [30]. Therefore, miR-9 may also function in modulating the paracrine angiogenic capacity of cardiomyocytes in response to stress. miR-9 appears to be a versatile miRNA, with its functional role(s) in hypertrophy remains in need of further characterization in vivo.

17.2.2 Pro-hypertrophic miRNAs

Conversely, many miRNAs are known pro-hypertrophic (Fig. 17.1) (Table 17.1). The cardiac sarcomeric myosin heavy chains (MHCs) are the primary regulators of muscle strength and contractility. In mouse, the Myh7 and myh7b genes encode the β-isoforms of MHC, with relatively low actomyosin ATPase activity, while myh6 encodes the fast-switch α-MHC. Cardiac hypertrophy is usually accompanied by an altered ratio of these two myosin isoforms [13]. Intriguingly, three miRNAs, miR-208a, miR-208b, and miR-499, termed "myomiRs," are encoded within the introns of myh6, myh7, and myh7b genes, respectively. These myomiRs function in modulating myosin gene expression and muscle contractility [31–33]. Given its unique expression pattern, it is not surprising that miR-208a is a key regulator of cardiac hypertrophy. In response to pressure overload and calcineurin signaling, miR-208a knockout mice failed to upregulate β-MHC and exhibited resistance to cardiac hypertrophy [32]. Overexpression of miR-208a results in increased expression of β-MHC and induction of hypertrophic growth in cardiomyocytes [31]. Recent studies have demonstrated that the functions of miR-208a in regulating myosin expression may be attributed to its direct repression of numerous transcription regulators, including thyroid hormone receptor associated protein 1 (Thrap1), Sox6, Hp-1β, and Sp3 [32, 33]. However, the mechanisms by which miR-208a modulates the upregulation of β-MHC in vivo and in hypertrophy need to be further investigated. For instance, it will be important to test the inhibitory effects of miR-208 on its targets: Thrap1, Sox6, and Hp-1β in hypertrophic heart vs. normal heart. miR-208 potently induced the expression of β-MHC in cardiomyocytes, but intriguingly, the induced expression of β-MHC in myocardium is heterogeneous in miR-208a transgenic mice [31]. This may be due to the "mosaicism" of transgene expression. However, the possibility that individual cardiomyocytes might differentially respond to miR-208 overexpression, or that the upregulation of α-MHC in vivo could be secondary to miR-208 induced cardiac abnormalities, cannot be excluded.

Previous studies have demonstrated that elevated expression of β-MHC alone is not sufficient to induce hypertrophy. Thus, the pro-hypertrophic function of miR-208a may not be solely attributed to its upregulation of β-MHC expression. The study by van Rooij et al. suggested that miR-208a might also regulate how the heart responds to stress during hypertrophy. Genetic deletion of miR-208a significantly altered the mRNA levels of genes encoding early response factors and heat shock proteins. However, none of these mRNAs contained predicted binding sites of miR-208a. Thus, the regulation on these cardiac stress response genes by miR-208a may be indirect [32]. Myostatin, a negative regulator of muscle growth, has been shown to be directly targeted by miR-208a. Inhibition of myostatin may contribute to the function of miR-208a in hypertrophy [31]. On the other hand, miR-208a also repressed the expression of Gata4, which is required for cardiac hypertrophy and appears to be pro-hypertrophic. Thus, miR-208a could target both anti-hypertrophic and pro-hypertrophic factors. The effects of miR-208 on these targets with opposing functions are likely dependent on cellular context. Growing evidence has suggested that miRNAs may regulate the expression of different subsets of downstream genes in different conditions. Comprehensive and systemic identification of targets of miR-208a in relevant physiological/pathological contexts is a key step to identify and understand the molecular mechanisms underlying its function in cardiac hypertrophy.

The three identified myomiRs, miR-208a, miR-208b, and miR-499, contain nearly identical seed sequences and may share certain common downstream targets and act redundantly. Consistent with this idea, transgenic expression of miR-499 in the hearts of miR-208a null mice was sufficient to restore the expression of β-MHC in the presence of propylthiouracil [33]. miR-499, like miR-208a, when overexpressed at a high level, can induce cardiac hypertrophy in vivo [34]. However, miR-499 was also shown to have anti-hypertrophic effects. CnA, a strong pro-hypertrophic factor, was found to be one of the direct targets of miR-499 in this setting. As shown by Wang et al. inhibition of miR-499 with an antagomir significantly elevated the signaling activity of the CnA–NFAT pathway and enhanced isoproterenol (ISO)-induced hypertrophy in vivo [35]. Thus, the functional roles of miR-499 in cardiac hypertrophy are still elusive.

More interestingly, miR-208a appears to act upstream of and regulate the expression of two other myomiRs in the adult mouse heart. These miRNAs form a "myomiR network" and regulate muscle myosin content and myofiber identity in the heart [33]. However, it is still a mystery how these myomiRs act together to modulate muscle strength and contractility in the process of cardiac hypertrophy. Are they friends or foes? In the myomiR network, we might predict that miR-499 cooperates with miR-208 to repress the expression of a set of common target genes, therefore synergistically or additively regulating muscle performance. On the other hand, miR-208 and miR-499 could also inhibit subsets of distinct downstream targets, which might be functionally unrelated or opposite, therefore resulting in complicated consequences. The study by van Rooij et al. demonstrated that the three myomiRs, though sharing a nearly identical seed sequence, might differentially repress their common target genes. The differences may be attributed to the significant dissimilarity in sequences

outside the seed region of mature miR-208a, miR-208b, and miR-499, which may influence their targeting capacities and preferences [33]. The mechanisms of the myomiR network could be even more complicated than mentioned above, since miR-499 and miR-208a/b, having different targeting capacities on their common targets, could each function as a "dominant negative miRNA" of the other. Obviously, such complexity will confound the study of individual myomiRs.

miR-195 is a stress-responsive miRNA that has been found upregulated in mouse models of cardiac hypertrophy induced by TAB or active CnA overexpression as well as in human patients with cardiac hypertrophy [4, 6]. Overexpression of miR-195 induced hypertrophic growth of isolated rat neonatal cardiomyocytes in vitro. Consistently, transgenic expression of miR-195 in cardiomyocytes driven by the α-MHC promoter also resulted in hypertrophy and cardiac dysfunction [6, 36]. Though the mechanisms are still not clear, the pro-hypertrophic effects of miR-195 suggest that this miRNA is a key regulator of hypertrophy and could be a potential therapeutic target of anti-hypertrophic treatment in the heart.

The miR-199 family has two members: miR-199a and miR-199b. miR199b has been found to be upregulated in heart failure of both mice and humans. It has also been demonstrated that the level of miR-199a was elevated in hypertrophic hearts but decreased in the later stages of heart failure in mice [36]. Overexpression of miR-199a in neonatal rat cardiomyocytes was sufficient to induce hypertrophic growth in vitro, whereas inhibition of miR-199a attenuated PE-induced hypertrophy [36]. A pro-hypertrophic function was also confirmed for miR-199b. Martins et al. reported that silencing of endogenous miR-199b by antagomirs significantly inhibited hypertrophy and fibrosis in vivo in mice hearts. Intriguingly, the promoter region (~2.7 kb upstream) of the miR-199b gene contained a conserved and functional NFAT binding site, so transcription of this miRNA can be regulated by CnA–NFAT signaling. miR-199b could directly target the dual-specificity tyrosine (Y) phosphorylation-regulated kinase 1a (Dyrk1a), which has been reported to downregulate NFAT activity [37]. Thus, miR-199b apparently forms a signal circuit with NFAT to promote cardiac hypertrophy. Such a notion is not unique to miR-199b, given increasing evidence that many miRNAs and their targets function in a feedback or feed-forward regulatory network to modulate gene expression.

miR-23a is another miRNA that has been consistently found to be upregulated in numerous hypertrophic models. miR-23a is a direct target of NFATc and may act as a feed-forward factor to elevate the signaling output of the CnA–NFAT pathway in hypertrophy. Lin et al. reported that a miR-23a antagomir could abolish CnA–NFATc3-induced cardiomyocyte hypertrophy in vitro. Transgenic expression of miR-23a, though not sufficient to cause obvious cardiac abnormality, significantly enhanced PE- or TAC-induced hypertrophy. Conversely, downregulation of miR-23a significantly attenuated isoproterenol-induced cardiac hypertrophic growth in vivo [38]. Thus, miR-23a appears to be a key regulator for the induction of cardiac hypertrophy. It has been reported that miR-23a can target and repress Murf1 and Foxo3a, negative regulators of cell growth. This may contribute to the pro-hypertrophic function of miR-23a [39]. Clearly, genetic studies in which miR-23a is mutated will allow us to further define the function of this miRNA.

Another question is how much redundancy miR-23a has with other members of the miR-23 family (miR-23b and miR-23C). Nevertheless, miR-23a appears to be a potential therapeutic target for the development of anti-hypertrophic drugs.

17.3 miRNAs in the Postinfarction Heart

Myocardial infarction (MI) is among the major causes of both debilitating illness and death in the world. Interruption of the local blood supply in heart ventricle, usually due to coronary artery occlusion, results in death of cardiomyocytes. The loss of myocardium induces a unique pattern of postinfarction remodeling, including ventricle dilation, eccentric hypertrophy, scar formation in the infarct area, interstitial fibrosis of adjacent myocardium, and alteration of ventricle geometry. At the cellular level, the remodeling process is usually accompanied with cell death (necrosis and apoptosis), hypertrophic growth of myocytes, as well as altered proliferation/migration of fibroblasts and endothelial cells (Fig. 17.2). Upregulation or downregulation of miRNAs has been found in myocardial infarction and postinfarction remodeling. Multiple studies have shown that alteration of miRNA expression contributes to cardiac injury and/or protection in MI by regulating key signaling elements (Fig. 17.2) (Table 17.2).

Numerous studies have documented the significantly altered expression pattern of miR-1 in myocardial infarction and/or ischemia/reperfusion (I/R). miR-1 is dramatically elevated in rat hearts upon acute myocardial I/R [40]. In the hearts of patients who died of myocardial infarction, miR-1 levels appear to be reduced in the infarcted area but elevated in remote non-infarcted regions [41]. The altered temporal and spatial expression pattern suggests that miR-1 might play key role(s) in myocardial infarction. Mechanistically, miR-1 appears to function as a proapoptotic miRNA. Overexpression of miR-1 is sufficient to induce apoptosis in H9c2 cells, while myocytes treated with a miR-1 inhibitor were more resistant to oxidative stress. miR-1 might exert its proapoptotic functions cumulatively by repressing the expression of multiple antiapoptotic regulators, including HSP60, HSP70, and Bcl-2 [40, 42].

As discussed earlier, miR-133 and miR-1 may function cooperatively or synergistically to regulate hypertrophic growth in myocytes. However, during myocyte apoptosis, these two miRNAs appear to play opposite roles. Unlike miR-1, miR-133 blunted oxidative stress responses and inhibited apoptosis when overexpressed in H9c2 cells. Inhibition of miR-133 promoted apoptotic cell death in H9c2 cells as well as in isolated neonatal rat ventricular cardiomyocytes. miR-133 repressed the expression of caspase-9, a key regulator of apoptosis, likely by directly targeting the 3′ UTR of this gene. Thus, miR-133 could inhibit proapoptotic factors, including caspase-9, and protect cardiomyocyte from apoptosis [42]. Together, these studies suggest that miR-1 and miR-133 could play pivotal roles in maintaining myocardial homeostasis by balancing cardiomyocyte death and survival. However, further investigations in vivo are needed to properly define their functional roles in infarcted hearts.

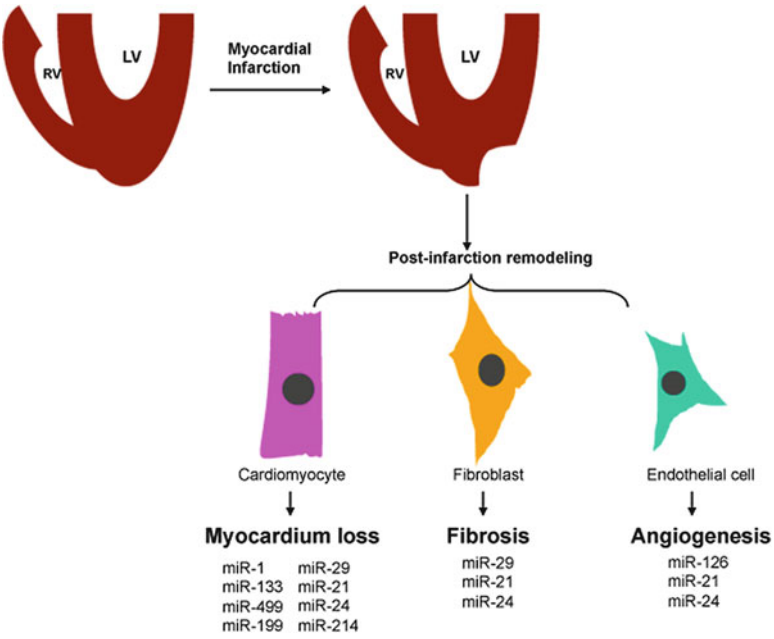

Fig. 17.2 The schematic of postinfarction remodeling. The loss of myocardium induces a unique pattern of postinfarction remodeling accompanied by cell death and altered cell growth and proliferation and migration of cardiomyocytes, fibroblasts, and endothelial cells. miR-1, miR-133, miR-499, miR-199, miR-29, miR-21, miR-24, and miR-214 are involved in cardiomyocyte death and survival. miR-29, miR-21, and miR-24 in fibroblasts have also been found to modulate fibrosis. miR-126, miR-21, and miR-24 in endothelial cells may function in regulating angiogenesis during postinfarction remodeling

Table 17.2 miRNAs in myocardial infarction

Cell type	miRNA	Biological functions	Targets
Cardiomyocyte	miR-1	Proapoptotic	HSP60, HSP70, and Bcl-2
	miR-133	Antiapoptotic	Caspase-9
	miR-499	Antiapoptotic	CnAα and CnAβ
	miR-29	Proapoptotic	Mcl-1, DNMT3, Tcl-1, YY1, P85α, CDC42
	miR-21	Antiapoptotic	FasL, Pcd4
	miR-24	Antiapoptotic	Bim
	miR-199	Regulate hypoxia-induced cell death	Hifα, Sirt1
	miR-214	Antiapoptotic, regulate Ca^{2+} influx	Ncx1
Fibroblast	miR-29	Antifibrotic	Fibrillins and elastins
	miR-21	Antifibrotic	Spry1
	miR-24	Antifibrotic	Furin
Endothelial cells	miR-126	Proangiogenic	Spred-1
	miR-21	Antiangiogenic	RhoB
	miR-24	Antiangiogenic	Gata2 and PAK4
	miR-214	Antiangiogenic	Quaking

miR-499 was found downregulated in the heart in ischemic conditions. As demonstrated by Wang et al. this muscle-enriched miRNA regulates left ventricular remodeling in hearts subjected to I/R injury. miR-499 represses the expression of CnAα and CnAβ by directly targeting their 3′ UTRs, resulting in reduced dephosphorylation of Drp-1, thereby inhibiting mitochondria fission and apoptosis of cardiomyocytes. Transgenic overexpression of miR-499 reduced deleterious cardiac remodeling and exhibited beneficial effects in infarcted heart, whereas knockdown of endogenous miR-499 enhanced I/R-induced collagen deposition and resulted in more pronounced cardiac dysfunction [35]. It is intriguing to note that miR-499, when overexpressed at a high level, can induce cardiac hypertrophy in vivo [34]. Thus, it is evident that the cellular steady-state level of a miRNA can dramatically influence cellular functions.

After myocardial infarction, the expression of the miR-29 family, miR-29a, miR-29b, and miR-29c, is downregulated in the border zone, suggesting that miR-29 family members may have regulatory functions in postinfarction cardiac remodeling [43]. Ye et al. reported that downregulation of miR-29 by antagomirs inhibited I/R-induced myocyte apoptosis/necrosis and reduced myocardial infarct size. miR-29 may repress the expression of numerous apoptotic factors. Mcl-1, a member of the antiapoptotic Bcl-2 family, appears to be directly targeted by miR-29 [44]. Consistently, inhibition of miR-29 elevated the level of Mcl-1 in mouse hearts [45]. Transcripts of several other antiapoptotic genes, including DNMT3, Tcl-1, YY1, P85α, and CDC42, contain putative miR-29 binding sites and are likely direct targets of miR-29 [46, 47]. Given its proapoptotic property in cardiomyocytes, miR-29 could be a potential postinfarct anti-remodeling therapeutic target. Interestingly, higher miR-29 expression is detected in cardiac fibroblasts. Numerous fibrotic genes, including collagens, fibrillins, and elastins, appear to be direct targets of miR-29. These observations suggest that miR-29 not only regulates myocyte apoptosis/survival, but it also modulates cardiac fibrosis in hearts subjected to myocardial injury. Indeed, inhibition of miR-29 in the heart elevated the expression of collagens and induced fibrosis in vivo in mice [43]. Thus, global inhibition of miR-29 in the heart may represent a double-edged sword during postinfarction cardiac remodeling [48]. It could be beneficial by inhibiting cardiomyocyte apoptosis, but it could also be detrimental due to the enhanced fibrosis in the infarcted heart.

miR-21 is widely expressed in various cell types in the heart, including fibroblasts, endothelial cells, and cardiomyocytes. Altered expression of miR-21 in myocardial remodeling has been observed in numerous studies; however, the functional roles of this miRNA in cardiac stress response are still controversial. In cardiomyocytes, miR-21 has been shown to target several proapoptotic genes, including FasL and Pcd4, thereby functioning as a pro-survival, cardiac-protective miRNA [49–51]. Indeed, knockdown of miR-21 resulted in elevated expression of proapoptotic genes and enhanced oxidative stress-triggered cell death. miR-21 also exhibited cardioprotective effects in vivo in that mice with cardiac-specific overexpression of miR-21 displayed more resistance to I/R-induced myocardial injury [51]. Consistently, Dong et al. reported that adenovirus-mediated overexpression of miR-21 in hearts improved left ventricle remodeling in the early phase of acute myocardial infarction [52].

In spite of its beneficial effects in cardiomyocytes, miR-21, when upregulated in endothelial and fibroblast cells, could be detrimental in cardiac remodeling, as shown in several other studies [53–55]. In endothelial cells, miR-21 might function as an antiangiogenic factor, since miR-21 represses the expression of RhoB, a positive regulator of vessel growth. Knockdown of miR-21 results in enhanced endothelial migration and tube formation, whereas overexpression of miR-21 inhibits angiogenesis both in vitro and in vivo [55]. However, the function of miR-21 in cardiac angiogenesis in postinfarction remodeling remains unknown.

Enhanced cardiac fibrosis appears to be closely correlated with an elevated expression of miR-21 in infarcted heart. miR-21 is upregulated in the fibroblast-rich region adjacent to the infarct after myocardial I/R [54]. Thum et al. showed that knockdown of miR-21 with a specific antagomir inhibited interstitial fibrosis in a mouse pressure overload-induced heart disease model [53]. However, such a view is not supported by Patrick et al. who showed that downregulation of miR-21 either by genetic deletion or LNA administration did not result in any alteration of cardiac fibrosis after pressure overload or myocardial infarction [56]. Thus, the functional roles of miR-21 in cardiac fibrosis are still controversial.

miR-126 is an endothelial cell-enriched miRNA. Genetic deletion of this miRNA resulted in leaky vessels and partial embryonic lethality [57]. Compared to their wild-type littermates, the surviving miR-126 null mice were more susceptible to cardiac injury and exhibited severe ventricular rupture and defective cardiac neovascularization following MI [57]. Consistently, a study by van Solingen et al. demonstrated that knockdown of miR-126 impaired ischemia-induced angiogenesis [58]. Thus, this endothelial cell-specific miRNA, which is required for normal vessel growth, may function as a proangiogenic factor and appears to be cardiac protective in postinfarction remodeling. Sprouty-related protein-1 (Spred-1) has been found to be one of the direct targets of miR-126 [57, 59]. Spred-1 is an inhibitor of the Ras/MAP kinase pathway and can repress vascular endothelial growth factor (VEGF)-/fibroblast growth factor (FGF)-mediated angiogenesis. Thus, Spred-1 appears to be a downstream mediator of the angiogenic action of miR-126. Intriguingly, miR-126 is also highly expressed in endothelial cell-derived apoptotic bodies, where it mediates the induction of CXCL12 expression, likely by repressing RGS16. In response to tissue injury, CXCL12, a CXC chemokine, functions as a positive regulator of regeneration by counteracting apoptosis and recruiting progenitor cells to repair the damaged tissue [60]. Although the role of miR-126 in CXCL12 induction needs to be further investigated in heart injury models, the potential cardiac-protective effects of miR-126 in MI may be due to its direct role in angiogenesis as well as in regulating apoptosis and progenitor cell mobilization. Thus, miR-126 may rise up as a promising cardiac protection reagent or drug.

Like miR-21, miR-24 can be detected in endothelial cells, cardiomyocytes, and fibroblasts of the heart. I/R dramatically upregulates the expression of miR-24 in endothelial cells of the parainfarcted region, but not in cardiomyocytes or fibroblasts [61]. Overexpression of miR-24 in endothelial cells triggers apoptosis and represses angiogenesis in vitro. Consistently, endothelial miR-24 antagonism in vivo exhibited inhibitory effects on apoptosis and resulted in increased capillary and arteriolar

density in the parainfarcted region, thus significantly reducing the infarct size and improving heart function after MI [61]. Intriguingly, the function of miR-24 in cardiomyocytes appears to be different from that in endothelial cells. As shown by Qian et al. in vivo delivery of miR-24 significantly inhibited cardiomyocyte apoptosis, attenuated infarct size, and reduced cardiac dysfunction in a mouse MI model [62]. Recently, another study showed that miR-24 regulated cardiac fibrosis after myocardial infarction. In vivo delivery of miR-24 after MI attenuated fibrosis in the infarct border zone and improved heart function. Overexpression of miR-24 by synthetic miR-24 precursors also decreased the differentiation and migration of cardiac fibroblasts in vitro. The antifibrotic effects of miR-24 could be at least partially attributed to its repression of furin, a protease which controls processing which activates latent TGF-β [63]. Thus, the functional effect of miR-24 on myocardial infarction appears to be cell type dependent. The presence of miR-24 appears to be beneficial to cardiomyocytes and fibroblasts but detrimental to endothelial cells. This miRNA could be a promising therapeutic target; however, careful design will be required to ensure miR-24 is targeted in desired cell types, not others. Furthermore, the clinical consequences of miR-24 on different cardiac cell types need to be thoroughly evaluated.

miR-199 is another important regulator of MI. A study by Rane et al. demonstrated that miR-199a could repress the expression of Hif-1α in cardiomyocytes. Hypoxia acutely downregulated miR-199a expression in cardiomyocytes. This reduction of miR-199 causes rapid induction of Hif-1α and is apparently required for hypoxia-induced apoptosis. Intriguingly, knockdown of miR-199a during normoxia recapitulated HPC (hypoxia precondition) and protected cardiomyocytes against hypoxia-induced cell death [64]. Additionally, miR-199 appears to be a key oxygen sensor as well as a sophisticated regulator of apoptosis in cardiomyocytes. Rane et al. also showed that Sirt1 is another direct target of miR-199a. Sirt1 can downregulate prolyl hydroxylase2 (PHD2) and is required for the stabilization of Hif-1α. Thus, miR-199 could repress Hif-1α directly by targeting its 3′ UTR or indirectly by inhibiting Sirt1 and consequently inducing PHD2 [64]. Recently, several groups uncovered a "competitive endogenous RNA" (CeRNA) mechanism, in which mRNA targets sharing common miRNA binding sites cross talk with each other and form a large-scale regulatory network [65–68]. Although CeRNAs are yet to be discovered in the cardiovascular system, it is very interesting to speculate that Sirt1 and Hif-1α, which are functionally related and both directly targeted by miR-199a in cardiomyocytes, could act as CeRNAs to modulate each other's expression.

Upregulation of miR-214 has been observed during ischemic injury and in heart failure [6, 43]. However, the potential role of miR-214 in cardiac remodeling remained unknown until very recently. Aurora et al. showed that genetic deletion of miR-214 in mouse resulted in loss of cardiac contractility, increased apoptosis, and excessive fibrosis in response to I/R injury, indicating that miR-214 may play a role in cardiac protection. miR-214 appears to exert its cardioprotective function by regulating cardiomyocyte Ca^{2+} homeostasis and cellular survival during myocardial injury [69]. While the study by Aurora et al. provides the genetic evidence for

miR-214's function in vivo, additional investigation is necessary to determine in which cell types miR-214 functions to exhibit its cardiac protection. Since miR-214 appears to be expressed not only in cardiomyocytes but also in endothelial cells and fibroblasts, it may modulate angiogenesis and fibrosis. For instance, a study by van Mil et al. has shown that miR-214 in endothelial cells could repress the expression of Quaking to inhibit the secretion of proangiogenic growth factors and negatively regulate angiogenesis [70]. Aurora et al. used ubiquitously expressed CAG-Cre to drive the global deletion of miR-214 in all cell types, which could result in tangled phenotypic effects and prevent the precise assessment of its cell type-specific functions. Thus, it would be interesting to assay the phenotypic consequences of a cardiomyocyte-specific deletion of miR-214 or test if cardiomyocyte-specific transgenic overexpression of miR-214 would result in cardioprotective effects and/or rescue the defects observed in miR-214 mutants after I/R injury.

17.4 Biological Implications and Future Prospects

Identification of miRNAs as novel modulators of gene expression adds a new dimension to our understanding of gene-regulatory networks. However, several fundamental questions regarding the basic biological principles of this class of small noncoding RNAs still need to be addressed. For instance, despite the global contribution of miRNAs to development and disease, most miRNAs appear to be "nonessential," since animals with genetic deletions of individual miRNAs appear to be phenotypically normal in unstressed conditions [12]. This apparent "phenotype gap" may be attributed to functional redundancy among related miRNAs in a family, which may share similar seed sequences and/or repress common downstream target genes. From an evolutionary standpoint, such functional dispensability and potential redundancy may imply less stringent selective constraints acting on miRNA genes. However, this does not seem to be the case, because most miRNAs are highly conserved across species [1]. The evolutionary selective advantages of this class of small RNAs appear elusive.

Although "nonessential" in normal conditions, many miRNAs play crucial roles in stress responses and disease models. In some cases, animals with genetic deletion of individual miRNAs are more susceptible to stress. For instance, genetic deletion of miR-214 in mice did not cause detectable cardiac defects at physiological baseline, but resulted in loss of cardiac contractility, increased apoptosis, and excessive fibrosis in response to I/R injury [69]. In some other cases, miRNAs appear to be required for stress-induced pathogenesis. As shown by van Rooij et al. miR-208a null mice did not display obvious abnormalities in size, shape, or structure of the heart at normal physiological conditions. Intriguingly, mutant mice exhibited strong resistance to cardiac hypertrophy upon pressure-overload stress [32]. Given the fact that most miRNAs are "nonessential" in normal conditions but play pivotal roles in stress responses, it is speculated that most, if not all, of the selective advantages of miRNA genes during evolution may be contributed by their significant functions in regulating pathogenesis.

Previous studies have suggested that regulation of the processing of a single pre-miRNA hairpin could not only affect the level of mature miRNA but also result in the modulated production of numerous miRNA isoforms with distinct biological functions [71]. Humphreys et al. reported that miRNAs in HL-1 cardiomyocytes exhibit pronounced sequence diversity [72]. It will be important to profile the cardiac miRNA population at single nucleotide resolution during heart development and/or cardiac pathogenesis in order to understand the functional relevance of such variation.

17.5 Clinical Implications and Prospects

As consistently shown in multiple studies, miRNAs are also present in mammalian serum and plasma. In many cases, human pathogenesis, including cardiac remodeling, appears to be accompanied with an altered population of miRNAs in these biological fluids, suggesting that the circulating miRNAs may potentially serve as biomarkers for disease diagnosis [73]. Although it is still debated whether the dysregulation of circulating miRNAs is adaptive or causative and whether they have potential functions in cardiac pathogenesis, growing evidence suggests that miRNAs are promising and attractive biomarkers for cardiac defects. First, an altered population of circulating miRNAs has been found to be associated with multiple heart diseases, including myocardial infarction and heart failure [73]. Second, using circulating miRNAs as biomarkers would allow for minimally invasive diagnosis of cardiac pathogenesis. Third, many miRNAs in serum or plasma, often protected in vesicles or exosomes, are very stable [74]. The high stability of these small RNAs can minimize potential technical variations and provide a firm grounding for clinical diagnosis. Moreover, circulating miRNAs in collected samples also appear quite resistant to temperature variation and repetitive freeze–thaw cycles, therefore allowing storage and comparison of patient samples between point-of-care sites [75]. Together, the unique characteristics of miRNAs make them promising biomarkers for diagnosis of human diseases, including cardiovascular disorders.

Numerous studies have suggested that miRNAs could also serve as potential therapeutic targets for the treatment of heart diseases. Given the fact that miRNAs play crucial roles in cardiac remodeling and stress responses, with little to no functional effect under normal unstressed physiological conditions, miRNA inhibition or delivery may provide an efficient and potent means to modulate cardiac pathogenesis with minimal side effects on normal tissues. Moreover, pharmaceutical manipulation of miRNAs is becoming more and more feasible. Systemic delivery of LNA-modified antimiRs, synthetic miRNA duplexes/miRNA mimics, or shRNAs by adeno-associated virus (AAV-shRNA) has been successfully developed and applied in mouse models of human diseases [76–78].

Despite the promise of miRNA-based therapeutics, one should be aware of the limitations and challenges that need to be overcome prior to full clinical application. For instance, functions of certain miRNAs (miR-29, miR-21), whether beneficial or detrimental in cardiac remodeling, are apparently cell type dependent. Thus, our

current miRNA-manipulating strategies need to be optimized in order to achieve efficient and selective delivery of miRNA inhibitors or mimics into the desired tissue types or specific population of cells.

17.6 Summary

Cardiac hypertrophy and postinfarction remodeling are regulated by complex signal networks and involve multiple cellular processes and molecular events (Figs. 17.1 and 17.2). miRNAs, the novel noncoding small RNA modulators of gene expression, add a new dimension to the regulatory networks governing cardiac remodeling (Tables 17.1 and 17.2). miRNAs have clearly reshaped our understanding of the pathogenesis of heart diseases. Studies on miRNAs also provide important insight into the development of innovative clinical approaches. It is expected that in the future miRNAs will become ideal biomarkers for disease diagnosis and will have great potential as strategic targets for clinical therapeutics in human diseases, including cardiovascular disorders.

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Chapter 18
Negative Regulators of Inflammation as Endogenous Protective Mechanisms in Postinfarction Remodeling

Amit Saxena and Nikolaos G. Frangogiannis

Abstract Myocardial infarction triggers an intense inflammatory reaction that serves to clear the wound from dead cells and matrix debris while promoting cardiac repair and formation of a scar. Dysregulated inflammation following cardiac injury has adverse consequences on the reparative response enhancing dilative remodeling and causing contractile dysfunction. Inhibitory molecular signals and suppressive pathways that prevent excessive or uncontrolled inflammation are activated in the infarcted myocardium and may protect from the development of adverse remodeling. This chapter discusses the cellular effectors and molecular signals responsible for suppression and containment of the postinfarction inflammatory response. Neutrophils, monocytes/macrophages, and lymphocyte subpopulations with suppressive properties, dendritic cells, vascular cells, fibroblasts, and extracellular matrix proteins contribute to inhibition of the inflammatory signals by producing soluble suppressive mediators (such as transforming growth factor-β, interleukin-10, and lipid-derived mediators) and through activation of intracellular inhibitory signals. We propose that dilative remodeling in patients with myocardial infarction may reflect impairment of suppressive anti-inflammatory pathways. Selective inhibition of inflammatory mediators in patient subpopulations with overactive postinfarction inflammation may protect from the development of heart failure.

Keywords Myocardial infarction • Remodeling • Transforming growth factor (TGF)-β • Interleukin-10 • Toll-like receptors • Cytokine • Regulatory T cells • Monocytes • Fibroblasts • Neutrophils

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18.1 Introduction

18.1.1 The Postinfarction Inflammatory Response and Cardiac Remodeling

Over the last 30 years, advances in acute coronary care and development of early reperfusion strategies significantly improved survival rates in patients suffering an acute myocardial infarction leading to a decline of over 60% in all-cause mortality during the first 30 days after the acute event [1]. This impressive success in care of patients with acute infarction has resulted in a growing pool of patients who, having survived the acute infarction, are at risk of developing heart failure [2]. Thus, in patients with acute myocardial infarction, the decreasing acute mortality rates are associated with an increased incidence of postinfarction heart failure [1]. The pathogenesis of postinfarction heart failure is linked with profound alterations in cardiac geometry, function, and structure, a process termed "ventricular remodeling." Adverse dilative remodeling is a predictor of arrhythmias, heart failure, and mortality in patients surviving an acute infarction [3]. The extent of postinfarction remodeling is dependent not only on the amount of lost myocardium but also on the qualitative characteristics of the reparative process [4].

The adult mammalian myocardium has negligible endogenous regenerative capacity; as a result, loss of a large number of cardiomyocytes leads to their replacement with collagen-based scar. In acute infarction, sudden necrosis of millions of cardiomyocytes triggers an intense inflammatory reaction that not only serves to clear the wound from dead cells and matrix debris but also provides key molecular signals for cardiac repair. In healing wounds timely repression and containment of inflammatory signals are needed to ensure optimal tissue repair. Because cardiac function is dependent on preservation of architectural integrity, defective temporal and spatial regulation of the postinfarction inflammatory reaction may have catastrophic consequences on cardiac geometry and function. A dysregulated and overactive inflammatory reaction may result in impaired cardiac repair through several distinct mechanisms. First, exaggerated inflammation may augment matrix degradation causing cardiac rupture. Second, prolongation of the inflammatory reaction may perturb collagen deposition leading to formation of a scar with reduced tensile strength, thus promoting chamber dilation and adverse remodeling. Third, enhanced expression of proinflammatory mediators may activate proapoptotic signals inducing further loss of cardiomyocytes in the non-infarcted remodeling myocardium. Finally, defective containment of the inflammatory reaction may lead to extension of the inflammatory infiltrate into the non-infarcted myocardium enhancing fibrosis and worsening diastolic function [5].

A growing body of evidence has demonstrated that inhibition and resolution of inflammation are biosynthetically active processes that require recruitment of cellular effectors and activation of molecular mediators that suppress inflammation. This chapter will discuss the pathways responsible for negative regulation, resolution, and containment of inflammation in the infarcted myocardium. These STOP

signals are crucial for cardiac repair following myocardial infarction. Defects in repression and resolution of the postinfarction inflammatory reaction may be responsible for adverse remodeling and development of heart failure in a large number of patients surviving a myocardial infarction.

18.2 Molecular Signals Involved in Regulation of Postinfarction Inflammation

18.2.1 Initiation and Activation of the Innate Immune Response Following Myocardial Infarction

In the infarcted myocardium, necrotic cardiomyocytes release their intracellular contents initiating an intense inflammatory response through activation of innate immune mechanisms. Generation of damage-associated molecular patterns by necrotic cells and matrix fragments activates membrane-bound Toll-like receptors (TLRs) [6]. A growing body of evidence implicates TLR signaling in initiation of the postinfarction inflammatory response. TLR4- and TLR2-mediated pathways promote inflammation in the infarcted myocardium [7, 8], thus contributing to the development of adverse remodeling [9]. High-mobility group box 1, the receptor for advanced glycation end products [10], and the complement system are also activated after necrotic myocardial injury and participate in the early stages of the inflammatory response in the infarcted heart [11]. Reactive oxygen species (ROS) are also generated and induce inflammatory signals while exerting direct inhibitory effects on myocardial function.

18.2.2 Negative Regulation of the Innate Immune Response

Several distinct pathways have been identified as negative regulators of innate immune responses [12, 13]; however, their involvement in limiting myocardial inflammation following infarction remains unknown. Disruption of some of these negative regulators results in uncontrolled inflammation suggesting their essential role in restraining TLR-mediated immune responses. The TLR-inducible zinc finger proteins A20 and Zc3h12a have also been identified as essential negative regulators of TLR signaling in vivo [14]. A20 is dynamically regulated in the infarcted and pressure-overloaded myocardium [15]. Myocardial A20 overexpression attenuates postinfarction inflammation by reducing nuclear factor (NF)-κB activity and exerts potent anti-hypertrophic effects [16]. Interleukin-1 receptor-associated kinase (IRAK)-M is the only member of the IRAK family that lacks kinase activity, thus functioning as a decoy that negatively regulates TLR- and IL-1-mediated inflammatory responses. IRAK-M null mice exhibit accentuated inflammatory

responses following bacterial and viral infections [17, 18] and IRAK-M-deficient macrophages display enhanced activation of IL-1/TLR signaling [19]. Ongoing research in our laboratory has identified IRAK-M as an important negative regulator of the postinfarction inflammatory response; in addition to its established role in limiting macrophage-derived inflammation, IRAK-M expression in cardiac fibroblasts may limit their matrix-degrading potential. Several other pathways are involved in negative regulation of the innate immune response; however, their role in myocardial infarction has not been investigated. MyD88s, a lipopolysaccharide-inducible spliced form of MyD88 that lacks the IRAK-4-interacting domain, is involved in negative regulation of TLR signaling by inhibiting IRAK-4-mediated IRAK-1 phosphorylation [20]. Receptor-interacting protein 3 (RIP3 - a kinase that inhibits RIP1-induced NF-κB signaling) [21], activating transcription factor 3 (ATF3), the autophagy-related molecule Atg16L1, and the TAM receptor protein tyrosine kinases are also involved in inhibition of TLR signaling.

18.2.3 Cytokine and Chemokine Signaling Following Myocardial Infarction

Innate immune signaling pathways ultimately activate the NF-κB system driving expression of proinflammatory cytokines and chemokines [22, 23]. As prominent and early mediators of inflammation, proinflammatory cytokines critically regulate the response to cardiac injury. Interleukin (IL)-1, the prototypical proinflammatory cytokine, mediates chemokine synthesis in the infarcted myocardium and promotes infiltration of the infarct with leukocytes [24]. Generation of active IL-1β requires processing of pro-IL-1β, an inactive precursor, by the converting enzyme caspase-1. Caspase-1 activity is tightly regulated within the "inflammasomes," multiprotein molecular platforms that control maturation and processing of IL-1β [25]. Inflammasome activation in the infarcted myocardium is localized in both leukocytes and resident fibroblasts and drives IL-1-mediated inflammatory activity [26, 27].

The proinflammatory cytokine tumor necrosis factor (TNF)-α is also released in the infarcted myocardium [28] and may play an important role in regulation of the postinfarction inflammatory response. TNF-α null mice were protected from cardiac rupture and chronic dysfunction following infarction and exhibited suppressed inflammation and attenuated matrix metalloproteinase (MMP) activity in comparison with wild-type animals [29]. However, TNF-α may also induce cytoprotective signals capable of preventing or delaying the development of myocyte apoptosis following myocardial infarction [30]. It has been suggested that TNF-α may exert distinct biological effects through the TNFR1 and TNFR2 receptor. Actions mediated through TNFR1 may be deleterious, inducing cardiac dysfunction, whereas TNFR2-mediated actions may be protective by attenuating adverse remodeling [31].

Chemokine induction is also a consistent and prominent feature of the postinfarction inflammatory response [32]. Chemokines provide directional signals for recruitment of leukocyte subpopulations into the infarcted myocardium.

CC chemokines, such as monocyte chemoattractant protein (MCP)-1 and the macrophage inflammatory proteins (MIP-1α and MIP-1β) [33], function as potent mononuclear cell chemoattractants, whereas ELR motif-containing CXC chemokines (such as IL-8) play an important role in neutrophil chemoattraction [34]. Through interactions with chemokine receptors expressed by leukocyte subpopulations, the chemokine expression profile in the infarcted myocardium determines the composition of the leukocytic infiltrate.

18.2.4 Negative Regulation of Proinflammatory Cytokine and Chemokine Responses

Negative regulation of cytokine signaling may involve several distinct mechanisms:

(a) Upregulation of endogenous cytokine inhibitors may suppress specific cytokine responses. IL-1Ra, an endogenous competitive inhibitor of IL-1-driven inflammation, plays an important role in preventing autoinflammatory responses. Although the role of endogenous IL-1Ra in suppression and resolution of the postinfarction inflammatory response has not been dissected, IL-1Ra overexpression exerts protective actions on the infarcted heart [35].
(b) Decoy receptors (such as the type II IL-1 receptor) may be upregulated in the infarct serving as molecular sinks for cytokines.
(c) Endocytosis of signaling-competent receptors followed by proteasomal degradation may play an important role in preventing uncontrolled cytokine actions.
(d) Active termination of cytokine signaling through dephosphorylation of Janus-activated kinases (JAKs) [36]. Most of the cytokine-activated intracellular proteins belong to the family of signal transducers and activators of transcription (STAT) and after tyrosine phosphorylation by JAKs, then dimerize and translocate to the nucleus where they initiate transcription. Several phosphatases have been implicated in negative regulation of cytokine signaling, including SHP-1, the protein tyrosine phosphatase PTP1B and the T cell protein tyrosine phosphatase (TCPTP).
(e) Perhaps the best-studied family of cytokine inhibitors is the Suppressor of Cytokine Signaling (SOCS) family of inhibitory proteins. Although members of the SOCS family are likely to play an important role in regulation of the postischemic myocardial inflammatory response, their contribution to suppression of inflammation in the infarcted heart has not been investigated.

Termination of chemokine signaling may also play an important role in regulating the postinfarction inflammatory reaction [32]. Suppression of chemokine synthesis by inhibitory mediators, such as transforming growth factor (TGF)-β [37], IL-10, or lipid-derived substances, may be the predominant mechanism for deactivation of chemokinetic signals. Two additional pathways may operate for negative regulation of chemokine responses. First, posttranslational modifications of mature

chemokine proteins through protease-mediated actions may generate molecules with reduced activity or endogenous chemokine antagonists [38]. Second, silent chemokine receptors that bind to their ligand without signaling may function as decoy or scavenging receptors [39]. Whether chemokine decoy receptors are expressed and play a crucial role in myocardial inflammation remains unknown.

18.2.5 Soluble Inhibitors of the Postinfarction Inflammatory Response: The Role of IL-10 and TGF-β

Induction of soluble inhibitory mediators may play an important role in suppression and resolution of the postinfarction inflammatory response. Extensive evidence has identified IL-10 and TGF-β as important negative regulators of immune responses [40]. Both mediators have been implicated in regulation of the postinfarction inflammatory response; however, their role is complex as they affect most cell types involved in cardiac repair in a context-dependent manner.

IL-10 mRNA and protein expression are markedly induced in reperfused canine and murine myocardial infarction [34, 41]. T cell subsets and macrophage subpopulations appear to be responsible for IL-10 synthesis in the infarcted myocardium [41, 42]. In a canine model, IL-10 upregulation in the infarcted myocardium was associated with decreased expression of proinflammatory cytokines; in vitro, IL-10 released in the cardiac interstitium was responsible for induction of tissue inhibitors of metalloproteinases (TIMP-1) mRNA by isolated canine mononuclear cells suggesting a role in matrix stabilization [41]. Two independent loss-of-function studies in mouse models of reperfused infarction have produced somewhat contradictory results. Yang and coworkers suggested that IL-10−/− mice had markedly increased mortality and exhibited an enhanced inflammatory response following reperfused myocardial infarction showing accentuated neutrophil recruitment, elevated plasma levels of TNF-α, and higher tissue expression of intercellular adhesion molecule 1 (ICAM-1) [43]. In contrast, Zymek and coworkers found much more subtle differences in the postinfarction inflammatory response between IL-10 null and wild-type animals [44]. Infarcted IL-10−/− mice and wild-type littermates exhibited comparable survival following infarction. Although IL-10 disruption was associated with higher peak TNF-α and MCP-1 mRNA levels in the infarcted heart, repression of proinflammatory cytokine and chemokine synthesis and resolution of the neutrophil infiltrate were not affected. IL-10 gene disruption did not alter fibrous tissue deposition and dilative remodeling of the infarcted heart [44]. Thus, IL-10 induction in the infarcted heart may play a limited role in controlling the acute inflammatory response, but is not involved in clearance of the inflammatory infiltrate and resolution of inflammation.

TGF-β may regulate immune and inflammatory responses by modulating leukocyte phenotype and activity. The effects of TGF-β on lymphocytes, monocytes, and macrophages can be either stimulatory or inhibitory, depending on

microenvironmental cues, the state of cellular differentiation, and the tissue origin of the cells highlighting the pleiotropic nature of the cytokine. TGF-β has a deactivating effect on macrophages, suppressing proinflammatory cytokine and chemokine synthesis [45] and decreasing ROS generation. These TGF-β-mediated effects may be important in suppression and resolution of the inflammatory response. In addition to its effects on leukocytes, TGF-β is a potent modulator of fibroblast phenotype and function. TGF-β stimulation induces myofibroblast transdifferentiation [46] and enhances synthesis of extracellular matrix proteins. Moreover, TGF-β suppresses the activity of proteases that degrade extracellular matrix by inhibiting MMP expression and by inducing synthesis of protease inhibitors, such as plasminogen activator inhibitor 1 (PAI-1) and TIMPs [47, 48]. Thus, through its broad immunomodulatory and anti-inflammatory properties and its profibrotic/matrix-preserving actions, TGF-β is an excellent candidate for a role as the "master switch" mediating suppression of postinfarction inflammation while promoting fibroblast activation and scar formation [49].

TGF-β is markedly upregulated in experimental models of myocardial infarction [50] where it is predominantly localized in the infarct border zone, associated with expression and activation of its downstream intracellular effectors, Smad2, Smad3, and Smad4 [51, 52]. Systemic inhibition of TGF-β signaling by injection of an adenovirus harboring soluble TGF-β type II receptor in the hind limb muscles resulted in attenuated left ventricular remodeling by modulating cardiac fibrosis [53, 54]. However, early TGF-β inhibition increased mortality and exacerbated left ventricular dilation enhancing cytokine expression, suggesting that during the resolution phase of the inflammatory response, TGF-β signaling may play an important role in suppression of inflammatory mediator synthesis [53].

Because of the pleiotropic effects of TGF-β on the inflammatory and reparative response following infarction, identification of therapeutic targets requires dissection of the signaling pathways responsible for its actions in the infarcted myocardium. TGF-β is capable of activating the canonical Smad-mediated cascade as well as a variety of Smad-independent pathways. Smad3 null animals had markedly suppressed peak chemokine expression and decreased neutrophil recruitment in the infarcted myocardium and showed timely repression of inflammatory gene synthesis and resolution of the inflammatory infiltrate. Although myofibroblast density was higher in Smad3 null infarcts, interstitial deposition of matrix proteins in the peri-infarct zone and in the non-infarcted myocardium was markedly reduced. In vitro, Smad3 signaling not only was essential for TGF-β-mediated myofibroblast differentiation and matrix synthesis but also exerted antiproliferative effects [55] on cardiac fibroblasts. In the absence of Smad3, the infarct was filled with a large number of dysfunctional fibroblasts. Thus, TGF-β/Smad3 signaling does not appear to be involved in suppression and resolution of inflammation in healing infarcts, but mediates interstitial fibrosis in the infarct border zone and in the non-infarcted myocardium. The potential role of Smad-independent signaling in mediating TGF-β-induced suppression of postinfarction inflammation has not been investigated.

Growth differentiation factor (GDF)-15, another member of the TGF-β superfamily, has been recently identified as a crucial endogenous mediator involved in suppression of the postinfarction inflammatory response [56]. GDF-15 restrains inflammation by counteracting conformational activation of neutrophil β2 integrins, thus preventing excessive chemokine-activated leukocyte arrest on the endothelium [57].

18.2.6 The Potential Involvement of Lipid Mediators in Resolution of Postinfarction Inflammation

Lipid-derived mediators play an important role in regulation of the inflammatory response: prostaglandins and leukotrienes are released early following tissue injury and exert proinflammatory actions [58], whereas specialized proresolving mediators (SPM) are induced during the resolution phase of inflammation [59]. Four distinct families of SPMs have been identified: the lipoxins, resolvins, protectins, and maresins. These mediators have potent and direct anti-inflammatory and proresolving functions, limiting inflammatory leukocyte recruitment while promoting clearance of apoptotic neutrophils by macrophages. Administration of exogenous resolvin E1 exerted protective actions on the ischemic and reperfused myocardium [60]. However, the role of endogenous lipid mediators in negative regulation of the postinfarction inflammatory response has not been studied.

18.3 The Cellular Effectors of the Anti-inflammatory Response

Timely repression of inflammatory gene synthesis and resolution of the inflammatory infiltrate following myocardial infarction require the coordinated actions of several different cell types and involve the participation of the extracellular matrix (Fig. 18.1). Neutrophils, mononuclear cells/macrophages, vascular endothelial cells, fibroblasts, and pericytes undergo dynamic phenotypic changes contributing to suppression and resolution of the inflammatory reaction. Moreover, the extracellular matrix dynamically participates in regulation of the inflammatory cascade.

18.3.1 The Neutrophils

One of the most important cellular mechanisms responsible for resolution of the inflammatory response is clearance of apoptotic cells in the injured tissue [61]; this process is associated with active suppression of inflammation, as phagocytes

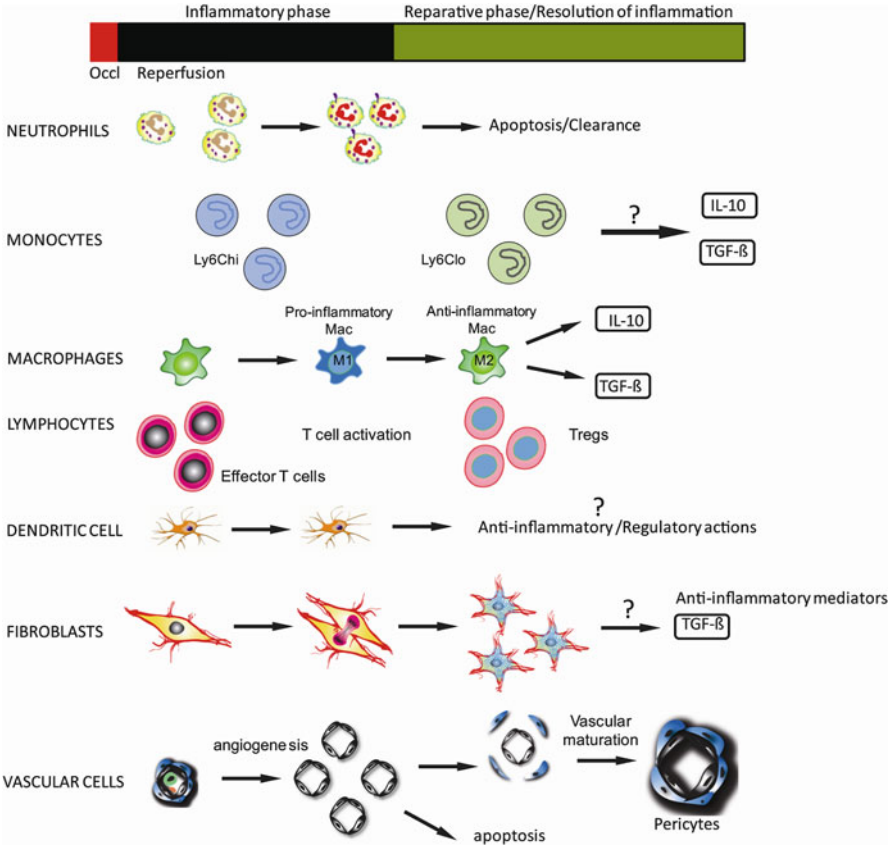

Fig. 18.1 All cells involved in cardiac repair contribute to resolution of postinfarction inflammation. During the reparative phase of infarct healing neutrophils, monocytes/macrophages, lymphocytes, dendritic cells, fibroblasts, and vascular cells undergo phenotypic changes secreting soluble inhibitory mediators and suppressing the inflammatory reaction

ingesting apoptotic cells release large amounts of inhibitory mediators [62, 63]. The infarcted myocardium is infiltrated by abundant neutrophils; these short-lived inflammatory cells represent a cellular population programmed to undergo apoptosis. Their clearance by professional phagocytes is likely to activate an anti-inflammatory program in phagocytotic macrophages, characterized by increased expression and release of IL-10, TGF-β, and proresolving lipid mediators [64]. However, this hypothesis has not been tested in experimental models of myocardial infarction.

18.3.2 Monocyte Subpopulations and Macrophages

Over the last 10 years, it has been widely recognized that monocytes are functionally heterogeneous cells [65]. In mice two major subsets of monocytes have been documented: (a) Ly6C^{hi} inflammatory monocytes that migrate into injured tissues and exhibit high levels of expression of the CC chemokine receptor CCR2 while expressing low levels of the fractalkine receptor CX3CR1 (Ly6C^{hi}CCR2^{hi}CX3CR1^{low}) and (b) Ly6C^{low}/CCR2$^{low/neg}$/CX3CR1^{hi} "resident" monocytes [66] that exhibit less pronounced proinflammatory responses. Monocyte subpopulations with distinct functional properties have also been described in humans: human CD16 monocytes express high levels of CCR2 and have proinflammatory properties resembling murine Ly6C^{hi} cells [65]. Following myocardial infarction, early induction of the CC chemokine MCP-1 [33], a CCR2 ligand, drives recruitment of proinflammatory Ly6C^{hi} monocytes; these cells scavenge debris and secrete proinflammatory cytokines and matrix-degrading proteases [67]. Repression of inflammatory signals may be associated with recruitment of Ly6C^{lo}/CX3CR1^{hi} monocytes that produce angiogenic mediators and contribute to infarct healing. Whether timely repression of postinfarction inflammation is dependent on active recruitment of suppressive monocyte subpopulations in the healing myocardium, driven by specific chemokine/chemokine receptor interactions, remains unknown. Monocyte heterogeneity has also been demonstrated in human patients with acute myocardial infarction. Circulating proinflammatory CD14+/CD16− cells show an early peak in patients with myocardial infarction and are negatively associated with recovery of function [68] after the acute event.

In the proinflammatory environment of the healing infarct, upregulation of macrophage colony-stimulating factor (M-CSF) induces monocyte to macrophage differentiation [69]. In the infarcted myocardium, the spatially and temporally regulated expression of cytokines, chemokines, and growth factors is likely to affect macrophage phenotype leading to sequential generation of many distinct subsets. During resolution of postinfarction inflammation, macrophages are capable of exerting both inhibitory actions by secreting mediators that suppress inflammation and proresolving effects through the removal of inflammatory leukocytes. Our knowledge on the role of macrophage-mediated interactions in repression of inflammatory signals and in resolution of the leukocyte infiltrate in the infarcted myocardium remains extremely limited. During the resolution phase of inflammation, macrophages that phagocytose apoptotic neutrophils may acquire a regulatory phenotype, expressing large amounts of inhibitory mediators and promoting suppression of the inflammatory response.

18.3.3 T Lymphocytes

Effector T cells are recruited in sites of injury where they release proinflammatory mediators. In addition, the immune system also produces regulatory T cells (Tregs), a population of T cells with suppressive properties that serves to limit the activation

and expansion of the immune response [70]. Recent evidence suggests that Tregs may play a role in suppression of the postinfarction inflammatory response [71]. Mice with genetic disruption of the chemokine receptor CCR5 exhibited accentuated inflammatory response and enhanced MMP activity following reperfused myocardial infarction. Increased inflammation in CCR5-null animals was associated with reduced infiltration of the infarcted myocardium with CD4+/CD25+ Tregs [71]. Although a causative relation between defective recruitment of Tregs and enhanced remodeling has not been established, Tregs may modulate macrophage phenotype and suppress postinfarction inflammation by secreting soluble mediators, such as IL-10 and TGF-β, or through contact-dependent pathways [71].

18.3.4 Dendritic Cells

Recent studies have demonstrated that dendritic cell ablation enhanced the postinfarction inflammatory reaction [72]. The anti-inflammatory effects of the dendritic cells were presumed due to regulation of monocytes and macrophage phenotype in the infarcted heart.

18.3.5 Vascular Cells

As the inflammatory infiltrate is replaced by granulation tissue, extensive angiogenesis occurs resulting in formation of neovessels that provide oxygen and nutrients to the highly dynamic and metabolically active cells of the healing wound. Early neovessels in the infarcted heart lack a pericyte coat and are hyperpermeable and proinflammatory [73], perhaps due to the high levels of vascular endothelial growth factor (VEGF) in their microenvironment. As the scar matures, the infarct vasculature undergoes a maturation process that may play an important role in suppression of the inflammatory response in the wound [74]. Some of the infarct neovessels acquire a coat comprised of pericytes and smooth muscle cells, whereas uncoated endothelial cells undergo apoptosis. Acquisition of a mural cell coat reduces vascular permeability and decreases angiogenic potential of the vessels, contributing to the formation of a stable scar. The interactions between pericytes and endothelial cells that result in vascular coating involve platelet-derived growth factor (PDGF)-BB-PDGFR-β signaling [75]. Pericyte coating is an important step for suppression of granulation tissue formation following myocardial infarction and promotes resolution of inflammation and stabilization of the scar. Whether pericytes secrete soluble mediators, or directly signal to promote endothelial cell quiescence, is unknown.

18.3.6 Fibroblasts

Due to their abundance in normal and infarcted hearts and their phenotypic plasticity, fibroblasts are capable of playing an important role as regulators of the postinfarction inflammatory response [76]. Through activation of the inflammasome, resident cardiac fibroblasts play an important role in initiation of the inflammatory phase following cardiac injury [26]. Whether activated infarct myofibroblasts can participate in repression of inflammation and resolution of the leukocyte infiltrate in the healing wound remains unknown.

18.3.7 The Extracellular Matrix

In addition to its structural role, the extracellular matrix proteins also acts as a key regulator of the inflammatory response [77]. In the early stages following infarction, generation of matrix fragments enhances cytokine and chemokine synthesis, while deposition of a plasma-derived provisional matrix provides a scaffold for leukocyte infiltration and may transduce proinflammatory signals. Clearance of matrix fragments from the injured tissue appears to be an essential step for resolution of inflammation and, much like removal of apoptotic cells, may activate inhibitory pathways [78].

The temporally and spatially restricted expression of fibronectin and matricellular proteins in the infarcted heart may provide an additional matrix-related mechanism for regulation of inflammation [79]. The matricellular protein thrombospondin (TSP)-1, a crucial TGF-β activator with potent angiostatic and anti-inflammatory properties, is strikingly upregulated in the border zone of the infarct (Fig. 18.2) [80]. TSP-1−/− mice exhibited enhanced and prolonged expression of chemokines in the infarcted heart and showed expansion of the inflammatory infiltrate into the non-infarcted area. Prolonged and expanded postinfarction inflammation in TSP-1-null animals resulted in increased adverse remodeling of the ventricle [80]. These findings suggest an important role for TSP-1 in suppression and containment of the postinfarction inflammatory reaction. Localized induction of TSP-1 in the infarct border zone may form a "barrier" preventing expansion of the inflammatory infiltrate in the non-infarcted myocardium. The mechanisms involved in TSP-1-mediated inhibition of inflammation remain unknown and may involve impaired TGF-β activation or direct anti-inflammatory actions.

18.4 Anti-inflammatory Therapeutic Strategies in Myocardial Infarction

The idea of targeting the inflammatory response in patients with myocardial infarction is not new. Twenty to thirty years ago, extensive experimental evidence derived primarily from research in large animal models suggested that early infiltration of

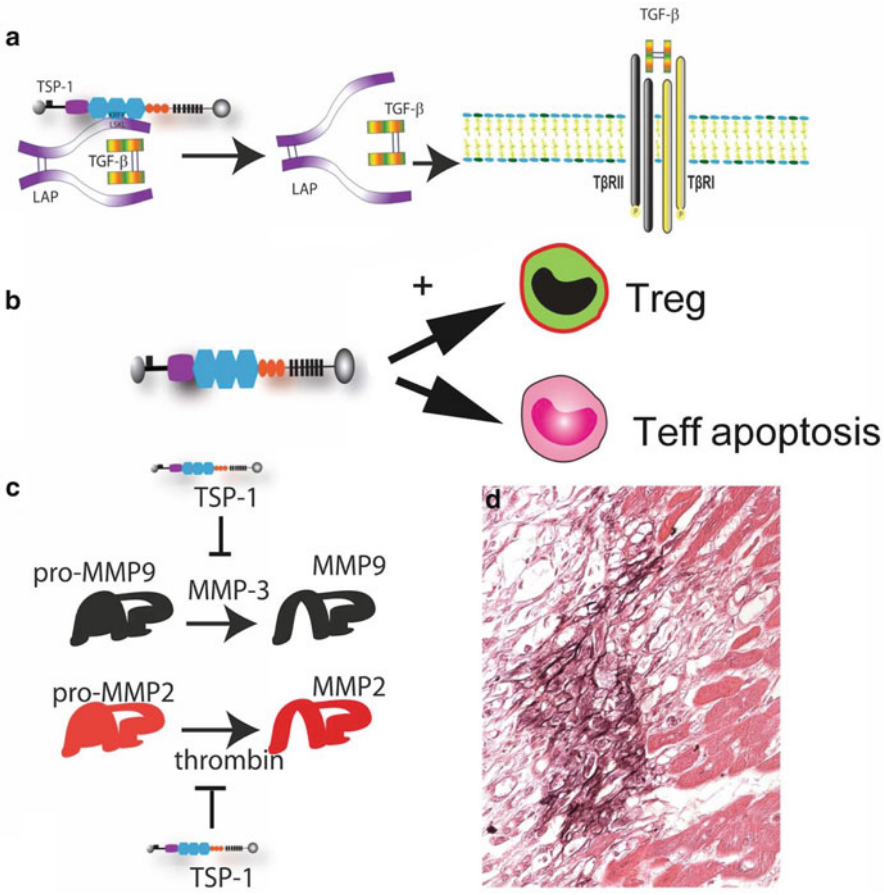

Fig. 18.2 Matricellular proteins play an important role in regulation of the postinfarction inflammatory reaction. Thrombospondin (TSP)-1, a prototypical matricellular protein, activates TGF-β (**a**), promotes regulatory T cell (Treg) formation and induces effector T cell apoptosis (**b**), and inhibits matrix metalloproteinase (MMP) activity (**c**). These actions may be important in modulating the postinfarction inflammatory response. TSP-1 shows a strikingly selective localization in the border zone of myocardial infarction (D-immunohistochemical staining for TSP-1 in canine infarcted myocardium after 1 h ischemia and 7 days of reperfusion, counterstained with eosin) where it may play a role in containment of the inflammatory reaction

the infarcted myocardium with leukocytes induces cytotoxic injury on viable cardiomyocytes extending ischemic damage [81]. These findings fueled several clinical trials aimed at protecting the ischemic myocardium through early inhibition of key inflammatory signals. Unfortunately, both anti-CD18 integrin approaches [82] and complement inhibition strategies [83] produced disappointing results raising concerns regarding the potential effectiveness of inflammatory targets in myocardial infarction. Later studies in murine models have challenged the concept of

"leukocyte-mediated cytotoxic injury" demonstrating that animals with genetic disruption of genes with a critical role in the inflammatory response (such as ICAM-1/P-selectin and IL-1RI) [24, 84] had no reduction in infarct size despite marked attenuation of leukocyte infiltration.

Thus, direct inflammatory cardiomyocyte injury may not be significant; however, a growing body of evidence suggests that disruption of inflammatory pathways may protect the heart by preventing adverse remodeling through effects on reparative cells and on matrix metabolism [5, 85]. Targeting the inflammatory reaction remains promising; however, the focus of our therapeutic strategies should be shifted to a new direction: to ensure optimal temporal and spatial regulation of the inflammatory response preventing uncontrolled or prolonged inflammation. From this perspective, the early disruption of key inflammatory signals attempted in some clinical trials may in fact prolong inflammation by preventing removal of dead cells and matrix debris leading to impaired wound healing, accentuated remodeling, and adverse outcome. Identification of novel therapeutic targets requires intensification of our research efforts in two main areas. First, using carefully selected experimental models, we need to understand the mechanisms involved in repression and resolution of the postinfarction inflammatory response and study the relation between specific defects in negative regulation of inflammation and cardiac remodeling. Second, using suitable biomarkers, we need to identify patients exhibiting defective resolution of inflammation following myocardial infarction; these subpopulations may be ideal candidates for targeted anti-inflammatory approaches to inhibit excessive or prolonged inflammation. Understanding the STOP signals involved in suppression of inflammation following infarction may be the key to prevent adverse remodeling and progression to heart failure.

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Chapter 19
TLR-Dependent Pathways and Akt/mTOR/P70S6K Pathways in Cardiac Remodeling After Myocardial Infarction

Lina Badimon and Gemma Vilahur

Abstract Left ventricular remodeling is a progressive process which starts immediately after acute myocardial infarction (MI) and evolves in the chronic phase of heart failure. The molecular and cellular changes associated with ventricular remodeling affect both the cardiomyocytes and the interstitial space and manifest clinically as increased ventricular size, altered shape of the ventricle, and worsened cardiac function. Specific therapy to optimize healing and prevent adverse post-MI remodeling is currently lacking. Understanding of the specific events (time and space) that occur in response to MI and are involved in infarct healing is crucial to abrogate postinfarction ventricular remodeling and/or stimulate the healing process. Inflammation and extracellular matrix remodeling are two key components associated with LV remodeling after MI. Myocardial injury upon MI activates an inflammatory response that orchestrates the cardiac repair process through a complex cascade involving cytokines, other inflammatory mediators, and growth factors. In this regard, the innate immune system, such as that mediated by the Toll-like receptors (TLRs), has appeared during the past decade as a major contributor to the pathogenesis of MI by modulating cell survival and tissue injury. Cardiac remodeling

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also encompasses many changes in the myocardial interstitium leading to fibrosis and hypertrophy. The mammalian target of rapamycin (mTOR) signaling has shown to exert a broad spectrum of functions including cell cycle progression, hypertrophy, protein synthesis, autophagy, and angiogenesis, all of which largely contribute to the fibrotic reparative scar.

This chapter aims to provide an in-depth analysis of the contribution of both the innate immune system (via TLR activation) and the activation of Akt/mTOR/P70S6K in the cardiac remodeling phase post-MI.

Keywords Myocardial infarction • Cardiac remodeling • Inflammation • Innate immune system • Toll-like receptor • mTOR • Hypertrophy • Autophagy • Fibrosis • Matrix remodeling

Abbreviations

HMGB	High-mobility group box
IL	Interleukin
IRF3	Interferon-regulatory factor-3
LV	Left ventricle
MI	Myocardial infarction
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PI3K	Phosphoinositide 3-kinase
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TORC	mTOR complex
TRAF6	Tumor necrosis factor receptor-associated factor-6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing type 1 interferons
VEGF	Vascular endothelial growth factor
WT	Wild type

19.1 Toll-Like Receptors

19.1.1 Toll-Like Receptors and the Innate Immunity

Innate immunity is our first line of defense against foreign intruders, and it is mainly driven by neutrophils, dendritic cells, and macrophages, as well as cytokines, chemokines, and the activation of the complement cascade [1]. The cells of the innate immune system recognize conserved motifs on pathogens termed

pathogen-associated molecular patterns (PAMPs). Recent evidence also suggests that the innate immune system recognizes endogenous danger signals, termed damage-associated molecular patterns (DAMPs). PAMP/DAMP in turn is recognized by pattern recognition receptors (PRRs). There are currently four known families of PRR: the transmembrane protein families of Toll-like receptors (TLRs), C-type lectin receptors (CLRs), cytoplasmic protein families such as nucleotide oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-like receptors (RLRs) [1].

Toll, meaning "amazing" and "wonderful" in German, was first described by Christiane Nüsslein-Volhard and colleagues in 1985 when they were studying the genetic mechanisms that control early embryonic development in the fruit fly *Drosophila* [2]. TLRs are type I transmembrane proteins composed of three structural domains: an extracellular C-terminal leucine-rich repeat (LRR) as ligand recognition domain, a central transmembrane domain, and an N-terminal cytoplasmic Toll/interleukin receptor homologous signaling domain (TIR) as an effector domain that mediates the homotypic interactions facilitating downstream signaling or substrate processing (Fig. 19.1). TLRs are expressed not only on immune cells but also in nonimmune cells including myocytes, fibroblasts, epithelial cells, keratinocytes, skeletal muscle, and neurons [3]. TLRs mostly detect viral and bacterial components; however, during the last years, increasing evidence indicates that TLRs may be turned on in the absence of infectious microorganisms by host-derived molecules (Fig. 19.1; detailed below).

There are at least 11 TLRs identified in humans (TLR1 to TLR11), and two new members TLR12 and TLR13 have been discovered in mice, but not much information is available about them. TLRs can be either located in the plasma membrane (TLR1, TLR 2, TLR4, TLR5, TLR6, and TLR10) or localized in the membranes of intracellular compartments (TLR3, TLR7, TLR8, and TLR9) such as endosomes. Newly discovered TLR11, TLR12, and TLR13 are also believed to be localized in the plasma membrane (Fig. 19.1). The location of the TLRs seems to be determined by the nature of their ligands. For instance, membrane-bound TLRs recognize pathogens and the breakdown products of extracellular matrix, whereas intracellular TLRs generally recognize nucleic acid structures (Fig. 19.1) [4]. The stimulation of TLRs leads to, through their specific intracellular signaling pathways, the activation of various downstream transcription factors and the ultimate production of inflammatory cytokines in host immune cells, a process that has been linked to myocardial infarction and cardiac remodeling. Indeed, myocardial infarction is associated with an inflammatory reaction that, although a prerequisite for healing and scar formation, when exacerbated may interfere with cardiac healing and collagen matrix deposition.

19.1.2 TLR Signaling Pathways

TLRs act as either homodimers (TLR4 and TLR9) or heterodimers with other TLRs (TLR2 with TLR1 and TLR6) [4]. The different dimmers recognize distinct molecules that include lipids, lipoproteins, nucleic acids, and proteins (Fig. 19.1).

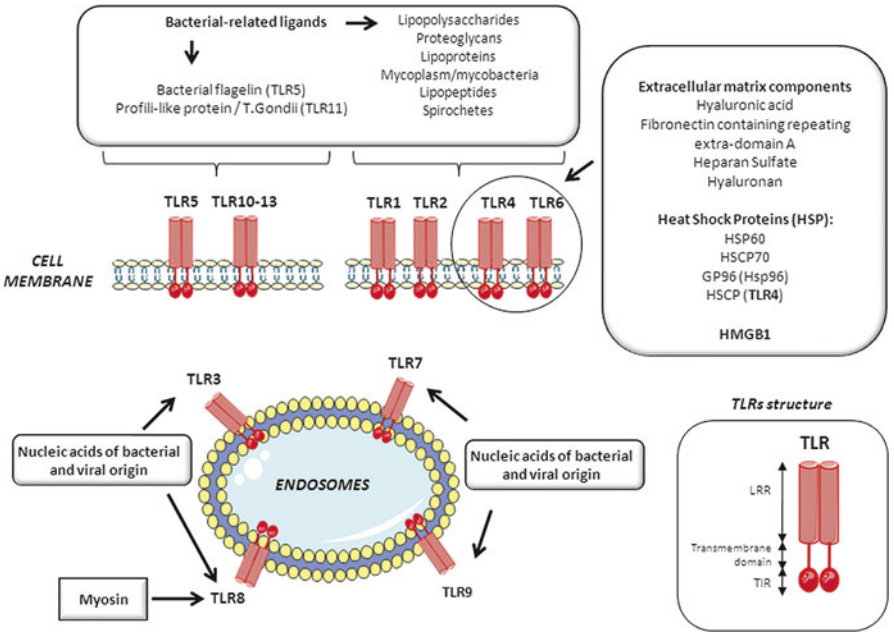

Fig. 19.1 *TLRs structure and ligands/activators of TLRs*. *HMGB* high-mobility group box, *HSP* heat shock protein, *HSCP* heat shock cognate protein, *LRR* extracellular C-terminal leucine-rich repeat (ligand recognition domain), *TIR* N-terminal cytoplasmic Toll/interleukin receptor homologous signaling domain

Upon dimerization of TLRs, the cytosolic TIR changes conformation to facilitate the recruitment of adaptors that initiate TLR downstream signaling. Myeloid differentiation factor 88 (MyD88) is a nearly universal TLR adapter protein (except with regard to TLR3) that also functions as an adaptor in signaling initiated by the IL-1 receptor. Thus, the signaling pathways activated by TLRs are broadly classified into MyD88-dependent or MyD88-independent signaling pathways (Fig. 19.2). TLRs, however, also involve the recruitment of other adaptors including MyD88 adaptor-like [(MAL) also known as TIR-domain-containing adaptor protein (TIRAP)], TIR-domain-containing adaptor protein inducing type 1 interferons (TRIF), TRIF-related adaptor molecule (TRAM), and sterile-α and armadillo motifs (SARM).

TLR–MyD88-dependent pathway. In some TLR signaling, such as TLR2 and TLR4, MAL is required for recruiting MyD88 to the receptor. As shown in Fig. 19.2, once recruited the MyD88/MAL complex activates the downstream interleukin-1 receptor-associated kinase 4 (IRAK4) which, in turn, induces IRAK1 phosphorylation. TRAF6 (tumor necrosis factor receptor-associated factor 6) is also recruited to the receptor complex by associating with phosphorylated IRAK1. Phosphorylated TRAF6 then dissociates from the receptor and forms a complex with TAK1 (TGF-β-activated kinase-1), TAB1 (TAK1-binding protein-1), and TAB2 (TAK1-binding

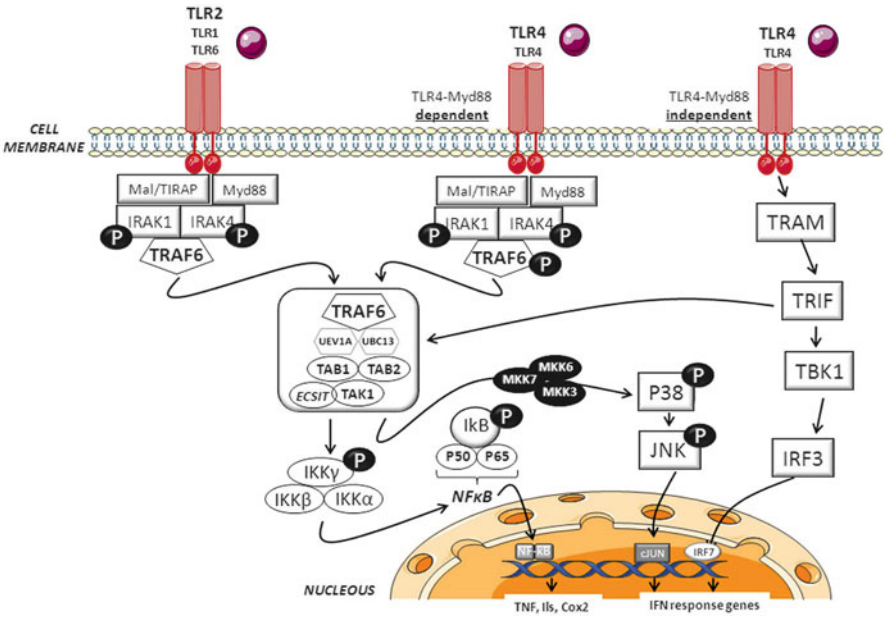

Fig. 19.2 *Both myeloid differentiation primary response protein 88 (MyD88)-dependent and independent TLR downstream pathways and eventual gene induction*. This figure depicts the signaling pathways activated by the two most important cardiac TLR receptors, TLR2 and TLR4. Only the signaling components discussed in the chapter are depicted in this figure. *IRAK1* interleukin-1 receptor-associated kinase, *TRAF6* tumor necrosis factor receptor-associated factor 6, *TAK1* TGF-β-activated kinase-1, *TAB1* TAK1-binding protein-1, *TAB2* TAK1-binding protein-2, *UBC13* ubiquitin-conjugating enzyme-13, *UEV1A* ubiquitin-conjugating enzyme E2 variant 1, *IKK* inhibitor of κ-light polypeptide gene enhancer in B-cell kinase, *NF-κB* nuclear transcription factor-κB, *MKK* mitogen-activated protein kinase, *TRIF* TIR-domain-containing adaptor protein inducing type 1 interferons, *TRAM* TRIF-related adaptor molecule, *TBK1*, TANK-binding kinase-1, *IRF* interferon regulatory factor

protein-2). TRAF6, TAK1, TAB1, and TAB2 associate with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme-13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1). TAK1 phosphorylates the IKK complex (inhibitor of κ-light polypeptide gene enhancer in B-cell kinase), which consists of IKK-α, IKK-β, and IKK-γ. The IKK complex then phosphorylates I-κB, which leads to its ubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of its target genes (proinflammatory cytokines) (Fig. 19.2). TAK1 also phosphorylates both p38 kinases and c-Jun N-terminal kinase (JNKs) by activating MKK3 (mitogen-activated protein kinase-3), MKK6, and MKK7. p38 and JNKs then enter the nucleus and induce the expression of their target genes (Fig. 19.2) [5].

TLR–MyD88-independent pathway. TLR4 can also signal through a MyD88-independent pathway (also known as the TRIF-dependent pathway) (Fig. 19.2). In the TLR–MyD88-independent pathway, TLR4 signals via TRIF-related adaptor

molecule (TRAM) and TRIF which activates TBK1 (TANK-binding kinase-1). TBK1 then stimulates the downstream interferon regulatory factor 3 (IRF3) initiating expression of type 1 IFNs while translocation into nucleus occurs (Fig. 19.2) [5]. TRIF, in turn, may indirectly activate NF-κB through TRAF6.

TLR5, TLR7/8, TLR9, and TLR11 use only MyD88 as its signaling adaptor and MAL is not required, whereas TLR3 signals through the adaptor TIR-domain-containing adaptor protein inducing interferon-β (IFN-β)-mediated transcription factor (TRIF), which recruits and activates TNF receptor-associated factor family member-associated NF-κB activator-binding kinase 1 (TBK1).

19.1.3 TLR Ligands in the Infarcted Heart

The mechanisms involved in TLR activation remain to be fully described. Upon ischemia, TLRs can respond to various endogenous cellular components released from damaged myocytes and extracellular components whose structure is altered as a result of released cellular enzymes (Fig. 19.1). Indeed, the endogenous tissue debris released from injured and inflamed tissues including the highly conserved nuclear protein high-mobility group box (HMGB)1 and breakdown products of the cardiac extracellular matrix (hyaluronan, S100 proteins, heat shock proteins (HSP), and the spliced extra domain A of fibronectin) have been shown to activate the TLR4 signaling pathway (Fig. 19.1) [6, 7]. HMGB1 is a damage-associated molecule passively released by necrotic cells and activated macrophages capable of activating TLR2, TLR4, and TLR9 [8]. HMGB has shown to accumulate in the ischemic myocardium, and peak serum levels have shown to correlate with worse outcome after MI. Another recent study has supported that 70 kDa heat shock cognate protein (HSCP70) released from cardiac cells during ischemia is able of activating TLR4 in resident cardiac driving myocardial inflammatory recruitment [9].

19.1.4 TLRs in Myocardial Infarction

TLR-mediated signaling has been robustly associated with atherosclerotic plaque formation, vascular remodeling, and cell death. During the past years, however, evidence has emerged that clearly indicates that TLR signaling may also play a critical role in acute MI and further remodeling process by modulating the inflammatory response, myocyte survival/death, and the fibrotic scar resolution. Cardiac myocytes express six receptors involved in TLR signaling (TLR2, 3, 4, 5, 7, and 9) [10] although the most studied TLRs in the heart are TLR2 and TLR4. However, the mechanisms by which TLRs exert their cardiovascular effects are incompletely defined [11]. Therefore, defining the critical role of TLR, particularly that of cardiac origin, in the setting of MI may have important therapeutic implications for the posterior inflammatory reaction and cardiac remodeling response.

A short period (5 min) of coronary artery occlusion has not shown to trigger an inflammatory reaction [12]. Yet, a longer ischemic insult may result in a more intense inflammatory response. We have reported in a pig model of closed-chest coronary balloon occlusion that 30 min of ischemia triggers systemic cytokine release and renders platelets and circulating leukocytes more susceptible to activation (higher cycloxigenase-2, monocyte chemoattractant protein-1, and tissue factor protein expression). Such inflammatory response is enhanced upon prolongation of the ischemic period [13]. Interestingly, such systemic inflammatory reaction is associated with a marked upregulation of myocardial TLR4–MyD88-dependent and TLR4–MyD88-independent signaling pathways in the ischemic myocardium leading to NF-κB nucleus translocation and IRF3 activation [14]. These observations support a prompt activation of the cardiac innate immune response upon an ischemic insult that may rapidly translate into a proinflammatory systemic response through TLR4/cytokine networks [13]. Besides, such inflammatory response may have stimulated the detected homing of leukocytes to the site of MI which, in turn, may have contributed to the detected TLR4 increase upon reperfusion [14]. In line with these observations, hearts from TLR4 mutant mice have demonstrated lower myocardial proinflammatory cytokine (TNF-α and IL-1β) levels and NF-κB activation, reduced cardiac inflammatory infiltration, smaller infarctions, and improved postischemic functional recovery compared with their wild-type (WT) counterparts [15–17]. Moreover, mice pretreated with eritoran, a specific TLR4 antagonist, have shown reduced nuclear NF-κB translocation and proinflammatory cytokine expression, when compared with mice treated with vehicle alone, leading to significantly smaller infarcts [18].

Blockade of TLR downstream effectors has also shown to contribute to attenuate cardiac injury upon MI. For instance, rats transfected with Ad5-dn-MyD88 (inactive form) into the myocardium have shown significantly reduced infarct size after MI compared with those transfected with Ad5-GFP (control green fluorescent protein) suggesting a deleterious effect of MyD88-dependent signaling on the postischemic myocardial recovery [19]. Inhibition of NF-κB using molecular (inhibition of p65 by double-stranded oligonucleotides [20] and use of an inhibitor of NF-κB (IkB) triple mutant (S32A, S36A, Y42A) [21]) as well as pharmacological methods (IkB kinase (IKK) inhibition [22]) have also shown to reduce ischemia/reperfusion injury.

In contrast, however, other studies have supported a protective role of TLR4–MyD88 signaling in the setting of MI by preventing myocyte death [23]. Human studies have also shown conflicting results. Population-based studies designed to determine the impact of TLR4 polymorphism on the risk of MI have reported conflicting data. Some studies suggest that among symptomatic men with documented coronary artery disease, the TLR4 Asp299Gly polymorphism is associated with a lower risk of cardiovascular events compared to the control population [24, 25], whereas others suggest an increased risk of MI [26] or no association [27]. It is noteworthy that TLRs are expressed in both compartments (cardiac and vascular), and the exact contribution of TLR signaling of cardiac versus circulatory origin is not clearly defined and may help to explain these opposing findings.

19.1.5 TLR in Healing Infarcts: Cardiac Remodeling

The stimulation of cytokine secretion in response to MI has profound effects on myocardium that provokes at least four changes directly in cardiac myocytes that contribute to cardiac remodeling: progressive myocyte apoptosis, myocyte hypertrophy, defects in contractility, and inflammatory signal transduction [28]. Among the cytokines, TLR-induced TNF-α, IL-1β, and IL-6 are most commonly associated with the remodeling process post-MI [29]. Although data on myocardial remodeling and TLRs are very limited, evidence has been gathered documenting that the levels of TNF-α and IL-1β are increased in hearts of patients with heart failure as well as in the hearts of animals with experimental cardiac dysfunction [30]. Moreover, cytokine levels in the non-infarcted region correlated with the eventual LV end-diastolic diameter measured at 20-week postinfarction [29]. We further evidenced that, although both NF-κB and IRF3 activation represent a rapid and intrinsic response upon MI, there is a sustained increase, for up to 6 days post-reperfusion, in TLR4 gene and protein expression in both infarcted and non-infarcted/remote myocardium. All together, these observations suggest that timely interception of TLR4 might be critical to prevent adverse myocardial scar formation and remodeling [14]. Frantz and colleagues [31] first documented that there was an enhanced TLR4 expression in remodeling murine myocardium remote from sites of ischemic injury and in heart tissue from patients with idiopathic dilated cardiomyopathy which was not observed in control myocardium. Later studies have also shown that TLR4 is increased in the myocardium of patients with heart failure [32]. What is unclear, however, is the functional significance of TLR4 upregulation under these cardiac conditions. Studies conducted in TLR4 knockout mice have shown improved left ventricular function and attenuated adverse left ventricular remodeling as indicated by reduced levels of atrial natriuretic factor, collagen, and heart weight–body weight ratio [15, 33].

Recent data in mice has evidenced a supporting role of the TLR downstream signaling in cardiac remodeling. As such, pharmacologic MyD88 inhibition has shown to protect against pathologic LV remodeling despite no changes were detected in infarct scar formation [34]. Yet, targeted deletion of the NF-κB subunit p50 has shown conflicting results. Some reports have documented that deletion of NF-κB subunit p50 markedly increases the extent of expansive remodeling and functional deterioration following MI [35]. In fact, NF-κB subunit p50 is generally considered to be an inhibitory subunit of the NF-κB complex. However, in contrast, studies published in p50 knockout mice have shown protection against LV remodeling following MI [36]. These discrepancies may derive from the imaging technique used for cardiac remodeling assessment, using a 3D high-resolution MRI 9.4 Tesla in the former and 2D short axis echocardiography in the latter.

As to the role of TLR2 in cardiac remodeling, conflicting results have also been reported. Shishido et al. [37] reported that 4 weeks after permanent coronary ligation, myocardial fibrosis in the non-infarcted area of TLR2 knockout mice was found to be reduced as compared to their WT counterparts and was accompanied by

reduced transforming growth factor 1 and collagen type 1 mRNA expression [37]. Furthermore, left ventricular dimensions at end diastole were smaller, and fractional shortening was higher in TLR2 knockout mice than in WT mice at 1 week and 4 weeks after surgery. However, infarct size and the degree of inflammatory cell infiltration in the infarcted area were similar between WT and TLR2 knockout mice. These findings suggested a critical role for TLR2 signaling in ventricular remodeling by modulating fibrous tissue deposition rather than affecting the inflammatory response [37]. In contrast to these pioneering findings, Mersmann et al. [38] found that TLR2-null animals had a marked left ventricular dilation which was associated with pronounced matrix remodeling characterized by reduced collagen and decorin density in the infarct scar as compared to their WT littermates.

In summary, during the last years emerging evidence has indicated that TLRs mediate the inflammatory response upon MI driving the subsequent remodeling process. Yet, the critical role of TLR2 and TLR4, especially that of cardiac origin, in cardioprotection and prevention of adverse left ventricle remodeling remains unclear.

19.2 PI3K/Akt/mTOR/P70S6K

As described above, following the first acute inflammatory phase, cellular and interstitial changes occur in the left ventricle, including myocyte hypertrophy and enhanced protein synthesis (interstitial collagen deposition) that govern the evolving cardiac fibrotic scar. Several studies have suggested that collagen quantity and quality in the infarcted LV plays a critical role in heart failure progression [28, 39]. An important pathway related to the activation of the protein translation machinery is the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR)/P70S6K signaling pathway [14]. The contribution of this pathway to cardiac remodeling is supported by several studies in mice reporting that whereas cardiac-specific overexpression of constitutively active PI3Kα results in enlarged hearts owing to increased cardiomyocyte size, overexpression of dominant-negative PI3Kα produces smaller hearts with reduced cardiomyocyte size without affecting cell number [40]. These changes in heart size are associated with corresponding alterations in Akt phosphorylation and activity and its downstream effectors mTOR/P70S6K [40].

19.2.1 PI3K/Akt/mTOR/P70S6K Signaling

As depicted in Fig. 19.3, oxidative stress-related activation of PI3K results in the activation of 3-phosphoinositide-dependent kinase-1 (PDK1) which in turn phosphorylates Akt, also called protein kinase B. Yet, full Akt activation requires Akt phosphorylation both on threonine 308 (by PDK) and on serine 473 by the mTOR

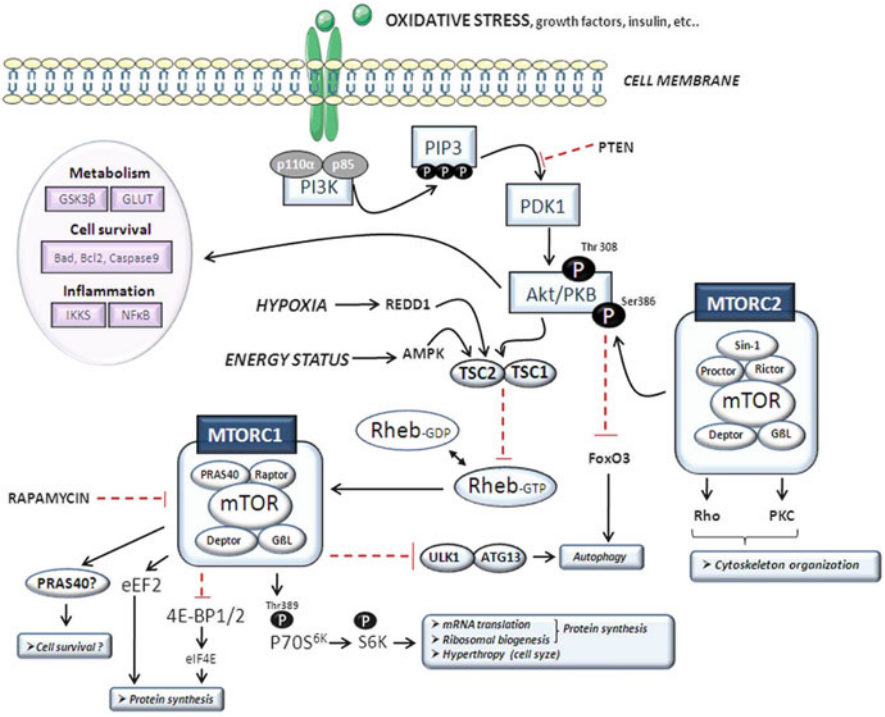

Fig. 19.3 *Diagram of key features of the PI3K/Akt/TOR signaling network*. The lipid kinase PI3K is activated downstream of oxidative stress signals (*continuous arrows*). PI3K generates the second messenger PIP3 which activates PDK1. The TOR serine–threonine kinase is present in two distinct multiprotein complexes, termed TORC1 and TORC2. Key substrates are shown, and their activities are either increased (*block arrows*) or decreased (*pointed red lines*) by TOR-mediated phosphorylation. The resultant functional outcomes are included. *PDK1* phosphoinositide-dependent kinase-1, *GSK3β* glycogen synthase kinase 3β, *GLUT* glucose transporter, *mTOR* mammalian target of rapamycin, *TSC* tuberous sclerosis complex, *mTORC* mTOR complex, *eEF2* elongation factor 2

complex 2 (see below for a more detailed description). Once activated, Akt/PKB phosphorylates several downstream targets including glycogen synthase kinase 3β (GSK3β), glucose transporter (GLUT), several antiapoptotic effectors, inflammatory mediators, and, last but not the least, the mammalian target of rapamycin (mTOR; Fig. 19.3). mTOR is a large (290 kDa) serine–threonine protein kinase belonging to the phosphatidylinositol kinase-related kinase (PIKK) family [16]. Akt/PKB regulates mTOR activation via tuberous sclerosis complex (TSC)-1/2 and Rheb. Phosphorylation of TSC2 by Akt inhibits TSC2 function, but disinhibits Rheb, resulting in mTOR activation. mTOR exists in two different complexes: TORC1 (mTOR complex 1) and TORC2 (mTOR complex 2). Both these complexes bind mLST8/GβL and deptor, whereas TORC1 contains PRAS40 and raptor, and TORC2 contains protor, rictor, and mSin1 (Fig. 19.3). mTORC2 is mainly involved in Akt activation an actin cytoskeleton organization.

mTOR also functions downstream of Akt via mTORC1 which has been implicated in mRNA translation, ribosomal biogenesis, autophagy, and hypertrophy. Akt-related activation of mTORC1 leads to P70S6 kinase (P70S6K, a short isoform of the ribosomal S6 kinase) phosphorylation at threonine 389. By phosphorylating P70S6K, this results in the phosphorylation of the ribosomal protein S6K which in turn phosphorylates the ribosomal S6 protein, a component of the 40S ribosomal subunit. S6 regulates protein translation, ribosomal biogenesis, and cell growth (hypertrophy) (Fig. 19.3). Protein synthesis is also triggered by mTOR negative regulation of the translation repressor proteins 4E-BP1/2. Blockade of 4E-BP1/2 activates the translation initiator eIF4E leading to increased protein translation and the progression of the cell from G0/G1 to S phase [41]. mTOR also enhances the cellular capacity for protein synthesis (i.e., ribosomal biogenesis) by activating elongation factor 2 (eEF2 kinase).

19.2.2 mTOR in Cardiac Remodeling

The signaling mechanism regulated through mTOR largely supports its central role in cardiac remodeling post-MI. In fact, mTOR mRNA and protein levels have found to be elevated in cardiac tissue from heart failure patients compared to healthy subjects [42]. Moreover, the data presented so far also emphasizes that control of mTOR-dependent pathway may potentially prevent post-MI adverse cardiac remodeling. In this regard, mTOR signaling pathway can be regulated by PTEN (physiological inhibition) or rapamycin (pharmacological inhibition). PTEN is a lipid phosphatase and tensin homologue deleted on chromosome 10 which dephosphorylates PIP3, preventing Akt activation (Fig. 19.3) [43]. Rapamycin is a macrocyclic antibiotic discovered in the Easter Island ("Rapa Nui") with high affinity to block mTORC1 (mTORC2 is insensitive to rapamycin) [44]. Inhibition of mTOR through systemic rapamycin treatment upon reperfusion of the ischemic heart has shown to prevent adverse LV remodeling, a protective effect not detected when rapamycin was given before the onset of ischemia or 3-day post-MI [45]. In line with these observations, we have reported in a preclinical model of MI that activation of PI3K/Akt and its downstream effectors mTOR/P70S6k occurs during early reperfusion (within the first 2.5 h) and its maintained over late phase reperfusion (up to 6 days), and not during severe/persistent ischemia [14]. Moreover, Akt/mTOR/P70S6k activation was timely associated with myocyte hypertrophy and collagen interstitial deposition in the forming reparative fibrotic scar. According to these data, rapamycin has shown to attenuate left ventricle hypertrophy and cardiac remodeling by reducing P70S6k and S6K activity in mice [46]. Moreover, rapamycin has also shown to attenuate cardiac hypertrophy in humans [47].

Finally, further data has also suggested that there may be a TLR-mediated signaling pathway that cross talks with the PI3K/Akt signaling pathway concomitantly contributing to cardiac hypertrophy. In fact, inhibition of mTOR by rapamycin in TLR4-deficient mice has shown to result in an additional decrease in the development

of cardiac hypertrophy [19]. A growing body of evidence suggests that reactive oxygen species (ROS) may play a link in the pathogenesis of cardiac remodeling since ROS can activate transcription factors, such as NF-κB, and stimulate the PI3K/Akt/mTOR signaling pathway.

19.2.3 New Horizons for mTOR

mTOR and autophagy. Autophagy is essential in the heart for protein turnover and the homeostasis of organelles. If autophagy is reduced, abnormal proteins accumulate and lead into apoptosis, enhanced endoplasmatic reticulum stress, and cardiac dysfunction. In addition to its well-established role in translation, the mTOR pathway has been suggested to play a role in autophagy. mTOR, through both TORC complexes, has shown to negatively regulate autophagy in the context of MI and cardiac remodeling. Recent studies have revealed that this is achieved via TORC1—through the phosphorylation and subsequent repression of the autophagy-promoting factors unc-51-like kinase 1 (ULK1) and autophagy-related gene 13 (ATG13)—and/or via TORC2 by repressing the transcription factor FoxO3 (Fig. 19.3). mTOR inhibition and the subsequent upregulation of autophagy in the critical border zone (periinfarction) have shown to act as protective mechanisms in the failing heart by limiting infarct size and preventing left ventricular remodeling [48]. Nonetheless, caution in mTOR sustained repression should be taken since unrestricted augmentation of autophagy may be detrimental.

mTOR and angiogenesis. Heart failure is associated with a decrease in myocardial capillaries in part by concomitant fibrosis. Recent studies have suggested that mTOR downstream activation beneficially contributes to angiogenesis by increasing VEGF and angiopoietin (Ang)-2 expression via hypoxia-inducible factor (HIF)-1α [49, 50]. These findings indicate that disruption of the mTOR signal may promote pathological remodeling.

19.3 Conclusions and Perspectives

Due to improvement of therapeutic strategies after MI, mortality has significantly decreased in the past decades. Consecutively, there have also been an increasing number of patients whose prognosis depends on optimal treatment after MI. Robust clinical data supports that the extent of adverse myocardial remodeling contributes essentially to the prognosis after MI. Despite the widespread use of ACE inhibitors (angiotensin-converting enzyme inhibitors), β-blockers, and aldosterone antagonists, the incidence of heart failure as the end stage of left ventricular remodeling still remains high. Novel strategies to reduce cardiac remodeling are required. However, better understandings of the fundamental processes that occur upon MI/revascularization are needed to help achieve this goal. Underlying mechanisms of

remodeling are manifold [28] as detailed along the book, including the activation of the innate immune system (inflammation) and protein translation machinery (fibrosis), highlighted throughout this chapter. A large body of evidence suggests that innate immunity is triggered through TLR in models of acute MI. However, the importance of TLR and its downstream effector NF-κB in the chronic context of myocardial remodeling is far from determined. Likewise, whether the activation of the innate immunity is just an attempt to repair damaged tissue and is a beneficial process counteracting ischemic damage should also be regarded. Finally, the role of innate immunity in the heart versus the periphery needs to be ascertained.

On the other hand, increasing evidence supports that the signaling mechanisms regulated through mTOR have shown to play a major role in cell growth and proliferation, protein synthesis, and autophagy, hallmarks of cardiac remodeling. However, new venues of mTOR signaling such as angiogenesis stimulation open the question as whether mTOR inhibition may mediate beneficial or negative effects in the cardiac healing response following MI.

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Chapter 20
The STAT3 Pathway and Downstream Mechanisms in Cardiac Remodeling: Friend or Foe

Melanie Ricke-Hoch, Britta Stapel, Irina Gorst, Arash Haghikia, and Denise Hilfiker-Kleiner

Abstract Multiple in vitro and in vivo studies have shown that the signal transducer and activator of transcription 3 (STAT3) protein regulates key mechanisms in cardiac physiology (exercise, pregnancy) and pathophysiology (pressure overload, ischemia/reperfusion, myocardial infarction (MI), myocarditis, and cardiotoxic agents). STAT3 is activated in various cardiac cell types including cardiomyocytes, endothelial cells, fibroblasts, and cardiac progenitor cells by a multitude of factors including cytokines, growth factors, neurohormones, mechanical load, and ischemia. It acts as a signaling molecule, a transcription factor, and a mitochondrial protein involved in energy production, and it controls autocrine and paracrine pathways. While the majority of data imply rather beneficial roles of STAT3 in the heart, newer studies implicate that this is mainly the case when the expression and activation of STAT3 is precisely regulated. In contrast, continuous uncontrolled activation of STAT3 in cardiomyocytes seems to promote adverse cardiac remodeling processes especially after MI. Here, we provide an overview on STAT3 signaling and summarize the current understanding of the role of STAT3 for cardiac inflammation, metabolism, remodeling, and regeneration based on experimental and clinical studies. Finally, we highlight the consequences of targeting STAT3 for future therapeutic approaches in the setting of cardiac remodeling.

Keywords STAT3 • Cardiac remodeling • gp130–STAT3 signaling • Inflammation • Metabolism • Angiogenesis • Hypertrophy • Sarcomere structure

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Abbreviations

ADMA	Asymmetric dimethylarginine
Bcl-3	B cell lymphoma 3
CCL2	Chemokine ligand 2
CCR2	Chemokine ligand receptor 2
CLC	Cardiotrophin-like cytokine
CNTF	Ciliary neurotrophic factor
CT-1	Cardiotrophin 1
CTGF	Connective tissue growth factor
CVF	Cobra venom factor
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp130	Glycoprotein-130
HK2	Hexokinase 2
IL	Interleukin
JAK	Janus kinase
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LV	Left ventricle
MBL	Mannose-binding lectin
MHC	Myosin heavy chain
MI	Myocardial infarction
MMP	Matrix metalloproteinase
OSM	Oncostatin M
miRNA	microRNA
NF-κB	Nuclear factor-κB
PAI-1	Plasminogen activator-1
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PKB	Protein kinase B, also known as AKT
PPAR	Peroxisome proliferator-activated receptor
PPCM	Peripartum cardiomyopathy
PIAS	Protein inhibitors of activated STAT
PTPRT	Protein tyrosine phosphatase receptor T
ROS	Reactive oxygen species
SH2	Src homology 2 domain
SHP2	SH2 domain-containing cytoplasmatic protein
SOCS	Suppressor of cytokine signaling
STAT3	Signal transducer and activator of transcription 3

STAT3-KO	STAT3-knockout
TAC	Transverse aortic constriction
TIMP-1	Tissue inhibitor of metalloproteinase-1
TNC	Tenascin C
TNF-α	Tumor necrosis factor-α
TSP-1	Thrombospondin-1
Ube	Ubiquitin-conjugating enzyme
UPS	Ubiquitin–proteasome system
VEGF	Vascular endothelial growth factor

20.1 Introduction

The adult heart experiences numerous insults of physiological (aging, pregnancy, exercise) and pathophysiological [ischemia/reperfusion, myocardial infarction (MI), pressure overload, infections, and cardiotoxic agents] nature, which can induce alterations in the cardiac homeostasis and architecture. These alterations may be adaptive and protective to a certain stress situation or can be maladaptive, thereby inducing, driving, and contributing to heart failure [1].

Recent findings in experimental in vitro and in vivo models implicate important intermediate signal-transduction pathways in the coordination of remodeling processes following cardiac stress stimuli [2]. It is a key issue for the clinical arena to identify both detrimental as well as protective pathways and to transfer this knowledge into clinical practice. In this regard, the STAT3 signaling system has been shown to critically impact on changes in cardiac inflammation, vasculature, extracellular matrix (ECM) composition, energetics and metabolism, and cardiomyocyte survival and architecture [3–13].

As a transcription factor, STAT3 participates in the regulation of numerous genes involved in cell survival, hypertrophic growth, proliferation, angiogenesis and neovascularization, development and regeneration, and anti-oxidative pathways [3, 4, 6–11, 13, 14]. Furthermore, STAT3 has been reported to modulate ECM response to mechanical overload and to influence inflammatory processes [4, 6–8, 10]. While many of these effects of STAT3 are achieved by its own direct transcriptional activity, recent findings identified the novel function of STAT3 to directly interact with mitochondrial function [12], which adds to the complexity of STAT3-mediated regulatory processes.

Altogether, recent studies support the conclusion that STAT3 functions as part of an integrated signaling network in the heart. Here, we provide an overview of established STAT3-related biological mechanisms in cardiac physiology and pathophysiology, we illustrate its particular role in directing cardiac cellular processes that modulate ventricular remodeling, and finally we challenge the question whether STAT3 signaling conducts beneficial or detrimental effects with respect to cardiac function and heart failure.

20.2 Canonical gp130-STAT3 Signaling, a Central Regulatory Circuit in Cardiac Physiology and Pathophysiology

Interleukin (IL)-6-type cytokines such as IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and cardiotrophin-like cytokine (CLC) are a family of helix bundle cytokines that play a key role in cellular and tissue homeostasis [15, 16]. They are involved in inflammatory and immunologic processes as well as in hematopoiesis, liver and neuronal regeneration, embryonic development, and cardiovascular physiology [16–18].

All IL-6-type cytokines interact with the glycoprotein-130 (gp130) receptor, and upon ligand binding, the homodimerization of gp130 (upon binding of IL-6 and IL-11) or heterodimerization of gp130 with LIF receptor (following binding of LIF, CNTF, CLC, OSM, and CT-1) or OSM receptor (for OSM), respectively, occurs [19–21] leading to the activation of three major downstream pathways: the Janus kinase (JAK)-STAT, the SH2 domain-containing cytoplasmic protein tyrosine phosphatase (SHP2)–extracellular signal-regulated kinase (ERK), and the phosphatidylinositol-3-kinase (PI3K)–protein kinase B (PKB, AKT) pathways [4, 18, 22–26]. The induction of the gp130-JAK-STAT pathway through IL-6-type cytokines leads mainly to activation of STAT3 and to a lesser extent to STAT1 activation [17]. Following dimerization of the gp130 receptor complex, JAK1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2) constitutively connected to the intracytoplasmic membrane-proximal regions of the receptor subunits are catalytically activated and themselves transphosphorylate tyrosine residues in the gp130 receptor intracellular domain [27, 28]. This allows the recruitment of STAT proteins to the gp130 receptor complex via recognition of its phosphotyrosines by the STAT Src homology 2 domains (SH2) resulting in binding of monomeric STAT to the Y-X-X-Q motives and in phosphorylation of STAT proteins at a single carboxy-terminal tyrosine residue at amino acid position 705 (Y705) [29]. The phosphorylated STAT proteins then detach from their receptor and undergo homo- or heterodimerization through reciprocal interactions between the SH2 domain of one monomer and the phosphorylated tyrosine residue of the other [30]. Dimeric STAT proteins translocate in an importin-α-dependent manner to the nucleus, bind to specific DNA elements in the promoters of immediate early target genes, and activate specific gene expression programs.

Ligand-dependent STAT activation is a tightly controlled transient process, lasting for minutes up to several hours, during which nuclear STAT proteins are eventually inactivated by tyrosine dephosphorylation leading to their nuclear export [31]. Additional regulatory mechanisms are known to negatively control STAT activity, that is, gp130 receptors are rapidly internalized upon binding of their ligands and are degraded by the ubiquitin–proteasome pathway [32]. Furthermore, SHP-1/2 can interact with the intracytoplasmic portion of cytokine receptors and dephosphorylate JAK proteins, thereby lowering their activity [33, 34]. In addition, the suppressor of cytokine signaling (SOCS) family represents a specific negative regulatory

feedback element of STAT signaling [35], since, for example, SOCS3 is a direct transcriptional target of STAT3. SOCS1, SOCS3, and SHP-1/2 interact with the kinase domain of various JAK proteins or the cytoplasmic phosphotyrosine residue (phospho-Y759 in humans, phospho-Y757 in mice) of the gp130 receptor resulting in the inhibition of STAT protein phosphorylation, thereby leading to termination of JAK-STAT signaling [36–38]. Additional negative regulators consisting of the group of protein inhibitors of activated STAT (PIAS-1, PIAS-3) inhibit the binding of phosphorylated STAT1 and STAT3 dimers to DNA, thereby reducing the transcriptional activation of STAT target genes. Finally, the protein tyrosine phosphatase receptor T (PTPRT) specifically dephosphorylates the Y705 residue of STAT3 and thereby regulates target gene expression but also cellular localization of STAT3 [39]. Importantly, the activation state of STAT3 seems to play a major role whether IL-6-gp130 signaling acts in a more anti-inflammatory or a more proinflammatory manner.

Not only is gp130-STAT3 signaling tightly controlled, but also activation of the other gp130-triggered signaling pathways is regulated to avoid unlimited cytokine signaling from the gp130 receptor with possible detrimental effects. For example, SHP2 limits gp130-SHP2-ERK signaling [18, 40, 41]. In addition, SOCS3 competes with SHP2 for binding to phosphorylated Y757 of the gp130 receptor, thereby limiting gp130-SHP2-ERK signaling and STAT activation [18, 40, 41]. Thus, simultaneous activation of SHP2-ERK and STAT maintains a balanced gp130 signaling, which appears crucial for beneficial responses mediated by this receptor system [40, 42]. Indeed, mice with a systemic deletion of all STAT-binding sites at the C terminus of the gp130 receptor, $gp130^{\Delta STAT}$, are characterized by impaired induction of the STAT1 and STAT3 activation and sustained activity of the SHP2-ERK pathway, which is associated with impaired intestinal wound healing and immune response and reduced life spans [18, 40, 41]. In contrast, $gp130^{Y757F}$ mice harboring a phenylalanine substitution at Y757 (Y757F) abolish binding of SHP2 and SOCS3 leading to a prolonged and continuous activation of STAT1 and STAT3 in response to IL-6 and IL-11 without activation of ERK or AKT signaling. As a consequence, $gp130^{Y757F}$ mice spontaneously develop gastric adenomas, splenomegaly, and an exaggerated immune response [18, 40, 41]. Moreover, mice with a cardiomyocyte-specific Y757F mutation ($\alpha MHC\text{-}Cre^{tg/-}$; $gp130^{fl/Y757F}$) display a high inflammatory reaction and LV rupture rate with increased mortality after MI [10].

Albeit an unrestricted high activation of gp130-STAT3 signaling seems to be detrimental in many organ systems including the heart, activation of STAT3 is absolutely required for many protective mechanisms in the heart including compensatory hypertrophy, cardiomyocyte protection, and wound healing after MI [4, 24, 43, 44].

In conclusion, it seems that a well-controlled and balanced activation of IL-6-type cytokine gp130-STAT3 signaling is beneficial for most organs including the heart, but sustained and prolonged gp130-STAT3 appears to be detrimental and is responsible for enhanced inflammation and high mortality, for example, after MI [10].

20.3 STAT3 in Cardiac Inflammation

STAT3 was initially described as a DNA-binding protein that recognizes the IL-6-responsive element in the promoter of the α_2-macroglobulin gene whose gene product belongs to the acute-phase response of the liver [45, 46]. Further evidences for STAT3 being a mediator of inflammation include the discovery of overlapping DNA-binding sites for STAT3 and the proinflammatory nuclear factor-κB (NF-κB) in the promoter of acute-phase proteins and the finding that it is activated by most proinflammatory agents, such as IL-6-type cytokines [47, 48]. Experiments with IL-6-knockout and cardiac-specific transgenic mice reveal its role in mediating cardiac inflammation after burn injury in combination with sepsis [49].

To ensure resolution of inflammation, the infiltration of inflammatory cells and the expression of proinflammatory cytokines in response to injury are balanced by the production of the anti-inflammatory cytokine IL-10 [50]. In an ischemia/reperfusion model, IL-10 is shown to be induced in the myocardium with concomitant downregulation of IL-6 mRNA expression [51, 52]. Furthermore, IL-10 suppresses the inflammatory response by inhibiting the expression of several cytokines such as IL-1, IL-6, IL-12, and TNF-α in activated macrophages and dendritic cells and mediates its effects indirectly through activation of STAT3 [52–54]. Hereafter, the induced STAT3 activates the expression of other genes, which in turn are required for a selective control of the transcription of inflammatory genes [53]. One of those intermediate genes might be *B cell lymphoma (Bcl-3)*, whose gene product blocks TNF expression after IL-10-mediated STAT3 activation in human macrophages [54].

Interestingly, infiltrating CD5-positive T cells have been identified as a predominant source of reperfusion-dependent IL-10 production, which express the cytokine upon induction by IL-6 [52, 55]. Another proinflammatory cytokine, tumor necrosis-α (TNF-α), is as well able to induce IL-10 synthesis by monocytes [55].

20.3.1 STAT3 and Inflammation Following Myocardial Ischemia

STAT3 is rapidly and transiently activated in response to myocardial ischemia per se or by reperfusion following ischemia. Mice harboring a cardiomyocyte-restricted deletion of STAT3 (α*MHC-Cre*$^{tg/-}$; *STAT3*$^{fl/fl}$: STAT3-KO) display increased cardiac injury and lower survival rates after MI mainly caused by adverse remodeling suggesting that STAT3 is needed for protective mechanisms after ischemia [7]. Likewise, mice lacking the gp130 receptor and therefore having reduced STAT3 activation in the acute phase after infarction show increased mortality rates [10]. However, not only lack of STAT3 activation but also sustained uncontrolled cardiac activation of STAT3 after MI as it is present in mice carrying the mutation of *gp130*^{Y757F} only in cardiomyocytes (α*MHC-Cre*$^{tg/-}$; *gp130*$^{fl/Y757F}$ mice) leads to lower survival rates post-MI. The high mortality under unrestricted gp130-STAT3 activation derives mainly from enhanced and prolonged ventricular inflammation and high rupture rates [10].

In search for adverse downstream effectors of STAT3 signaling in infarcted *αMHC-Cre*$^{tg/-}$; *gp130*$^{fl/Y757F}$ left ventricles (LVs), the mannose-binding lectin (MBL)/lectin complement activation has been identified as a mediator of inflammation by hyperactivated STAT3 whose relevance is supported by the finding that attenuation of complement activation by the C3 antagonist cobra venom factor (CVF) reduces cardiac inflammation and improves cardiac function and survival in infarcted *αMHC-Cre*$^{tg/-}$; *gp130*$^{fl/Y757F}$ mice [10]. In addition, a murine SOCS3-knockout model, in which STAT3 is continuously activated because the negative feedback loop of the gp130-STAT3 signaling in cardiomyocytes is abolished, develops lethal cardiac arrhythmias and heart failure in response to pressure overload, supporting the hypothesis that too much of STAT3 activation seems to be harmful for the heart [56]. However, it seems that STAT3 not simply promotes inflammation, it can also act as a mediator of anti-inflammatory effects since it has been shown that in ischemia/reperfusion injury, the cardioprotective effects and the attenuation of myocardial inflammation by recombinant IL-10 given were lost if STAT3 activation was inhibited [57]. Likewise, intravenous administration of exogenous IL-11 evokes activation of STAT3 in cardiomyocytes in vivo and attenuates cardiac fibrosis and suppresses the expression of proinflammatory cytokines like IL-6 and TNF-α in a STAT3-dependent manner supporting the anti-inflammatory properties of activated STAT3 [58]. Here it is important to note that a majority of cytokine expression in the ischemic heart seems to derive from non-myocyte cells [59, 60].

Taken together, STAT3 plays an important role for protective mechanisms in the ischemic heart and after ischemia/reperfusion; however, the genetic and pharmacological dissection of gp130-mediated signaling indicates that only a well-controlled and balanced STAT3 activation is protective in the ischemic heart. Moreover, it seems specifically important in which cell type STAT3 is activated under ischemic condition, a feature that may explain the somehow contrary roles of STAT3 with regard to cardiac inflammation under ischemic conditions.

20.3.2 STAT3 and Myocarditis

It seems that STAT3 acts as a protective factor in viral myocarditis since cardiac-specific overexpression of SOCS3 increases the susceptibility to viral infection of the myocardium [61]. Further evidences for STAT3 regulating cardiac inflammation in responses to pathogens derive from STAT3-KO mice, which display a significantly higher TNF-α secretion and cardiac apoptosis rate in response to lipopolysaccharide (LPS)-induced inflammation [11].

With regard to cardiac inflammation, the question whether STAT3 is a friend or a foe cannot be answered in general. STAT3-mediated responses depend on the abundance of the particular cytokines, which activate the gp130-STAT3 signaling cascade, and on the cell type where these cytokines and the receptor system are expressed and activated in order to be either pro- or anti-inflammatory. Furthermore, the activation status of STAT3 is of major importance for the behaviors as a friend

or a foe, since too much STAT3 activation after MI or cardiac deletion of STAT3 during LPS-mediated inflammation could be detrimental. Thus, the key issue seems to be a balanced and well-controlled activation of STAT3 in all cells affected by inflammatory processes in the heart.

20.4 STAT3 in Angiogenesis and Extracellular Matrix Composition

One of the earliest evidence of a potential role of STAT3 in angiogenesis was derived from observations showing that granulocyte-macrophage colony-stimulating factor (GM-CSF) via activated STAT3 contributes to vessel formation [62].

However, genetic analysis in mice with an endothelial-specific deficiency of STAT3 (*Tie2-Cre*$^{tg/-}$; *STAT3*$^{fl/fl}$), where STAT3 is depleted at E8 in angioblasts, showed that these mice survive into adulthood without major vascular defects implicating that endothelial STAT3 is optional for vessel formation during development [63].

While it seems that STAT3 is not directly required for vessel formation by endothelial cells, it has been shown that STAT3 acts as a transcription factor for angiogenic mediators such as vascular endothelial growth factor (VEGF) and as a suppressor of angiostatic genes [64]. The mechanisms of vascular formation in the postnatal heart involve both auto- and paracrine circuits where specifically secreted paracrine factors from cardiomyocytes seem to play a pivotal role for cardiac angiogenesis [65]. Indeed, it has been shown that STAT3 participates in the upregulation of VEGF and VE-cadherin expression in cardiomyocytes [64, 66], a feature that is in line with the observation that STAT3 acts as a paracrine mediator in cardiomyocytes for vascular growth as, for example, demonstrated in mice with cardiomyocyte-specific overexpression of STAT3 (*αMHC-STAT3tg*) that displays increased myocardial capillary density [64, 67].

In contrast to *αMHC-STAT3tg* mice, the STAT3-KO (*αMHC-Cre*$^{tg/-}$; *STAT3*$^{fl/fl}$) in cardiomyocytes leads to a rarification of cardiac capillaries, an enhanced fibrosis, and the development of an age-dependent heart failure suggesting a crucial role of STAT3 in controlling paracrine mechanisms involved in postnatal angiogenesis [7, 11]. Surprisingly, STAT3-KO mice show no differences in the expression of VEGF or VEGF receptors. However, they display an increase in the expression of antiangiogenic factors, which are able to inhibit endogenous VEGF activity or release such as connective tissue growth factor (CTGF) and thrombospondin-1 (TSP-1) [7, 68, 69]. Other potent antiangiogenic factors are as well upregulated like tissue inhibitor of metalloproteinase-1 (TIMP-1), tenascin C (TNC), and plasminogen activator-1 (PAI-1), which act by promoting apoptosis of endothelial cells or alter ECM composition [7, 8, 70, 71]. In this regard, supernatants of STAT3-KO cardiomyocytes are able to repress the proliferation of endothelial cells [7]. Additionally, TIMP-1 is well known to enhance fibrosis by inhibition of matrix-degrading enzymes such as matrix metalloproteinases (MMPs) [72]. This regulatory alteration may explain the

age-dependent increase in interstitial fibrosis and enhanced deposition of collagens of STAT3-KO mice, indicating impaired ECM homeostasis [7, 11]. Thus, deterioration of angiogenesis in the aging myocardium of STAT3-KO mice is caused at least in part by altered structural and regulatory components of the matrix scaffold of the vascular bed and by enhanced collagen accumulation [7, 73]. Additionally, disturbance of ECM homeostasis may cause insufficient migration of angiogenic cells such as endothelial cell precursors or blood-derived primitive stem cells to the side of injured myocardium and thereby reduces the neoangiogenic capacity [74].

Moreover, STAT3 is involved in angiogenesis under pathophysiological conditions like MI. In this regard, activated STAT3 induces angiogenesis in the ischemic preconditioned infarcted myocardium [75]. In a STAT3-dependent manner, granulocyte colony-stimulating factor (G-CSF) is involved in cardiac remodeling after MI by increasing the number of endothelial cells in the border zone of the infarcted myocardium. This observation is diminished in dominant-negative STAT3 transgenic mice, suggesting that the enhanced vascularization is mediated STAT3-dependently in cardiomyocytes [76].

Furthermore, in $\alpha MHC\text{-}Cre^{tg/-}$; $gp130^{fl/Y757F}$ with a continuous activation of STAT3 in cardiomyocytes, the prolonged activation of STAT3 contributes to a higher capillary density in the nonischemic myocardium in the subacute phase after MI [10]. But the increase in capillarization is not sufficient to improve the outcome after MI. In fact, the high vessel density in the border zone of infarcted $\alpha MHC\text{-}Cre^{tg/-}$; $gp130^{fl/Y757F}$ mice may weaken the tissue or may promote further inflammation and thereby even contribute to adverse effects such as LV rupture and dilatation of the scar. During elimination of necrotic tissue and digestion of collagens in the border zone and later in the infarct scar, the adverse remodeling is mediated by MMPs released from inflammatory cells. In fact, in $\alpha MHC\text{-}Cre^{tg/-}$; $gp130^{fl/Y757F}$ mice, MMP-1 and MMP-13 expression is upregulated, but is not counterbalanced by upregulated expression of their inhibitors such as TIMP-1 [10].

Additionally, STAT3 signaling influences the fate of cardiac stem or progenitor cells by the modulation of paracrine circuits in the heart or the direct induction of differentiation of stem cells. In this context, STAT3 activation (mediated by LIF stimulation) is able to directly induce endothelial differentiation of cardiac Sca-1$^+$ stem cells contributing to neovascularization during cardiac remodeling [77, 78]. Moreover, cardiomyocyte-specific deficiency of STAT3 alters the cardiac microenvironment by the release of STAT3-dependent paracrine factors such as erythropoietin (EPO). The depletion of EPO within the cardiac microenvironment attenuates the endothelial differentiation of an endogenous cardiac progenitor cell population by arresting their chemokine ligand 2 (CCL2)/chemokine ligand receptor 2 (CCR2) system, which as a consequence impairs the vasculogenic regeneration of the heart [79]. Interestingly, the endothelial differentiation seems to be VEGF independent [79]. These aspects suggest a potential positive role of STAT3 signaling for the fate of cardiac stem or progenitor cells contributing to the homeostasis of the heart, especially after cardiac injury, and harbor an endogenous source for the generation of new capillaries and vessels. The formation of new vessels could contribute to the preservation of the cardiac function.

Finally, STAT3 seems to play a key role in the regulation of cardiac angiogenesis during pregnancy-induced adaptive processes such as physiological hypertrophy of the maternal heart. In fact, cardiac-restricted deletion of STAT3 leads to peripartum cardiomyopathy (PPCM) with a massive loss of cardiac microvessels as the major phenotype [9]. This pathologic decrease in blood vessels is mainly caused by unbalanced oxidative stress in cardiomyocytes that promotes the release of activated cathepsin D, which subsequently cleaves the nursing hormone prolactin in the angiostatic factor 16 kDa prolactin. It is important to note that STAT3 expression and activation is downregulated in cardiac tissue of patients with PPCM suggesting that STAT3 activation is also crucial for cardioprotection from pregnancy-related stress in humans.

Conclusively, these data suggest that STAT3 plays an important role in protecting the vasculature as well as inducing neoangiogenesis in the heart under physiological and pathophysiological conditions by controlling paracrine circuits.

The proangiogenic features of STAT3 seem to involve paracrine mechanisms in cardiomyocytes and non-myocytes including the expression and regulation of VEGF, which indicate that activation of STAT3 controls vessel growth in vivo. In addition, STAT3 seems important to suppress the expression of antiangiogenic factors such as CTGF, TSP-1, and TIMP-1 and the generation of angiostatic peptides such as 16 kDa prolactin. Especially in cardiac adaptation processes in response to physiological stress (pregnancy) or in response to injury, STAT3 is essential for the formation and preservation of blood vessels and ultimately for the maintenance of the cardiac function. However, too much proangiogenic activity induced by a continuous activation of STAT3, for example, in the infarcted heart may be detrimental since enhanced vessel formation in the border zone of the infarcted LV may weaken the tissue and promote inflammation, ventricular rupture, and scar dilatation. Therefore, a precise regulation of STAT3 expression and activity is also necessary for beneficial cardiac angiogenesis.

20.5 STAT3 and Heart Metabolism

It is inevitable for the cardiac muscle to consistently produce high amounts of ATP to meet the demand of energy required to maintain cardiac pump function. There is ample evidence that derangements in myocardial fuel selection and energetics occur in heart failure; however, causes and consequences of these defects remain poorly understood [80]. With its important role as a mediator of cardiac vessel growth, STAT3 is important to provide the pipes to transport energy and oxygen to the cardiac muscle. Apart from its role in angiogenesis, distinct forms of STAT3 actions have been implied in the direct modulation of substrate metabolism and energy generation. However, so far data are derived from liver and from tumor biology, while effects on the role of STAT3 on cardiac energy generation are less elaborated.

STAT3 has been shown to act as a master metabolic regulator in STAT3-dependent cancer cell lines where it enhances aerobic glycolysis and downregulates

mitochondrial activity [81]. For instance, in human breast cancer cells, IL-6 enhances glycolysis by STAT3-mediated upregulation of the glycolytic enzymes hexokinase 2 (HK2) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), a result that could be confirmed in mouse embryonic fibroblast and links inflammation to alterations in cancer metabolism [82, 83]. In liver, a link between STAT3 signaling and modulation of glycolysis has been suggested since hepatocyte-specific STAT3 deletion results in hepatic insulin resistance [84]. In the heart of a transgenic mouse model with a cardiac-specific overexpression of peroxisome proliferator-activated receptor (PPAR)-α, insulin-induced STAT3 activation measured as phosphorylation at Y705 is diminished and therefore associated with an elevated oxidation of fatty acids and downregulation of genes involved in glycolysis [85].

With regard to substrate use, there is a major difference between cancer cells which strongly rely on glucose oxidation as their preferred way of energy generation and striated muscle cells which mostly favor fatty acids as the substrate for ATP production. In this context, STAT3 was attributed to mediate leptin-induced lipid oxidation in skeletal muscle, while the effect of STAT3 on cardiac fatty acid oxidation has yet to be investigated [86].

However, it is known that with the development of heart failure, the cardiac muscle switches from fatty acid utilization to glucose oxidation as the preferred substrate for energy generation [87]. Whether this switch is protective as it adapts the heart to different energy demands or contributes to the development of heart failure is a matter of debate and not really understood. A contribution of STAT3 in the regulation of genes involved in cardiac substrate utilization has not been evaluated yet. However, a direct function of STAT3 in mitochondrial ATP production in the heart and in cardiomyocytes has recently been demonstrated [12]. In fact, evidence has been presented that STAT3 is present in mitochondria of B cells and cardiomyocytes, where it binds to complex I and II of the electron transport chain (ETC) [12, 88]. This direct protein–protein interaction of STAT3 with ETC components requires phosphorylation at S727 but seems to be independent from Y705 phosphorylation or DNA binding. Deletion of STAT3 in this model leads to a drastic reduction in complex I and II activities, a lower membrane potential and ATP production, and an increase in the production of reactive oxygen species (ROS) [12]. The mitochondrial localization of STAT3 could recently be confirmed, and additionally a Tom20-mediated import of the protein was described [88]. Genetic deletion as well as pharmacologic inhibition of STAT3 phosphorylation at both sites, Y705 and S727, leads to a decreased ADP-stimulated respiration in the presence of complex I substrates [88]. No effect of STAT3 on complex II, complex IV, or uncoupled respiration was detected in cardiomyocyte mitochondria [88].

Also, an interaction between the mitochondrial protein cyclophilin D that is part of the mitochondrial permeability transition pore complex and STAT3 is reported, and STAT3 deletion or inhibition is found to increase the susceptibility of pore opening [88]. Interestingly, mice overexpressing a transgenic STAT3 protein with a mitochondrial target sequence but a mutated DNA-binding site in cardiomyocytes also display reduced complex I and II activities. However, this reduction is, unlike in the STAT3-deficient situation, not associated with augmented ROS, or decreased

ATP production and cardiac function and left ventricular dimensions are comparable to wild-type mice at 1 year of age [89]. Mitochondria from these transgenic mice are protected against ischemia-induced ROS production via complex I and show diminished cytochrome c release in response to ischemic insult [89]. It therefore seems likely that mitochondrial-targeted STAT3 on top of the endogenous protein protects against ischemic damage by partially inhibiting electron flow through complex I [89].

In the aforedescribed studies, the effect of mitochondrial-located STAT3 on cellular respiration at the level of the ETC is attributed to direct interactions of STAT3 and ETC proteins. However, to generate sufficient amounts of ATP required for maximal myocardial performance, nearly equimolar amounts of STAT3 and complex I and II proteins would be necessary. Using different proteomic approaches, it was shown that the ratio of STAT3 to complex I/II is ~10^{-5} [90]. From these experiments it can be concluded that direct regulation of cellular respiration by protein–protein interaction of STAT3 and respiratory chain proteins is not feasible and that the observed modulations in ATP production by STAT3 might rather be due to regulation of the expression of mitochondrial genes [90].

Taken together, STAT3 plays an important role in the heart and in cardiac cells for upstream processes like transport of nutrition and oxygen, while little is known about the role of STAT3 in cardiac fuel selection and substrate oxidation. However, strong evidence exists that STAT3 is essential to mediate ATP production in mitochondria via the ETC. However, the exact mechanisms by which STAT3 influences ATP generation especially at the level of complex I and II are controversially discussed. Additional studies are therefore necessary to decipher the contribution of disrupted metabolism in adverse structural remodeling of the myocardium in general and to determine the role of STAT3 in this context.

20.6 STAT3 in Hypertrophy and Sarcomere Composition

The role of the JAK-STAT pathway for the development of cardiac hypertrophy and a putative therapeutic utility still remains a matter of debate. While early studies demonstrated that under massive STAT3 overexpression ($\alpha MHC\text{-}STAT3^{tg}$), mice develop spontaneous concentric cardiac hypertrophy [67], the role of physiological expression and activation of STAT3 for cardiac hypertrophy was until recently unclear [10]. Some studies have favored a decisive contribution of STAT3 to the development of cardiac hypertrophy, particularly in response to certain pathophysiological stimuli, such as MI and ischemia/reperfusion [7], pressure overload, and hormones [91]. In this regard, upregulation of STAT3 signaling in response to MI may contribute to an adaptive component of remodeling processes in an effort to maintain function and forward flow in the acute phase. However, prolonged and unbalanced activation of JAK-STAT3 signaling appears detrimental after MI due to enhanced inflammation and left ventricular instability with increased rupture rate as mentioned above in the $\alpha MHC\text{-}Cre^{tg/-}$; $gp130^{fl/Y757F}$ mice [10].

Further evidence for the involvement of STAT3 signaling in cardiomyocyte hypertrophy is provided by in vitro experiments using the activator of gp130 receptor signaling, LIF, which induces cell growth in cardiomyocytes, a feature that is prevented by concomitant overexpression of SOCS3 [38]. However, LIF mainly induces a distinct type of cell growth that is marked by thinning of the width and elongation of the length, a phenotype that is considered as an eccentric cell growth, rather than a concentric hypertrophy. A link between STAT3 and the ubiquitin–proteasome system (UPS) was identified in a microRNA-dependent manner. In this circuit, STAT3 negatively regulates the expression of miR-199a on the transcriptional level, which in turn fine-tunes the expression of at least two ubiquitin-conjugating enzymes, the Ube2i and Ube2g, on the posttranscriptional level [5]. Under circumstances of low STAT3 expression, for example, as it occurs in the end-stage failing heart [92], the expression of miR-199a is upregulated with a subsequent impairment of the UPS. Consequently, this leads to disturbed homeostasis of certain sarcomeric proteins, such as α- and β-MHC proteins, a severe derangement of the sarcomere ultrastructure, and an eccentric type of cardiomyocyte growth—a phenotype that corresponds to the architecture of the cardiomyocyte syncytium in dilated cardiomyopathy. Moreover, the derailed UPS activity and protein turnover also result in alteration of the cardiomyocyte secretome with accumulation of the endogenous endothelial nitric oxide synthase (eNOS) inhibitor asymmetric dimethylarginine (ADMA), which in a paracrine fashion impairs endothelial function of adjacent vessels [5].

Conclusively, STAT3 regulates sarcomere composition, cardiomyocyte growth, and hypertrophy and thus determines the transition from adaptive to maladaptive remodeling depending on the extent, time point, and duration of signaling activity. While under certain conditions a transiently enhanced STAT3 activity may promote adaptive concentric hypertrophy, prolonged STAT3 activation or deficiency result in ventricular instability or eccentric ventricular dilatation, respectively. Notably the STAT3-related processes with regard to regulation of sarcomeric gene composition via microRNAs and the UPS are not restricted exclusively to the cardiomyocyte itself but may also impact via release of metabolites such as ADMA in a paracrine manner on the non-myocyte compartment especially the myocardial vasculature.

20.7 Conclusion

In conclusion, STAT3 is involved in multiple biological mechanisms in the developing, the adult, and the injured heart. A timely regulated STAT3 activation appears cardioprotective by positively modulating anti-oxidative defense, angiogenesis, eventually metabolism and energy production, hypertrophy, and survival, as reported in previous studies [7, 8, 11, 12, 67, 93]. However, upregulation and sustained activation of STAT3 promotes ventricular inflammation and rupture after ischemic insults resulting in high mortality. These antithetic effects clearly demonstrate that a precise regulation of STAT3 expression and activity is required for beneficial effects of STAT3 in the heart.

This double-faced nature of STAT3 may offer an explanation for discrepancies existing between experimental findings that demonstrated mainly beneficial effects of gp130-STAT3 signaling for the cardiovascular system and clinical studies which found that high serum levels of gp130 ligands, that is, IL-6, predict a poor outcome in patients after MI or with heart failure [94–96]. Based on the data summarized in this report, it could be speculated that high IL-6 serum levels in patients indicate a high and continuous activation state of STAT3 which has been shown to be detrimental especially after MI. Therefore, it should be explored whether there is a link between high IL-6 serum levels, activation state of cardiac STAT3, and poor prognosis in patients after MI. If this is the case, targeting STAT3 by small molecule inhibitors as currently tested in cancer therapy [97] might be a novel therapy option in these patients. In turn, in other scenarios such as heart failure due to myocarditis, peripartum cardiomyopathy, or heart failure due to cardiotoxic treatment strategies, therapies that enhance STAT3 activation may be beneficial and promote healing and regeneration. Taken together, in most cardiac physiological and pathophysiological situations, balanced STAT3 expression and activation is essential for cardioprotection and behaves as a friend, but an unbalanced STAT3 activation can turn a friend into a foe. Therefore, therapeutic success of targeting STAT3 in positive or negative ways will require the possibility to precisely control the activation status of STAT3.

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Chapter 21
The Role of Growth Differentiation Factor 5 in Cardiac Repair Post-Myocardial Infarction

Eric A. Shikatani and Mansoor Husain

Abstract Progressive post-myocardial infarction (MI) remodelling of the heart is mediated by processes that include apoptosis, proliferation, fibrosis and hypertrophy. The regulation of pathways leading to these events can be mediated by diverse factors present in specific spatiotemporal expression patterns post-MI. The growth differentiation factors (GDFs) which are closely related to the bone morphogenetic protein (BMP) family of growth factors regulate diverse processes in many tissues including the heart. The GDF/BMPs regulate a cascade of intracellular signalling molecules to affect gene regulation in target cells. GDF/BMP signal transduction is mediated by specific receptor heteromer activation, extracellular inhibition and intracellular regulation of downstream signalling. Despite roles in cardiac development, the role of specific GDF/BMPs in post-MI processes is not well known. While GDF-5 does not appear to be involved in cardiac development, it is involved in a variety of processes mediating both acute and chronic remodelling post-MI. As a function of specific heteromeric receptor activation and resultant downstream signalling cascades, GDF-5 impacts specific pathways differentially in cardiomyocytes, cardiac fibroblasts and endothelial and vascular smooth muscle cells. GDF-5 spares cardiomyocyte apoptosis via a SMAD1/5/8-SMAD4-Bcl/Bcl-xL-mediated process, increases vascularity post-MI and decreases p38-mediated collagen production post-MI. Thus, a single growth factor is able to exert a multitude of cardioactive effects post-MI, which together represent a potential therapeutic target.

Keywords Growth differentiation factor • Bone morphogenetic protein • Mitogen-activated protein kinase • Transgenic mouse model of human disease • Fibrosis

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21.1 Introduction

While great progress has been made in the early recognition and treatment of myocardial infarction (MI), this disease remains a leading cause of morbidity and mortality [1, 2]. The myocyte necrosis that occurs as a consequence of restricted coronary perfusion is followed by acute inflammatory and chronic fibrotic responses resulting in progressive remodelling of the heart [3–10]. Maturation of the scar, with or without infarct expansion, and hypertrophy of adjacent cardiomyocytes (CM) contribute to post-infarct remodelling, creating potential for cardiac dysfunction and heart failure due to impaired contractility and conductivity of the scar tissue. During this chronic remodelling phase, lack of nutrient and oxygen supply causes apoptosis of the hypertrophied myocytes [11, 12] which results in gradual expansion of the infarct zone, dilatation of the left ventricle and subsequent heart failure. The development of heart failure is the most important determinant of subsequent survival and quality of life [1, 2].

Bone morphogenetic proteins (BMPs) and BMP-like growth differentiation factors (GDFs) are secreted proteins which function in an autocrine/paracrine manner. GDFs have overlapping members with the BMPs, together which form a subfamily within the transforming growth factor-β (TGF-β) superfamily of cytokines. Their interactions with specific receptors on target cells initiate signal transduction resulting in the regulation of gene expression. Expression of several BMPs has been documented in the heart, some of which are essential for cardiac morphogenesis [13–20]. Some BMPs have also been shown to promote stem cell differentiation into CMs in the peri-infarct area [21, 22]. However, little is known about the effect of BMP deficiency on chronic remodelling responses post-MI. Differences in the affinity of BMPs and GDFs for their receptors, specific spatiotemporal expression patterns and the presence of extra- and intracellular inhibitors and downstream signalling molecules in target cells together result in diverse and overlapping functions in different cells and tissues (Table 21.1).

Growth differentiation factor 5 (GDF-5), also known as BMP-14 or cartilage-derived morphogenetic protein 1, is a member of the GDF/BMP family that has mainly been studied in skeletal development and chondrogenesis. While GDF-5 expression has been reported in the developing heart, its function during development as well as its expression and role in adult heart post-MI was unknown. Although GDF-5-knockout (KO) mice survive to adulthood, mutations in other BMP ligands, their receptors and downstream signalling proteins often confer embryonic lethality. While this may be taken to suggest that loss of GDF-5 does not impact cardiovascular development, it in no way addresses the role of GDF-5 in adult cardiovascular diseases. Our work to date shows that GDF-5 is expressed in the adult mouse heart and cardiac vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) and that its expression is upregulated in a specific spatiotemporal pattern post-MI. Moreover, the deficiency of GDF-5 in KO mice results in impaired cardiac remodelling due to decreases in activity of downstream signalling cascades resulting in defects in cell survival, proliferation and matrix deposition [23].

Table 21.1 Role of GDF/BMP signalling molecules in cardiovascular development and disease

	Species	Cardiovascular role	References
Ligands			
BMP-2	m	KO has multiple developmental and cardiac defects	[20]
	r	Inhibits apoptosis in neonatal cardiomyocytes	[73]
		Regulates cardiomyocyte contractility	[48, 103]
BMP-4	m	Multiple developmental and cardiac defects	[15, 16, 93, 124]
	x, z	Essential in left–right asymmetry of heart	[13, 14, 18]
BMP-2/4	h	Expression found in adult calcified heart valves	[125]
		Elevated expression in atherosclerotic plaques	[126]
BMP-5	c	Involved in endocardial cushion formation	[37]
BMP-5/7	m	Cardiac defects in double mutants	[127]
BMP-6/7	m	Cardiac defects in double mutants	[38]
GDF-1/2 (BMP-9)	m	KO has left–right patterning defects, including cardiac	[27, 28]
	h	Congenital cardiac malformations	[29, 30]
BMP-10	m	Maintains cardiomyocyte proliferation in development	[39]
GDF-5	m	Reduced vascularisation and VSMC migration	[58, 63, 64]
		KO has defect in post-MI cardiac repair	[23]
GDF-8	m, b, s	Expressed in heart; upregulated after MI	[35]
	m, r	Inhibits cardiomyocyte hypertrophy	[107–109]
GDF-15	r	Increased expression post-MI	[105]
	m	Inhibits influx of inflammatory cells post-MI	[110, 111]
	h	Upregulated in cardiac pathologies	[104, 128]
Receptors			
BMPR1A	m	KO has multiple developmental and cardiac defects	[51, 92]
BMPR2	m	KO lethal due to gastrulation defects	[49]
		Outflow tract defects with hypomorphic BMPR2	[50]
ACVR2/2B	r	Increased expression post-MI	[105]
SMADs			
SMAD-3	m	KO has attenuated TGF-β-mediated fibrosis post-MI	[101, 102]
SMAD-5	m	KO lethal from defective angiogenesis	[46]
		EC- and VSMC-specific KO cause mild cardiac defects	[47]
SMAD-6	m	KO has vascular dilatation and outflow tract septation	[129]
SMAD-7	c	Misexpression causes vascular dilatation and malformations	[130]
Inhibitors			
NOG	m, c	Thicker myocardium and endocardial cushion in mutants	[40, 41]

Of the 20 identified GDF/BMPs, several of them have roles in cardiovascular development or disease. While the developmental role of many GDF/BMPs, their receptors, downstream signalling SMADs and extracellular inhibitors has been characterised, their roles post-MI remain largely unexamined in experimental systems. Species—*h* human, *m* mouse, *r* rat, *s* sheep, *b* bovine, *c* chicken, *x* xenopus, *z* zebra fish

21.2 GDF/BMPs in General and Those Expressed in the Heart

The GDF and BMP family of cytokines have various functions in embryonic development and tissue homeostasis through their roles in the regulation of cell differentiation, proliferation and survival. Like other TGF-β superfamily cytokines, GDF/BMPs are synthesised as a large precursor peptide which dimerises then has the mature cytokine cleaved from the N-terminal pro-domain [24]. Each monomer has the characteristic cystine-knot structure of TGF-β cytokines. With the exception of GDF-8 and 9, GDF/BMPs are secreted without the pro-domain, resulting in small protein dimers that are then able to exert autocrine and paracrine effects. Despite being small peptides fully capable of diffusing freely, because they are secreted as membrane-bound inactive pro-peptides, coupled with the specific localisation of peptidases that cleave their pro-domains, the actions of GDF/BMPs tend to be localised to specific tissue compartments [25].

GDF/BMPs mediate signal transduction through specific receptors that regulate target gene expression during development and adult life (Fig. 21.1). BMP signalling plays critical roles in various tissues including the cardiovascular and skeletal systems. GDF/BMPs are expressed with spatiotemporal specificity and as such only a subset of GDF/BMPs is known to be expressed in either of the developing, mature or pathological heart. GDF/BMPs are expressed in the developing heart in a distinct but partially overlapping manner, suggesting they play specific roles in cardiac morphogenesis [17, 19]. BMP-2 or BMP-4 deletion resulted in multiple developmental abnormalities including cardiac anomalies [13–16, 18, 20, 26]. Mutations in GDF-1 and 2 (also known as BMP-9) cause several cardiac defects involving left–right patterning [27, 28], which have also been characterised in human congenital cardiac malformations [29, 30]. Although their roles in cardiac development are not well characterised, under normal physiological conditions, GDF-5, GDF-8 (also known as Myostatin) and GDF-15 (also known as macrophage inhibitory protein 1) are all expressed at low levels in the developing and adult heart [31–36]. BMP-5 [37], 6 and 7 [38] have been shown to be expressed during endocardial cushion development. BMP-10 is specifically expressed in the developing heart and has a critical role in the maintenance of embryonic cardiomyocyte proliferation [39]. Hence, while several GDF/BMPs have been shown to be expressed during cardiac development, their roles in post-myocardial infarct remodelling remain largely unexplored.

In addition to the study of GDF/BMPs themselves, several studies have examined the role of inhibitors and downstream mediators of GDF/BMP function in the context of cardiac development. Misexpression of noggin, an inhibitor of BMPs, led to septal defects in the heart [40]. Additionally, noggin-KO embryos exhibited thicker myocardium and larger endocardial cushion compared to wild type [41]. Small mothers against decapentaplegic homolog 4 (SMAD-4) a downstream mediator of GDF-5 and BMP signalling in general has been implicated in endocardial cushion, outflow tract and cardiac valvular development [42–44]. Deletion of SMAD-5 caused embryonic lethality due to disorganised vasculature and decreased

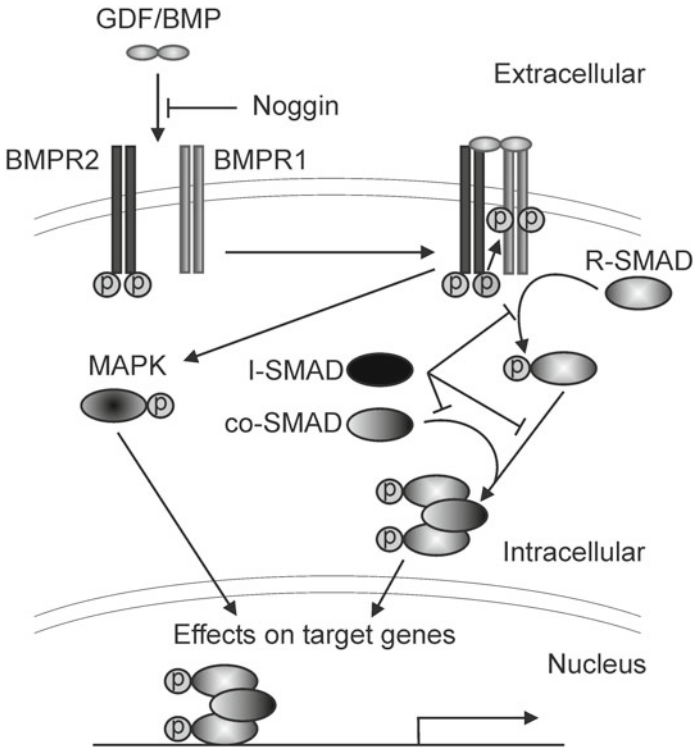

Fig. 21.1 *GDF/BMP signalling and regulation*. Homo/heterodimeric GDF/BMP binds to heteromeric complexes of type 1 BMPR (BMPR-1A and BMPR-1B) and type 2 BMPR (BMPR-2, ACVR-2A, ACVR-2B) to activate downstream signalling. Upon ligand binding, BMPR-2s transphosphorylate BMPR-1s, which then phosphorylate a regulatory SMAD (R-SMAD). The phosphorylated R-SMAD then complexes with a common-mediator SMAD (co-SMAD), and the whole complex translocates to the nucleus to target SMAD-responsive genes. BMPR activation by ligand binding can also result in the activation of MAPK pathways. Signalling by GDF/BMPs can be inhibited extracellularly by inhibitors such as noggin. SMAD signalling can be inhibited by inhibitory SMADs (I-SMADs), which can interfere with multiple signalling processes as well as enhance the degradation of signalling molecules

number of VSMCs [45, 46]. SMC- or EC-specific deletion of SMAD-5 did not affect angiogenesis during development [47]. Mice lacking SMAD-5 in both VSMC and CM exhibited increased left ventricular internal diameter and decreased fractional shortening, which manifested in adulthood. BMP-2, together with myocyte-specific enhancer factor 2A, was found to regulate cardiac ventricular contractility [48]. BMPR-1A-KO and BMPR-2-KO have been shown to cause multiple developmental defects, including cardiac malformations [8, 49–51].

These studies suggest that tight spatiotemporally controlled expression of BMPs, their receptors and their inhibitors is critical to cardiac development and possibly also normal cardiac function. In the embryonic mouse heart, GDF-5 is transiently expressed only at E13.5 [32]. While cardiac development has never

been examined in GDF-5-KO mice, these animals survive without overt cardiac malformations [33]. Indeed, as one of the few BMP mutants without overt cardiac malformation, the GDF-KO may be a good model for studying dysregulated BMP signalling.

GDF-5 is expressed in a variety of tissues including the heart [32, 33, 52–57]. In 13.5-day post-coitum mouse embryos, GDF-5 expression was seen throughout the ventricles and atria [32]. We have found that GDF-5 is expressed in adult heart, CMs and arterial SMC and ECs [23]. Since identification of GDF-5 mutations in humans with skeletal disorders [58–62], GDF-5 has been mainly studied in the context of chondrogenesis and osteogenesis. Vascular effects of GDF-5 have been described in some previous studies. These include findings of reduced vascularisation post-tendon injury in GDF-5-KO mice [58], angiogenesis [63], SMC migration [64], induction of vascular endothelial growth factor expression [65–67] and activation of SMAD-1/5 in human microvascular and pulmonary artery ECs [68]. Recently, GDF-5 was shown to prevent apoptosis in mouse embryonic fibroblasts but not in vascular SMCs via stabilising XIAP [69]. Our data also suggest a role for GDF-5 in proliferation and migration of SMC and ECs (unpublished data), cardiac fibroblasts, and in CM survival [23]. Together, these studies point to several potential effects of GDF-5 on cardiovascular tissues (Fig. 21.2).

21.3 Signalling Pathways Downstream of GDF/BMPs and Their Role in Remodelling Post-MI

GDF/BMPs regulate gene expression through a complex signalling cascade mediated by the existence of various inhibitors in the extracellular microenvironment, the combination of type 1 and 2 receptor heteromers activated, intracellular regulation of downstream signalling molecules and the recruitment of transcriptional coactivators or repressor to target gene promoters. GDF/BMP receptor heteromers are made of type 1 BMPR-1A and BMPR-1B and type 2 ACVR-2A, ACVR-2B and BMPR-2 [70, 71]. These receptors are expressed differentially in various tissues including the heart and blood vessels [56, 72, 73]. Although there is considerable receptor specificity for each GDF/BMP, it remains unknown to what extent activation of different combinations of type 1 and type 2 receptor complexes results in functional signal transduction cascades. Additionally, it is known that GDF/BMPs are able to heterodimerise, increasing the complexity of possible combinations of receptor–ligand interactions and downstream effects [74].

The crystal structures of several ligand–receptor complexes have been solved, showing a close and extensive interaction of ligands to their respective receptor complexes. Type 1 receptors have been found to bind the GDF/BMP dimer at several amino acid residues in the region where the two monomers interact, whereas type 2 receptors bind to a single monomer in a region distal from the interaction site. Receptor–ligand studies have shown that the receptor–ligand geometry is similar

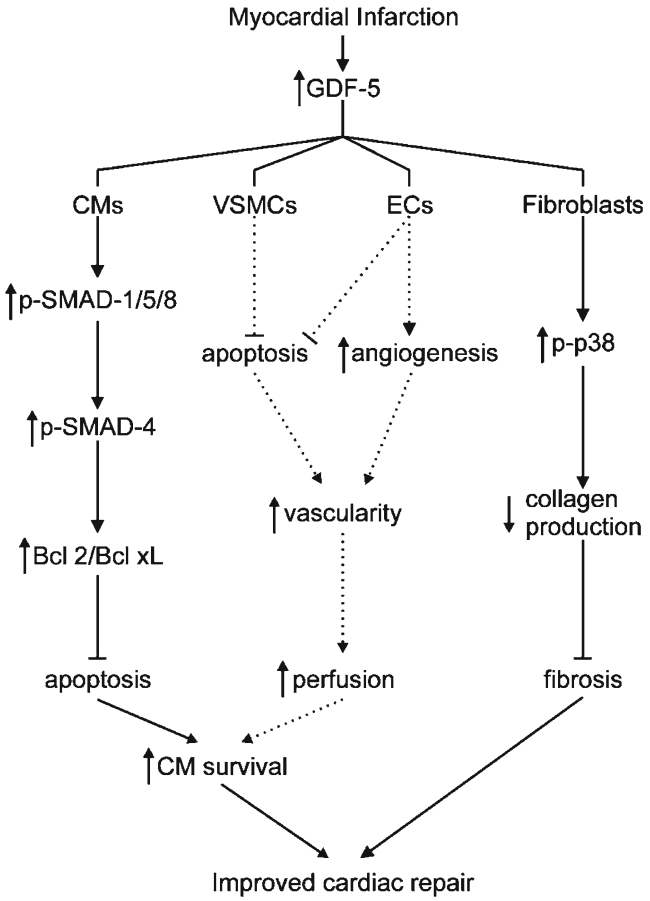

Fig. 21.2 *GDF-5 signalling affects multiple remodelling processes post-MI.* GDF-5 spares cardiomyocyte apoptosis post-MI via a SMAD-1/5/8-SMAD-4-Bcl/Bcl-xL-mediated process. In vascular cells, GDF-5 may inhibit apoptosis as well as promote angiogenesis, increasing vascularity and perfusion of infarcted myocardium post-MI. In fibroblasts, GDF-5 decreases p38-mediated collagen deposition preventing fibrosis of infarcted myocardium. Together, these effects result in improved remodelling post-MI. *CM* cardiomyocytes, *VSMC* vascular smooth muscle cells, *EC* endothelial cells. *Solid arrows* indicate experimentally validated pathways; *dashed arrows* indicate proposed mechanisms

regardless of the receptor–ligand pairing, suggesting that the specificity of receptor binding is mediated predominantly by ligand amino acid sequence [75, 76]. Since there is considerable sequence similarity of GDF/BMP family members [25], it is not surprising to find that single residue mutations result in the loss or gain of specificity of ligand–receptor specificity [75, 76]. Specific amino acids have been identified as being responsible for GDF-5 receptor specificity [77, 78], as well as interactions with specific BMP inhibitors [79]. Post-translational modifications such as glycosylation also confer further receptor specificity, as is the case with

BMP-6 where glycosylation at an asparagine residue is required for recognition by type 1 ACVR but not BMPR-1A and B [80].

Signal transduction by GDF/BMPs after binding and activation of receptor complexes is accomplished by transphosphorylation of the type 1 receptor by the type 2 receptor [81]. Upon phosphorylation, type 1 receptors phosphorylate regulatory SMAD (R-SMAD) proteins, resulting in subsequent nuclear localisation of R-SMADs. There are two antagonistic sets of R-SMADS: SMAD-1/5/8 and SMAD-2/3. Phosphorylation of R-SMADs depends on the type 1 receptor activated, with ACVR-1A, BMPR-1A and 2A phosphorylating SMAD-1/5/8, whereas ACVR-1B, ACVR-1C and ACRV-like kinase 4 phosphorylate SMAD-2/3 [82]. Upon phosphorylation, SMAD-1/5/8 binds to the common-mediator SMAD (co-SMAD) SMAD-4, where the whole complex translocates to the nucleus to interact with other transcription factors and SMAD target genes [83]. The inhibitory SMADs (I-SMADs) SMAD-6 and 7 regulate SMAD signalling by interfering with R-SMAD phosphorylation by type 1 receptors, R and co-SMAD promoter binding, as well as by regulating SMAD degradation. While members of the TGF-β superfamily (TGF-βs, GDF/BMPs and activins) share some receptors, use of different subsets of SMADs by BMPs (SMAD-1/5/8) and TGF-β, activins and GDF-15 (SMAD-2/3) segregates their pathways. Despite this complex network of potentially overlapping ligands, receptors and signal transduction pathways, our data show that specific deficiency of a single family member, namely, GDF-5, significantly hampers cardiac remodelling [23].

Mature GDF-5 preferentially signals through the heterotetrameric complex of BMPR-1A and BMPR-1B with BMPR-2, ACVR-2A and ACVR-2B with equal affinity [70, 84]. Since these receptors are expressed in the heart and type 2 ACVRs are upregulated after ischemia [85], GDF-5 could confer signalling through its preferred receptors and produce various cellular effects depending upon the presence of downstream signalling molecules as well the presence of extracellular inhibitors of GDF-5 signalling. There is also substantial evidence showing BMPs and their receptors transduce signals through SMAD-independent pathways. For example, interactions of several proteins with the cytoplasmic domain of BMPR-2 receptor suggest that this receptor, independent of SMAD, can regulate MAP kinases, PKC and PKA [86]. Indeed, GDF-5 has been shown to induce p38-MAP kinase phosphorylation during chondrogenesis [87, 88]. SMAD-independent gene regulation was also shown in pulmonary artery SMCs harbouring an inactive BMPR-2 mutant [89]. While BMP receptors are shared by several GDF/BMPs, their deletion in mice causes embryonic lethality [49, 51, 90–92], thus limiting their utility for studies of BMP signalling in cardiovascular disease.

Despite these studies on the developmental expression of various GDF/BMP family members, few studies have examined their roles in chronic post-infarction myocardial remodelling. In addition, the role of various extra- and intracellular GDF/BMP and SMAD regulatory proteins has not been examined in the context of cardiac remodelling post-MI. Understanding the role of these growth factors and associated signalling pathways will allow for the development of therapeutics to target the chronic remodelling post-MI, which could significantly improve morbidity and mortality post-MI.

21.4 Transgenic Studies of GDF/BMPs Post-MI

Although several GDF/BMPs and related regulatory proteins have been well studied in cardiac development [13, 14, 19, 40, 93, 94], their regulation in MI and their role in post-MI cardiac repair remain largely unknown. Among members of the TGF-β superfamily, the role of TGF-β in the induction of cardiac fibrosis post-MI has been studied in detail [95–98]. Although BMPR-regulated SMAD-1/5/8 expression has been observed in the infarct zone, their physiological importance has not been explored [99]. Cardiac over-expression of Smad1 in transgenic mice also conferred CM protection following ischemia–reperfusion injury [100]. Since GDF/BMPs and their receptors may regulate gene expression and confer signalling independent of SMADs [89], over-expression of SMAD-1 only partially determines the role of BMP signalling in cardiac injury. SMAD-3 deficiency has been shown to attenuate TGF-β-mediated induction of cardiac fibrosis post-MI, independent of any changes to acute inflammatory responses [101, 102]. Although fibroblast proliferation and migration were not affected, fibrosis was abrogated by inhibited collagen release by SMAD-3-KO myofibroblasts [102]. In neonatal CMs, BMP-2 was shown to suppress apoptosis [73] and regulate CM contractility [48, 103]. Among other members of the TGF-β pathway, GDF-8, activin, ACVR-2A, ACVR-2B and GDF-15 were also increased after MI [33, 35, 85, 104, 105]. It was recently shown that in MI, mesenchymal stem cell differentiation into CM-like cells required TGF-β/BMP-2 pathways [21]. Embryonic stem cells (ESC) were also shown to differentiate into functional CMs in the infarcted myocardium by signalling mediated through TGF-β/BMP-2 pathways [106]. Leukaemia inhibitor factor and BMP-2 synergistically promoted differentiation of ESC into CM and EC in a mouse model of MI [22].

GDF-8 has been shown to be expressed in murine, ovine and bovine hearts, localising to cardiomyocytes and Purkinje fibres [35], and is upregulated following MI in an ovine model of MI. Multiple studies have indicated that GDF-8 inhibits cardiomyocyte hypertrophy [107–109]; however, no studies have examined the utility of therapeutic inhibition of GDF-8 to repress post-MI cardiac hypertrophy.

After MI, GDF-15 is expressed in the myocardium and has a role in inhibiting the influx of inflammatory cells [110, 111]. In GDF-15-KO mice, lack of inhibition of the inflammatory response results in worse outcomes after MI due to increased cardiac rupture mediated by the increased recruitment of leukocytes to the infarcted myocardium [111]. Administration of recombinant GDF-15 reduced leukocyte recruitment in KO mice, but changes in post-MI survival was not reported. In this study, no long-term differences in cardiac remodelling were found in GDF-15-KO infarcted or peri-infarct areas versus wild-type (WT) mice, indicating that the role of GDF-15 is predominantly to mediate the inflammatory response in the acute post-MI phase without influencing the downstream pathways relevant in chronic post-MI remodelling events like cardiac hypertrophy and fibrosis.

21.5 The Role of GDF-5 in Chronic Remodelling Post-MI

While the expression of GDF-5 in adult heart has been previously described, the role of GDF-5 in post-MI responses had not been examined. The GDF-5-KO mouse does not develop any overt cardiac malformations, suggesting that the role of GDF-5 is not during developmental processes in the mouse heart. In order to examine the role of GDF-5 in MI, ligand as well as putative receptor expression was first determined in WT mouse hearts before and after MI. In uninfarcted WT mouse hearts, GDF-5 and the receptors ACVR-2A, ACVR-2B, BMPR and BMPR-1B were all expressed in uninfarcted tissues [23]. Following permanent ligation of the left anterior descending coronary artery causing an experimentally induced MI, GDF-5 mRNA and protein levels were most elevated 14 and 28 days post-MI, with protein levels sustained as much as 40 days post-MI, alluding to a possible involvement in chronic post-MI processes [23].

Histological analysis of infarcted hearts revealed no differences in infarct area at early time points between GDF-5-WT and KO hearts, suggesting that GDF-5 does not have a role in acute post-MI responses. This is in contrast to what is now known for GDF-15, which was shown to have a role exclusively in early, acute post-MI inflammatory processes [111]. GDF-15-KO mice did not have any differences with WT mice in terms of chronic post-MI remodelling processes. During chronic remodelling post-MI, infarcted myocardium is gradually replaced by scar tissue [112, 113]. During this process, proliferation of cardiac fibroblasts and deposition of extracellular matrix occur resulting in loss of contractility and conductivity of the infarcted area [112, 114, 115]. As a result, cardiac fibrosis contributes to the development and progression of heart failure [112, 113, 115, 116]. At 28 days, GDF-5-KO hearts had 42% greater infarct areas, 30% increase in the thinning of infarcted left ventricle (LV) and 156% greater infarct expansion compared to WT [23]. In GDF-5-KO hearts, increased fibrosis and collagen 1a1and 3a1 expression were observed post-MI compared to WT hearts [23]. Furthermore, p38-MAP kinase phosphorylation was further reduced in KO hearts post-MI. In mice, p38-MAP kinase phosphorylation suppressed COL1A1 and COL3A1 expression in the heart [117] and reduced cardiac fibrosis post-MI [118]. These data indicate that endogenous GDF-5 levels regulate collagen expression post-MI, a finding supported by recombinant GDF-5 (rGDF-5)-mediated p38-MAP kinase phosphorylation in cardiac fibroblasts [23]. GDF-5-KO infarcted hearts revealed expected losses in cardiac function by echocardiography consistent with increased scar expansion observed histologically.

Decreased scar thickness in GDF-5-KO mice and associated findings of increased infarct expansion and poor LV function [23] may be due to reduced proliferation of cardiac fibroblasts in GDF-5-KO hearts. As rGDF-5 was shown to stimulate proliferation of connective tissue fibroblasts [119] and mouse cardiac fibroblasts [23], it is necessary to examine the effect of rGDF-5 treatment on collagen gene expression and cardiac fibrosis. Despite a thinner scar in GDF-5-KO mice, no increase in mortality was seen in these mice. No differences in matrix metalloproteinase-2 and 9

levels or activity were observed in infarct tissue [23], suggesting that increased collagen may have prevented cardiac rupture in these KO mice. At this time, it is not known if the structural arrangement of the deposited collagen is more reticular than fibrillar, which may aid in preventing cardiac rupture despite thinner scar tissue.

A prominent phenotype of GDF-5-KO mice post-MI was reduction in the number of SMα-actin-stained (i.e. muscularised) precapillary arterioles in the infarct and peri-infarct areas [23]. This finding is consistent with the reported role of GDF-5 in angiogenesis [63]. In GDF-5-KO mice, revascularisation was also reduced following tendon injury [58]. Among cell types that play a role in vascularisation, GDF-5 has been shown to promote migration of bovine EC [63] and human umbilical vein SMC [64]. These data indicate that the GDF-5 deficiency may have contributed to reduced proliferation and migration of coronary arterial SMC and EC, resulting in decreased vascularisation in the infarct areas post-MI. Deletion of SMAD-5, which is a mediator of GDF-5 and other BMP signalling, resulted in defective angiogenesis and decreased number of vascular SMC [45, 46]. This is in agreement with our finding of reduced muscularised arteries in GDF-5-KO mice post-MI. GDF-5 and other BMPs were shown to induce SMAD-1/5 phosphorylation in human microvascular and pulmonary artery ECs [68]. Another major finding of the GDF-5-KO hearts post-MI was a large reduction in p38-MAP kinase phosphorylation compared to wild type [23]. Over-expression of p38-MAP kinase was shown to enhance angiogenesis in post-MI hearts [118]. Together, these observations suggest that GDF-5 may mediate vascularisation post-MI and that its deficiency results in decreased vascularisation leading to reduced supply of oxygen and nutrients and subsequent loss of CMs in the peri-infarct area. However, exhaustive characterisation of capillary density post-MI in infarct and peri-infarct areas was not determined, thus specific conclusions about the role of GDF-5 in post-MI angiogenesis may be somewhat limited.

Our finding of increased loss of CMs in GDF-5-KO mice post-MI, and our in vitro findings of rGDF-5-enhanced CM survival, via induction of the antiapoptotic Bcl2 and Bcl-xL genes mediated by SMAD4 suggests that GDF-5 also plays a role in CM survival in vivo [23]. While treatment of in vitro cultures of CMs with rGDF-5 spared them from apoptosis, the effects of rGDF-5 on apoptosis of cardiac fibroblasts, ECs and VSMCs were not examined, so it is unclear if GDF-5 also has a role in maintaining the survival of other cardiac cell types. In addition to the anti-apoptotic effect of GDF-5 on CMs, the combined loss of vascularity and resultant decrease in perfusion post-MI in GDF-5-KO mice may have increased the loss of peri-infarct tissue resulting in the expansion of the scar in GDF-5-KO mice. Examination of peri-infarct areas in post-MI hearts by cleaved caspase-3 staining showed a fivefold increase in apoptotic cells in GDF-5-KO 4 days post-MI versus WT [23], suggesting that the effects of GDF-5 also include acute post-MI sparing of cardiomyocyte apoptosis.

In contrast to GDF-15 which acts entirely as an acute post-MI anti-inflammatory stimuli [111], the aforementioned results indicate that GDF-5 acts both acutely and chronically post-MI as a pro-proliferative, antiapoptotic and antifibrotic stimuli to reduce adverse remodelling and improving healing post-MI.

21.6 Future Directions

With insights into the role of GDF-5 post-MI being involved in myocardial remodelling, future studies will be able to examine the therapeutic efficacy of post-MI treatment with exogenous GDF-5. Further studies should determine if other GDF/BMPs, BMP receptors, and intra- or extracellular inhibitors and downstream signalling molecules are also involved in chronic post-MI remodelling processes. While expression of GDF-5 does not seem to overlap with GDF-15, the spatiotemporal expression pattern and function of GDF-8 post-MI is not well known, nor is it established how the spatiotemporal specificity of the expression of these GDF and other GDF/BMP ligands impacts post-MI cardiac repair. Much attention has focused on treating acute post-MI symptoms with great success, significantly improving survivability of acute MI. Considering that chronic post-MI pathology still causes significant mortality and morbidity, therapeutics that target the remodelling resulting from fibrosis and apoptosis of surviving surrounding tissue needs to be further developed. Indeed, the use of mineralocorticoid receptor antagonists to reduce cardiac fibrosis post-MI [120, 121] has been validated as an effective means of preventing heart failure progression in clinical trials [122]. With the knowledge that GDF-5 deficiency causes defects in scar expansion and cell apoptosis, exogenous GDF-5 treatment may be a therapeutic strategy to abrogate cardiac fibrosis post-MI.

Interestingly, there was a differential effect of rGDF-5 treatment on p38 phosphorylation between CMs and fibroblasts [23], indicating that signalling cascades that are activated by GDF-5 in post-MI remodelling are cell-type specific even within the same tissues. These differences can be explored using tissue and cell-type-specific transgenic knockout systems to investigate cell-specific losses in GDF-5 to further clarify the roles of GDF-5 in post-MI repair and healing processes. Whether the specificity is derived from differential receptor complex activation or downstream signalling mediators remains to be determined. Since GDF-5 is able to bind to the heterotetrameric complex of BMPR-1A and BMPR-1B with BMPR-2, ACVR-2A and ACVR-2B with equal affinity [70, 84], activation of different receptor heteromers may be able to overcome specific defects in BMPR signalling. For example, in pulmonary hypertension mediated by haploinsufficiency of BMPR2 [123], GDF-5 treatment may be able to efficiently activate defective downstream signalling through alternate receptor complex activation. Understanding the effects of GDF-5 on various cell types of the cardiovascular system may also extend the utility of GDF-5 treatment in other vascular and cardiac pathologies.

It becomes increasingly important that pathways and genes that are involved in the late remodelling process are identified and characterised. With explicit knowledge of these factors, novel therapeutics could be developed to target the remodelling events that occur following myocardial infarction with the aim of decreasing morbidity and increasing quality of life for those that are affected by MI. Presumably, factors that are involved in the early growth and differentiation of cardiac myocytes and other cardiac tissues can be targets for intervention in the late remodelling process following an MI. We have demonstrated that the lack of GDF-5 causes adverse perturbations

in chronic post-MI remodelling. Demonstrating a therapeutic application for GDF-5 would open additional avenues in treating post-MI pathology to improve morbidity and mortality due to chronic remodelling and resultant heart failure.

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Chapter 22
Extracellular Matrix Biomarkers of Adverse Remodeling After Myocardial Infarction

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Abstract Approximately every minute, someone will die from a myocardial infarction (MI). A MI is the result of an obstruction of blood supply causing the heart to undergo complex structural and functional changes in the left ventricular wall, known as ventricular remodeling. Cardiomyocytes undergo necrosis rapidly after the onset of injury, leading to early accumulation of neutrophils, activation of metalloproteinases, and degradation of the stromal tissue, eventually leading to formation of a collagen scar. Circulating factors related to inflammatory and fibrotic responses are key predictors of extracellular matrix (ECM) changes that occur after MI. Changes in the collagen network of the ECM can alter myocardial stiffness, consequently leading to cardiac hypertrophy, fibrosis, and LV dysfunction. Proteins and peptides of the ECM are promising biomarkers for MI. This book chapter provides an overview of key ECM biomarkers involved in adverse remodeling post-MI and their practical applications.

Keywords Cardiac remodeling • Myocardial infarction • Extracellular matrix • Biomarkers

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22.1 Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the United States accounting for approximately 40% of all deaths. CVD includes a wide range of diseases that differ considerably in time scale and relative effects of genes and environment [1]. Of the different types of CVD, myocardial infarction (MI) accounts for the majority of morbidity and mortality. According to the latest American Heart Association statistics, each year an estimated 785,000 Americans will have a new coronary attack, 470,000 will have a recurrent attack, and an additional 195,000 will have a silent first MI [2]. Approximately every 25 s, an American will have a coronary event, and approximately every minute, someone will die of one [2].

22.1.1 What Is a Myocardial Infarction?

A MI is the result of an obstruction of blood supply to a section of the heart, causing myocyte death. Post-MI, the heart undergoes complex structural and functional changes in the left ventricular wall, known as ventricular remodeling. This is reflected by wall thinning, chamber dilatation, and impaired function of the left ventricle (LV). Cardiomyocytes undergo necrosis rapidly after the onset of injury, leading to early accumulation of neutrophils, activation of metalloproteinases, and degradation of the stromal tissue, eventually leading to formation of a collagen scar [3–5]. Diagnosis of MI traditionally includes analysis of three parameters: clinical history, electrocardiographic abnormalities, and increased serum concentrations of specific cardiac enzymes such as creatine kinase, aspartate aminotransferase, and lactate dehydrogenase [6–8]. The present goal in medicine is not only to treat MI patients effectively but also to identify individuals who are at risk for adverse events. One way to accomplish this goal is to develop novel cardiovascular biomarkers to more accurately aid in screening, diagnosis, and prognosis in the post-MI setting.

22.1.2 What Are Biomarkers?

Biomarkers are biological molecules used to indicate normal or pathological biological processes or to provide an index of pharmacological response to therapy [9, 10]. A biomarker can be a measurement found in a biosample (blood, urine, or tissue), a recording (blood pressure, ECG, or Holter), or an imaging test (echocardiogram or CT scan) [10]. It does not necessarily matter if an identified plasma protein marker has a cardiac origin (which would mean it is a primary indicator of response), as long as it has a cardiac action. For example, markers from the liver increase post-MI as part of a whole-body protective response to ischemia [11]. Because of the wide variety, biomarkers can be classified into three types: type 0,

Table 22.1 Considerations for biomarkers that reflect cardiac ECM remodeling following myocardial infarction

• Changes must reflect changes in cardiac ECM scan
• Changes must parallel the variation in other parameters reflecting cardiac fibrosis and LV function
• Positive gradient of concentrations: from coronary sinus blood toward peripheral vein blood, indicating cardiac origin
• High specificity and sensitivity
• Low cost
• Off the shelf/easy access
• Pain free/noninvasive
• Accurate and reproducible

type 1, and surrogate end point or type 2 biomarker [10, 12]. A type 0 biomarker demonstrates the progression of disease and correlates with known clinical indices, whereas a type 1 biomarker describes the effect a therapeutic intervention has in accordance with its mechanism of action. A surrogate end point or type 2 biomarker is intended to anticipate the clinical benefit of treatment on the basis of epidemiological, therapeutic, pathophysiological, or other scientific evidence [10, 12]. Cardiac biomarkers have to meet several criteria to be of use for both patients and clinicians (Table 22.1). Although circulating biomarkers offer the advantage of relative ease of collection, storage, and reduced cost, they may not prove as sensitive and specific as tissue or imaging markers in the diagnosis and progression of disease. Conversely, imaging technologies can detect and assess cardiovascular diseases with a high degree of sensitivity and specificity but may be limited by availability, technical difficulties, and cost.

22.1.3 Why Do We Need Biomarkers Post-MI?

The discovery and development of biomarkers has shed light on both the pathogenesis as well as the management of MI [13, 14]. In general, biomarkers have been independently linked with various phenotypes associated with high risk of MI [15–17]. For this reason, biomarker discovery has high potential for providing a real benefit for MI patients with applications in at least five areas: screening, diagnosis, prognosis, prediction of disease recurrence, and therapeutic monitoring [9, 10]. The main requirement with regard to the potential of a candidate biomarker is the strength of the correlation between the expression patterns of the biomarker and the clinical outcome [18].

Interest in using proteins and peptides of the extracellular matrix (ECM) as promising biomarkers for MI has increased within the last decade. One obvious reason is ECM biomarkers are able to detect early changes in the structure and function of the LV to provide important prognostic information [18, 19]. Having a normal connective tissue environment is a prerequisite for having normal myocardial

architecture and function. Changes in the collagen network of the ECM can alter myocardial stiffness, consequently leading to cardiac hypertrophy, fibrosis, and LV dysfunction [20, 21].

Circulating factors related to inflammatory and fibrotic responses are key predictors of ECM changes that occur after MI [15, 22]. Changes in circulating levels of proinflammatory cytokines and pro- and antifibrotic proteins have been observed in patients with heart failure [23–25]. The field of biomarker discovery holds great potential for improving clinical measurements of disease. This book chapter provides an overview of key ECM biomarkers involved in adverse remodeling post-MI and their practical applications.

22.2 Inflammatory Response

Inflammation is key in the LV response to MI. MI is associated with an inflammatory reaction which is a requirement for healing and scar formation [26–28]. While the activation of inflammatory mediators following MI is necessary for adequate wound healing, at the same time it can cause harmful effects on the LV to alter its size, shape, and function. Numerous studies have implicated several inflammatory markers, including C-reactive protein, the complement factor C5a, interleukin (IL)-8, and adhesion molecules such as intercellular adhesion molecule (ICAM)-1, in predicting the risk and progression of LV dysfunction post-MI [29–35]. These studies demonstrate the importance of anti-inflammatory strategies in post-MI treatments; however, in order to achieve the most effective treatment without interfering with healing and cardiac repair, an understanding of the molecular events associated with inflammation is required.

22.2.1 Neutrophils

Inflammation occurs in stages. In the absence of reperfusion, neutrophils are the first cells to respond to the site of injury. Neutrophils have four subsets of membrane-bound compartments: secretory vesicles, gelatinase/tertiary granules, specific/secondary granules, and azurophilic/primary granules. These granules contain antimicrobial proteins, proteases, ECM proteins, and soluble mediators of inflammation [36, 37]. The order of release is as follows: secretory vesicles, gelatinase granules, specific granules, and finally azurophilic granules [38, 39]. The strength of the stimulus will affect the release of the particular granules.

22.2.1.1 Secretory Vesicles

Contents of secretory vesicles are secreted more readily than other granules, making these molecules important in understanding the early role neutrophils have on progression of LV dysfunction post-MI. Secretory vesicles are formed by endocytosis

and mostly contain serum proteins such as human serum albumin. The membrane of secretory vesicles is enriched with adhesion molecules, such as Mac-1 (CD11b/CD18), complement receptor 1, and urokinase-type plasminogen activator receptor. When incorporated into the plasma membrane in response to inflammatory mediators, secretory vesicles enable the neutrophil to interact with endothelial cells, ECM, and microorganisms [37]. It is for this reason that secretory vesicles are believed to play a major role in early neutrophil activation [40].

In addition to serum proteins and adhesion molecules, heparin-binding protein (HBP) and proteinase-3 are also shown to reside within secretory vesicles [41, 42]. HBP, also known as azurocidin, exists in both secretory vesicles and azurophilic granules [43]. An association between HBP and an increase in neutrophil influx has been observed, mostly likely caused by cytoskeleton rearrangements of endothelial cells [44]. HBP has also been shown to be elevated in plasma of patients with severe sepsis and increases before circulatory collapse [45].

Similar to HBP, proteinase-3 is expressed in both secretory vesicles and azurophilic granules [42]. Ng *et al.* indicated proteinase-3 as being a potential marker for patient prognosis post-MI as part of a multimarker strategy [46]. Although the exact role of proteinase-3 post-MI has not yet been discovered, there is evidence to suggest it can induce apoptosis through caspase-like activity [47], cleave angiotensinogen to form angiotensin I and II [48], and activate tumor necrosis factor-α [49] in addition to other pro-inflammatory mediators [50, 51]. In addition, proteinase-3 has been shown to degrade ECM components such as fibronectin, type IV collagen, and the core protein of proteoglycans [52] implicating it in LV remodeling post-MI.

22.2.1.2 Gelatinase Granules

In human neutrophils, gelatinase granules account for 25% of the total peroxidase-negative granules [53]. Exocytosis of gelatinase granules liberates matrix degrading enzymes such as matrix metalloproteinases (MMPs), signifying their importance for neutrophil extravasation and diapedesis [54]. Collectively, MMPs degrade major ECM components including collagens, fibronectin, proteoglycans, laminin, and gelatin. Neutrophils are the early source of MMP-9, also known as type IV collagenase or gelatinase B [55]. MMP-9 is secreted as a zymogen and becomes active upon cleavage of the N-terminal domain or treatment with oxygen metabolites [56]. Macrophages and endogenous LV cells including myocytes, endothelial cells, and vascular smooth muscles cells also express MMP-9 [57], making it a key player in understanding the progression and recovery of patients post-MI. For this reason, MMP-9 will be discussed in greater detail later in this chapter.

22.2.1.3 Specific Granules

The second, larger component of peroxidase-negative granules is the specific granules. These granules contain mostly antimicrobial substances and are released into

the phagocytic vacuole or the exterior of the cell upon encounter with inflammatory mediators. Contents of specific granules represent an oxygen-independent antimicrobial system enhancing the oxygen-dependent activities of the NADPH oxidase [36].

Of the substances released from activated neutrophils, the majority of researchers focus on myeloperoxidase (MPO) because of its involvement in the pathogenesis of atherosclerosis. Recently, interest in lactoferrin has increased due to its unique antimicrobial, immunomodulatory, and antineoplastic properties [58–61]. Lactoferrin, an iron-binding protein, has antimicrobial activity against a broad spectrum of Gram-positive and Gram-negative bacteria [62]. *In vitro* stimulation of adherent neutrophils with phorbol myristate acetate releases a significant amount of the total cell content of lactoferrin, compared to a lower percentage of released MPO, indicating lactoferrin is a more dynamic marker of early neutrophil activation [63]. In contrast to MPO, lactoferrin inhibits the upregulation of adhesion molecules on endothelial cells and limits iron-mediated damage at sites of inflammation [59, 64, 65]. Lactoferrin also inhibits proinflammatory cytokine production in monocytes decreasing inflammation by hindering neutrophil recruitment and activation [66].

Another important peptide found in specific granules of neutrophils is neutrophil gelatinase-associated lipocalin (NGAL). NGAL plays an important role in the iron-depleting strategy of innate immunity [67] and also promotes matrix degradation and myocardial remodeling [68]. NGAL is responsible for regulation of MMP-9 activity by preventing its degradation, leading to enhanced proteolytic activity with prolonged effects on collagen degradation [69]. Recent studies have revealed a significant increase of NGAL in plasma as well as in necrotic and healthy tissue of patients diagnosed with acute MI [70, 71]. Enhanced NGAL expression may thus contribute to the increased MMP-9 activity seen post-MI, potentially promoting ECM remodeling [55, 72–74].

22.2.1.4 Azurophilic Granules

Azurophilic granules are packed with acidic hydrolases and antimicrobial proteins such as MPO and serine proteases [75]. Azurophilic granules contain many proteins in their proforms which undergo proteolytic cleavage on arrival at the granule compartment [76, 77]. Exocytosis of azurophilic granules are limited and are believed to contribute mainly in the intracellular degradation of microorganisms in the phagolysosome [78, 79].

MPO is a heme enzyme, released by activated neutrophils as well as monocytes. MPO accumulates in the infarct zone leading to the formation of reactive chlorinating species. Neutrophils have been demonstrated to release MPO into the coronary circulation, yielding elevated MPO plasma levels in patients with unstable angina and acute MI [80, 81]. Moreover, MPO release has been seen to actually precede myocardial injury, even before complete microvascular obstruction. Brennan *et al.* assessed the plasma levels of MPO as a predictor of the risk of cardiovascular events

in 604 sequential patients and noted that a single initial measurement of plasma MPO independently predicts the early risk of MI as well as the risk of major adverse cardiac events in the following 30-day and 6-month periods [82].

Studies in MPO-null mice using a chronic coronary artery ligation model demonstrated a marked reduction in leukocyte infiltration and LV dilatation, associated with delayed myocardial rupture and preservation of systolic function [83]. MPO and oxidants released by activated neutrophils have been found to enhance the degradation of ECM through proteases [81] consequently promoting the development of abnormal ventricular geometry. MPO levels may thus act as a prognostic test for the likelihood of MI, revascularization, and death.

Azurophilic granules also contain three structurally related serine proteases: proteinase-3, cathepsin G, and elastase. Collectively, these serine proteases degrade a variety of ECM components, such as elastin, fibronectin, laminin, type IV collagen, and vitronectin [84]. Serine proteases are also known to stimulate coagulation during systemic infection resulting in arterial thrombosis and possibly contributing to the progression of CVDs such as MI [85]. Of note, serine elastase is a potent activator of MMP-9 and may be the primary activator during the early post-MI response [55].

In addition to azurophilic granules, proteinase-3 is also found in secretory vesicles and may induce apoptosis through caspase-like activity [47]. Elastase is a key player in protease degradation of ECM. Bidouard *et al.* determined inhibition of elastase prior to reperfusion causing reduction in the infarct size [86]. This effect could be due to the ability of elastase to activate MMP-2, MMP-3, and MMP-9 and inactivate TIMP-1 resulting in an increase in ECM degradation [87]. In contrast to proteinase-3 and elastase, cathepsin G plays a protective role post-MI by inactivating bradykinin and IL-6 both of which are well-known inflammatory mediators [88, 89]. Although cathepsin G does have this protective effect post-MI, it also is a potent platelet agonist and degranulator [90]. Thus, the ability to directly activate platelets can lead to intravascular thrombosis, irreversible ischemia, and tissue death.

22.2.2 Macrophages

Following MI, circulating monocytes are recruited to the infarct zone. Upon extravasation into the tissue, the monocytes become macrophages. Macrophages exert crucial roles in wound healing after MI [91, 92]. Macrophages phagocytose tissue and cell debris; secrete cytokines, chemokines, and growth factors; regulate fibroblast function and collagen turnover; as well as modulate the angiogenic response [93]. There are a wide range of heterogeneous macrophage phenotypes with differential gene expression profile and functional characteristics [94]. A broad but perhaps oversimplified classification is the classical M1 and alternative M2 activation groups. A study by Troidl *et al.* suggests that macrophages are differently activated during different phases of MI remodeling process. During the acute inflammatory phase, classical M1 macrophages are primarily activated, while macrophages polarize into alternative M2 subtype during the scar formation period [95].

22.2.2.1 Classical M1 Activation

Post-MI, macrophage polarization toward the classic proinflammatory M1 is induced by Th1 cytokines, including interferon (IFN)-γ and tumor necrosis factor (TNF)-α. Activated M1 macrophages increase the production of several proinflammatory cytokines and chemokines, reactive oxygen species, and nitrogen intermediates. In the early inflammatory phase post-MI, M1 macrophages remove unwanted debris, boost acute inflammatory response, and promote ECM degradation.

Persistent M1 macrophage activation exacerbates post-MI ventricular remodeling and dysfunction. Hu *et al.* demonstrated that, enhanced M1 macrophage polarization was responsible for deteriorated LV malfunction and dilatation and cardiac fibrosis in class A scavenger receptor null mice. Additionally, the authors observed increased production of M1 cytokines including IL-1β, IL-6, and TNF-α [96]. IL-1β secretion in the M1 phase induces MMP-9, which facilitates the secretion of inflammatory cytokines and the degradation of ECM. Leuschner and colleagues reported that post-MI hearts showed rapid monocyte kinetics and the average residence time of monocytes in inflamed tissue is 20 h. After 20 h, most monocytes will undergo local death, and a smaller number will regress to circulation, liver, or other organs [97]. This study indicates that monocytes' transformation to macrophages or macrophage polarization occurs rapidly in response to the surrounding microenvironment. Another important concept revealed in this chapter is that macrophages originating from the spleen migrated to the LV post-MI, making the spleen a major site for macrophage production.

22.2.2.2 Alternative M2 Activation

In contrast to M1 macrophages, polarization toward the alternatively anti-inflammatory M2 during the later phase of inflammation is mediated by Th2 cytokines, including elevated production of IL-4, IL-10, and TGF-β1 post-MI [98, 99]. To date, three distinct M2 subtypes have been recognized: IL-4 and IL-13 induce M2a phenotype, immune complexes in combination with IL-1β or LPS promote M2b subset, and IL-10, TGF-β, or glucocorticoids drive M2c macrophages [100]. M2 macrophages show increased phagocytic activity and anti-inflammatory properties [101, 102]. M2 macrophages facilitate the active resolution of inflammation, ECM deposition, fibroblast proliferation, and angiogenesis [103], all of which are crucial for tissue repair [104]. Macrophage phagocytosis of apoptotic neutrophils, a marker of the active resolution of acute inflammation, triggers TGF-β production, suggesting that phagocytosis may stimulate the conversion to the M2 status [105].

22.2.2.3 Classical Versus Alternative Activation

The timely shift and balance between M1 and M2 macrophages is crucial for stable scar formation and adequate healing response post-MI. Prolonged and excessive activation of the M1 phenotype yields uncontrolled inflammation and ECM breakdown,

Table 22.2 Markers of M1 and M2 macrophage polarization phenotypes

Classical (M1)	Alternative (M2)
Proinflammatory	*Anti-inflammatory*
CCL3 (MIP-1α)	Acyl-malonyl condensing enzyme (AMAC)-1
CCL20	Arginase (Arg) 1, 2
CCR7	CCL2
CD11c	CCL17
CD16/CD32	CCL22
COX2	CCL24
CXCL9	CD36
CXCL10	CD163
CXCL11	CD206 (mannose receptor C type 1, MRC1)
iNOS	CD209a, CD209b, CD209c
IL-1β	Fizz1
IL-6	
IL-12	F13a1
INF-β	IL-10
MCP-1	IL-1R antagonist
Proplatelet basic protein (PPBP) RANTES	Macrophage galactose *N*-acetyl-galactosamine-specific lectin (Mgl)-1, 2
TNF-α	Pdcd1Ig2
	PPARγ
	Selenoprotein-1 (SEPP1)
	SOCS3
	Stabilin-1
	Ym1 (chitinase 3-like 3, Chi313)

which in turn contributes to adverse remodeling and cardiac rupture. Persistent M2 activation stimulates excessive fibroblast proliferation and ECM deposition, which is a hallmark of cardiac fibrosis and influences structural, biochemical, mechanical, and electrical properties of the myocardium [106]. Based on the crucial role of macrophage in wound healing, the M1/M2 ratio may be a potential marker for predicting long-term outcome post-MI. However, the accurate macrophage phenotype and regulation factors post-MI remains to be investigated. With true and reliable markers of macrophage activation now defined (Table 22.2), characterization of macrophage phenotypes post-MI is not only needed but is also possible.

22.2.3 Matrix Metalloproteinase-9

MMP-9 is expressed in cardiac myocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, neutrophils, lymphocytes, and macrophages [55, 72, 107, 108]. MMP-9 was first described as being able to process only collagen that was first

denatured or already cleaved by collagenases such as MMP-1. Recent literature, however, has shown that MMP-9 can process full-length interstitial collagens in addition to a wide array of other substrates [109]. Furthermore, numerous predicted substrates have been identified for MMP-9 [110]. In addition, MMP-9 does not require an activation cleavage step to proteolyze substrates. Bannikov and colleagues demonstrated that pro-MMP-9, in the presence of substrate, has enzymatic activity without the loss of the 10 kDa pro-domain [111]. While this feature is likely common in other MMPs, it has only been demonstrated for MMP-9 indicating that an active MMP-9 band on a gelatin zymogram may not be as useful as we have previously considered.

Recent developments in mass spectrometry-based proteomic approaches and novel emerging technologies hold particular promise for unbiased discovery and subsequent validation of new biomarkers of MI [112]. Didangelos *et al.* applied a subfractionation proteomic method for the identification of proteins present in the vascular ECM space in addition to post-MI samples [113, 114]. MMP-9 has been detected by mass spectrometry, and the presence of MMP-9 has been linked to the degradation of fibronectin in human aortic samples [113]. Zamilpa *et al.* identified multiple proteins that are differentially expressed in the infarct region of MMP-9-null mice compared to wild-type mice. Among previously known *in vitro* MMP-9 substrates, fibronectin was validated as an *in vivo* MMP-9 substrate in the post-MI setting using ECM-targeted proteomic approaches [115].

The MMP-9 promoter has several inflammatory response elements, including activator protein-1, specificity protein-1, and NF-κB sites [109]. For this reason, MMP-9 is associated with the inflammatory response to tissue injury. A striking increase in MMP-9 activity has been shown to occur at days 1 through 4 in the infarct regions of murine LV, consistent with neutrophil and macrophage infiltration [72, 73, 116–118]. The earlier initial increase in MMP-9 protein levels seen at day 1 post-MI is due to the release of preformed MMP-9 from infiltrating neutrophils, where it is stored in gelatinase granules [119]. Studies in both MMP-9-null mice as well as MMP-9 pharmaceutical inhibitors have shown a reduction in LV dilation and preservation of cardiac function [73, 120–123]. Interestingly, MMP-9 deletion also stimulates neovascularization in the post-MI infarct region [74], suggesting that MMP-9 serves both beneficial and detrimental roles in the post-MI response.

Blankenberg and colleagues performed the first comprehensive clinical study that implicated MMP-9 as a novel prognostic biomarker for cardiovascular mortality in patients with CVD. In this study, MMP-9 was found to correlate with acute-phase reactants (IL-6, hs-CRP, and fibrinogen) indicating that MMP-9 could have its own pathophysiological significance in cardiovascular mortality independent of an association with inflammatory markers [124]. Squire and coworkers extended these studies to demonstrate that increased MMP-9 correlates with larger LV volumes and greater LV dysfunction following MI [125]. Additional studies have indicated an increase in MMP-9 levels to be associated with increased wall thickness, lower ejection fraction, as well as persistent adverse LV remodeling [126–129].

22.3 Markers of Fibrosis

Several molecules have been put forward as candidate circulating biomarkers of cardiac fibrosis, but very few survive the test of time to become a useful clinical tool. Myocardial fibrosis results from a robust increase in collagen deposition, in the setting of an unchanged or lower rate of collagen degradation. Fibrosis is regulated by a number of processes mediated by mechanical and humoral mechanisms [130]. The increase in collagen content plays an important role in the deteriorating function of the heart and in progression to heart failure. Fibrosis leads to myocardial stiffness and promotes the onset of abnormalities in diastolic function. Whereas reparative fibrosis contributes to the preservation of LV morphology and systolic function [131], reactive fibrosis may contribute to the deterioration of LV function by increased myocardial stiffness [130, 132, 133] and impaired electrical activity [134].

22.3.1 Collagen-Related Biomarkers

In the heart, collagen turnover is regulated by fibroblasts and myofibroblasts [135]. These cells synthesize and secrete preprocollagen type I and III, the two more abundant subtypes of fibrillar collagen present in the heart, as a pro-α-collagen chain. In the endoplasmic reticulum, three pro-α-chains form a triple-helix structure, known as procollagen [136]. Procollagen molecules are secreted into the interstitial space where they undergo cleavage of their end-terminal propeptide sequences to enable collagen fiber formation [137]. Procollagen molecules initially contain two terminal propeptides: the amino (N)-propeptide and the carboxy (C)-propeptide; these are released into circulation by the action of specific procollagen N- and C-proteinases [138]. The fibrotic process may be indirectly characterized by measuring collagen propeptides, since quantification of cleaved propeptides correlate with the levels of fibrillar collagen deposited. Although these propeptides reflect collagen synthesis, degradation products of collagen and the MMPs responsible for the breakdown of the ECM can also be detected in blood and are used as biomarkers of collagen turnover (Fig. 22.1).

22.3.1.1 Collagen Synthesis

Procollagen Type I Carboxy-Terminal Propeptide

The most used biomarker for the quantification of collagen type I synthesis is the 100 kDa C-terminal propeptide of collagen type I. A stoichiometric ratio of 1:1 exists between the number of collagen type I molecules produced and the procollagen type I carboxy-terminal propeptide (PICP) molecules released [139]. Elevated

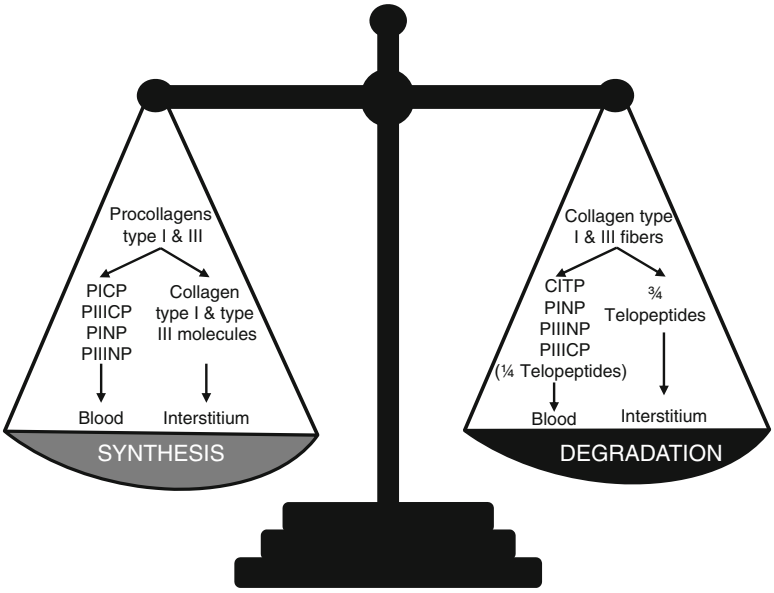

Fig. 22.1 Equilibrium between collagen synthesis and degradation dictates the quality of the LV scar after myocardial infarction. Collagen propeptides are formed during collagen synthesis and are found in circulation; the propeptide levels can be used as biomarkers for collagen synthesis. Similarly, the telopeptides originate during collagen breakdown and are present in the blood. These telopeptides can be used as biomarkers of collagen degradation

levels of PICP in serum have been associated with MI [140] and diastolic heart failure [129]. Animal models have revealed PICP is associated with the volume of myocardial tissue occupied by collagen fibers in left ventricular hypertrophy [141]. Further, changes in collagen metabolism are detected early after MI. In a study by Radovan and colleagues, increased accumulation of collagens I and III was observed within the first 48 h post-MI, and higher synthesis of collagen I persisted until 1 month post-MI [142].

Procollagen Type I Amino-Terminal Propeptide

Procollagen type I amino-terminal propeptide (PINP) is a 70 kDa propeptide that has been used as a marker for synthesis of collagen type I [143]. Contrary to PICP, PINP is not always cleaved from the procollagen molecule [144]; hence, PINP serum levels do not directly correlate to collagen synthesis. This may explain why PINP levels in the blood showed no significant difference between controls and patients with hypertrophic cardiomyopathy [145], heart failure patients [146], and hypertensive patients with or without diastolic heart failure [129].

Procollagen Type III Amino-Terminal Propeptide

Procollagen type III amino-terminal propeptide (PIIINP) is a 42 kDa N-terminal propeptide, cleaved from procollagen type III, and is widely used as a marker for collagen type III synthesis [143]. The N-terminal domain of collagen type III is sometimes removed incompletely, resulting in the incorporation of PIIINP in the collagen fibers [147]. This may lead to an underestimate of the synthesis of collagen type III. Nevertheless, PIIINP plasma levels can be used to predict cardiac events and mortality. Serum PIIINP levels were significantly correlated with relative wall thickness in hypertensive patients with LV hypertrophy [148]. Additionally, elevated serum levels of PIIINP have been positively correlated to increased mortality risk and heart failure [16, 149–151]. Patients with acute MI and with no improvement in LV function during follow-up had higher concentrations of serum PIIINP [142].

22.3.1.2 Collagen Degradation

Collagen Type I Carboxy-Terminal Telopeptide

The degradation of collagen fibrils is accomplished by interstitial collagenase as well as several other proteases. This enzyme cleaves all 3 α-chains of collagen at a single specific locus to generate a ¾ telopeptide and a ¼ telopeptide, in a 1:1 stoichiometry. Whereas the large telopeptide remains within the interstitial space for further degradation by gelatinases, the small C-terminal telopeptide (12 kDa) is found in an immunochemically intact form in the blood [152] and can be used as a biomarker of collagen degradation. In a clinical study that examined the balance between collagen degradation and synthesis 72 h post-MI, high levels of collagen type I carboxy-terminal telopeptide (CITP) in the plasma were associated with poor long-term clinical outcome [153]. A 1-year follow-up of the same clinical study revealed that poor outcome events such as death, heart failure, and recurrent MI were significantly greater for patients presenting the higher CITP levels. This trend is also observed for patients with hypertrophic cardiomyopathy [145], hypertensive heart disease [154], and heart failure with diastolic dysfunction [22, 129].

MMPs and TIMPs

Fibrillar collagens are substrates for MMPs. Of the 25 MMPs described to date, MMP-1 has the highest affinity for fibrillar collagen and preferentially degrades collagens I and III. Increased MMP-1 synthesis has been reported in the infarcted myocardium [155]. Inactivation of MMP-1 synthesis in the infarcted myocardium results in collagen accumulation, which in turn leads to a stiff and noncompliant LV and gradual heart dysfunction [156]. MMP-1 activity is dependent on the concentration of active enzyme and of a family of naturally occurring tissue inhibitors of

metalloproteinases (TIMPs) [157]. TIMP-1 (29 kDa) is a glycoprotein member of the TIMP family and co-localizes with MMP-1 in normal myocardium and is expressed by cardiac fibroblasts and myocytes [158–160]. TIMP-1 levels are closely associated with established markers of heart failure severity, parameters of systemic inflammation, and markers of LV remodeling [161]. The interaction between MMP-1, which initiates the degradation of collagen fibers within the heart, and TIMP-1 is of critical relevance in the maintenance of the integrity of the cardiac collagen network post-MI. The MMP-1/TIMP-1 ratio was inversely correlated with ejection fraction in patients with either systolic or diastolic heart failure [162]; additionally, high levels of TIMP-1 have been related to diastolic dysfunction [163]. Creemers *et al.* demonstrated that TIMP-1−/null mice showed a greater degree of LV dilation post-MI [164].

22.3.2 Galectin-3

Galectin-3 (Gal-3) is a 29–35 kDa soluble β-galactoside-binding lectin [165] that plays an important role in the regulation of cardiac fibrosis and remodeling. Gal-3 is expressed by activated macrophages and induces proliferation of cardiac fibroblasts and increased deposition of collagen type I in the myocardium [166]. Therefore, Gal-3 has an important role post-MI. mRNA levels for Gal-3 were significantly increased in the infarct region of mice 7 days post-MI [167]. Gal-3 expression is substantially upregulated in animal models of heart failure [168] and occurs before the development of clinical heart failure [169, 170].

Presently, all cardiac biomarkers of fibrosis present limitations mainly associated with specificity and accuracy. Collagen turnover is so dynamic—estimated to occur every 80–120 days in the normal myocardium and with much increased kinetics in the MI setting [171]—that not all circulating molecules proposed as biomarkers reflect the actual collagen metabolism at the cardiac level. Furthermore, the inconsistencies reported in the acquisition of Gal-3 levels from plasma and serum cannot be dismissed. Gal-3 may be an ideal biomarker of cardiac fibrosis, but methods for quantification need to be improved. Knowledge about fibrosis in the diseased heart will provide insight into the status and prognosis of a patient. New, better biomarkers of fibrosis are needed to improve diagnosis, prognosis, and therapy. Such biomarkers need to both address fibrous tissue formation and degradation in cardiac remodeling.

22.4 Cardiac Fibroblasts

Fibroblasts synthesize several ECM proteins, including collagen, and have a pivotal role in the formation of the structural framework of most animal organs. Fibroblasts are identified by their spindle-shaped morphology and lack of cell-specific markers that reflect their multiple cell lineages [172]. While fibroblasts remain poorly defined in molecular terms, their functional importance is unmistakable. For many years, studies on cardiac function and cardiac diseases have mainly been focused on the

cardiomyocyte and inflammatory cells. More recently, the cardiac fibroblast has been acknowledged as an extremely important cardiac cell with pivotal roles in cardiac physiology and pathology.

22.4.1 Configuration and Organization in the Heart

Cardiomyocytes embody approximately 75% of normal myocardial volume yet constitute only 30–40% of cell numbers [173, 174]. The remaining non-myocyte cells are predominantly fibroblasts. Cardiac fibroblasts surround myocytes and bridge the interstitial space between myocardial tissue layers; as a result, every cardiomyocyte is spatially close to a fibroblast in normal cardiac tissue [106]. Under physiological conditions, cardiac fibroblasts provide a mechanical scaffold for cardiomyocytes and coordinate pump function of the heart [175].

Cardiac fibroblasts are the primary cell type responsible for homeostatic maintenance of ECM in the normal heart. The major components of cardiac ECM are the fibrillar collagens I (80%) and III (10%) [176], with smaller amounts of collagens IV, V, VI, elastin, laminin, proteoglycans, and glycosaminoglycans [177, 178].

22.4.2 Myofibroblast

Myofibroblasts have been described as a sub-phenotype of activated fibroblasts in diseased hearts [179]. Myofibroblasts are identified by expression of contractile proteins, including α-smooth muscle actin, vimentin, and desmin [176]. Myofibroblast apoptosis has been linked to progression of the granulation tissue into a mature scar, whereas failure of myofibroblast apoptosis has been suggested to drive the progression to fibrosis [180]. At the same time, Blankesteijn and colleagues proposed that the continued presence of myofibroblasts in the infarct scar is necessary to maintain the ECM environment [181]. Cardiac myofibroblasts are particularly receptive to stimulation by proinflammatory cytokines and hormones, the levels of which are enhanced in the remodeling heart [182]. Fibroblasts respond to these stimuli by changing proliferative and migratory rates, altering secretion of ECM proteins and MMP synthesis and modulating the synthesis of cytokines (such as TNF-α, IL-1, IL-6, TGF-β) and growth factors (e.g., vascular endothelial growth factor) [182]. For the purposes of this book chapter, in the context of fibrosis post-MI, activated cardiac fibroblasts/myofibroblasts will simply be referred to as cardiac fibroblasts.

22.4.3 Function Post-MI: Remodeling and Fibrosis

Cardiac fibroblasts are activated in response to MI and have a key role in the wound healing response. The myocardium healing comprises a multitude of temporal and

spatial tightly regulated steps, including hemostasis, infiltration of immune and inflammatory cells, degradation and phagocytosis of necrotic cardiomyocytes and cellular debris, proliferation of cardiac fibroblasts within the injury area, synthesis and reconstruction of granulation tissue, and subsequent ECM remodeling to produce a mature scar [176].

While collagen deposition in adult hearts is normally low, in diseased hearts collagen deposition is robustly increased [183] causing myocardial stiffening. The levels of fibrotic tissue are dependent on the proliferative potential of fibroblasts and on synthesis and deposition of ECM proteins [184]. ECM degradation, resulting from increased MMP expression, dominates the initial phase of the injury response, whereas net ECM deposition, arising from enhanced collagen synthesis, dominates the later phase of healing. The mechanisms regulating the resolution of acute injury responses versus transition to chronic activation of cardiac fibroblasts ultimately ending in heart failure are not well understood.

Fibroblasts are possibly the most underestimated cell population in the heart. They contribute to cardiac development, structure, and function. It is important to note that in addition to being the main producers of ECM proteins in the heart, fibroblasts have been named "sentinel cells" that function as local immune modulators [185, 186]. The cardiac fibroblast has also been reported to contribute to cardiac electrophysiology [187]. Cardiac fibroblasts are sources and targets of biochemical and electromechanical signaling pathways, making these cells excellent candidates for target therapies for the diseased heart.

22.5 Therapeutic Targets

Several studies demonstrate the importance of biomarkers associated with various phenotypes associated with a high risk of disease [15–17, 146]. The discovery and development of biomarkers has shed light on the pathogenesis and management of MI [188–190] and has the potential to play a major role in early diagnosis, disease prevention, drug target identification, drug response, etc. In this section, we discuss recognized and emerging drug candidates acting on a preselected therapeutic target.

22.5.1 Renin–Angiotensin–Aldosterone Inhibitors

LV remodeling post-MI is believed to be associated with prolonged angiotensin stimulation [191]. In order to prevent the harmful effects of the renin–angiotensin–aldosterone system, three distinct paths of drug development have been developed: inhibitors of the angiotensin-converting enzyme (ACE), angiotensin receptor blockers, and aldosterone antagonists.

Myocardial levels of angiotensin II (Ang II), a potent vasoconstrictor, are elevated in a number of pathologies characterized by myocardial remodeling [192]. Although Ang II can be regulated through two different receptor subtypes (AT1R and AT2R), *in vitro* cardiac fibroblasts express predominantly the AT1R. In both animal [193, 194] and human models [195, 196], Ang II was shown to induce fibrosis in the myocardium via multiple mechanisms, including enhanced ECM protein synthesis [197, 198], decreased MMP activity [199, 200], and increased TIMP activity [199, 201]. Sun and Weber have reported enhanced angiotensin production, upregulation of AT1 receptors, and increased collagen mRNA in myofibroblasts associated with healing infarct scars [202].

Clinical trials have shown that ACE inhibitors reduce morbidity and mortality, reduce the progression of established heart failure [203], lessen cardiovascular events in patients at risk but without symptomatic heart failure [204], and reduce cardiac fibrosis and improves LV diastolic function [205]. Angiotensin AT1 receptor blockers offer clinical benefits comparable to ACE inhibitors in heart failure therapy [206–208]. Aldosterone, a mineralocorticoid, has also been heavily implicated in the myocardial fibrogenic response upon stimulation with Ang II or through Ang II-independent mechanisms [209].

22.5.2 Statins

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol synthesis pathway [182]. The pleiotropic anti-inflammatory properties of statins make them drugs of interest for targeting the inflammatory component in patients with advanced heart failure. Additionally, statins have unfailingly been shown to inhibit proliferation of cardiac fibroblasts, an attractive feature when targeting fibrosis. Animals treated with statins exhibit reduced LV remodeling, fibrosis, and collagen synthesis post-MI [210]. In heart failure patients undergoing statin treatment, levels of proinflammatory cytokines were reduced in plasma [211, 212], and clinical studies suggest that statins can decrease local expression of proinflammatory cytokines in the myocardium [213].

22.5.3 Cytokine Therapies

The complexity of cytokine signaling networks has posed a challenge for drug development. Initial efforts with anti-cytokine therapy in heart failure focused on TNF-α [4, 214, 215]. Etanercept is a recombinant human TNF receptor that binds to soluble TNF. Phase I safety studies showed that biweekly injections of etanercept for 3 months, in patients with advanced heart failure, resulted in a significant dose-dependent improvement in LV function and remodeling [216].

Interleukins, such as IL-6 and IL-11, have also been used as possible therapeutic targets in cardiovascular disease. Cardiotrophin-1 (CT-1) is a member of a family of cytokines including IL-6, which is capable of inducing cardiomyocyte hypertrophy in vitro [217]. CT-1 also has significant protective roles. CT-1 can protect cardiomyocytes when added either prior to simulated ischemia or at the time of reoxygenation following simulated ischemia [218]. In a murine model of MI, IL-11 treatment after coronary ligation attenuated cardiac fibrosis [219], and pretreatment with IL-11 reduced infarct area in a murine ischemia/reperfusion (I/R) model [220].

22.5.4 MMP Inhibitors

Whereas only a small number of studies have explored inhibitors of collagen biosynthesis to mitigate cardiac fibrosis [178, 221], numerous research has focused on MMP inhibitors' (MMPI) effects on pathologic remodeling. Rohde *et al.* showed that a nonselective MMPI decreased LV dilatation post-MI in mice [122]. In multiple animal models, MMP-9 levels increase early post-MI, and MMP-9 deletion in mice attenuates remodeling [73]. The effects of MMPI in progression to heart failure have also been examined. A porcine model of congestive heart failure showed attenuated LV remodeling and preserved cardiac function when treated with a nonselective MMPI, compared to non-treated controls [121]. Similar results were obtained in a study of chronic human heart failure [222].

22.5.5 Nuclear Receptor Agonists

The role of ligands of the peroxisome proliferator-activated receptors (PPAR), PPARα, PPARδ, and PPARγ, in LV remodeling post-MI is receiving widespread attention for their actions on the myocardium [223]. PPARs are nuclear receptors that regulate lipid storage and metabolism, and are expressed by multiple cell types in the cardiovascular system, including cardiomyocytes and fibroblasts [176]. Recent evidence suggests that PPARs may attenuate inflammatory responses, therefore influencing cardiac remodeling [224]. This function is mainly mediated through the ability of agonist-activated PPARs to form complexes with other transcription factors, thus cross inhibiting their transcriptional activity [225]. In animal models of acute MI, PPARα and PPARγ ligands showed cardioprotective properties [226], attenuated fibrosis, preserved diastolic function, and inhibited inflammatory activation in pressure overload cardiac hypertrophy [227].

22.6 Conclusions and Future Directions

One third of total global deaths are attributed to CVD, and the majority of deaths are due to MI [228]. The field of biomarker discovery holds great potential for improving clinical diagnosis by identifying individuals who are at risk, determining the severity of disease, and developing personalized treatment for patients diagnosed with MI. Advances in our understanding of the pathogenesis and consequences of MI have initiated the development of new biomarkers as well as expanded the role of biomarkers in classification and individualization of treatment [229]. Physicians still depend on invasive techniques for the diagnosis and treatment of MI. Recent advances in the development of gene arrays in addition to proteomic technologies have produced multiple new biomarkers providing hope for an increase in the number of new diagnostic markers. The future of biomarker use for the treatment of MI will depend on the production of cost-effective treatments, multimarker profiling to accurately stage progression of molecular disease, and the availability of simple diagnostic kits for both physicians and patients [10, 229–231].

An ideal biomarker must be accurate, fast, and more cost-effective than any current technique [230]. Because of limited healthcare budgets, only biomarkers with exceptional performance will find utility in primary care settings making a noninvasive procedure at a low cost an advantage [10, 232]. This is especially true of many current diagnostic standards, where cost often exceed $1,000 per test, and procedures are partially invasive and risk associated [232]. Developing biomarkers that are cost-effective will most likely improve health outcomes.

Development of simple tests that accurately and reliably predict the development of heart failure following MI should be a goal of biomarker research. In diseases where early diagnosis is key for survival, these tests can be vital in improving patient heath. To reach this goal, sensitivity and specificity are major issues that have to be improved. Developing diagnostic and screening tests that look at a range of biomarkers rather than relying on only one biomarker is a likely potential solution [229].

With the recent advances in technology, the use of multimarker profiling will be beneficial in the development of individualized treatment schemes for post-MI patients [10]. Although a multimarker approach has shown potential for comprehensive risk assessment, several limitations must be recognized. The ultimate test of a multimarker paradigm will be its value in therapeutic decision-making [229]. For example, the ability of a biomarker to predict relative risk and specific end points differ. The B-type natriuretic peptide (BNP) has been shown to be a potent predictor of mortality risk, but BNP exhibits a weak association with recurrent ischemic events [231].

The discovery of new potential biomarkers shows great potential for treatment post-MI. Circulating factors related to inflammatory and fibrotic responses are key predictors of early ECM changes in the structure and function of the heart providing important prognostic information for MI [15, 22]. Improvement on cost-effectiveness as well as multianalyte processing will move us in the right direction so that in the

future biomarkers will not only be available to treat, diagnose, or predict recurrence but also will be able to guide physicians in decision-making. Biomarkers may one day be available in over-the-counter testing [10].

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Chapter 23
Oxidative Stress in Cardiac Repair and Remodeling: Molecular Pathways and Therapeutic Strategies

Yao Sun

Abstract Cardiac remodeling occurs in a variety of heart diseases, contributing to ventricular dysfunction. Following myocardial infarction (MI), cardiac repair and remodeling appear in both infarcted and non-infarcted myocardium. Factors regulating cardiac repair/remodeling at different stages following MI are under investigation. There is growing recognition and experimental evidence that oxidative stress mediated by reactive oxygen species (ROS) plays a role in the pathogeneses of myocardial repair/remodeling in various cardiac diseases. After acute MI, oxidative stress is developed in both infarcted and non-infarcted myocardium. Accumulating evidence has demonstrated that ROS participates in several aspects of cardiac repair/remodeling following infarction that includes cardiomyocyte apoptosis, inflammatory/fibrogenic responses, hypertrophy, and angiogenesis. The exact pathways on ROS-mediated myocardial remodeling are under investigation. The therapeutic potential of oxidative stress-directed drugs in myocardial remodeling following infarction has not been fully realized.

Keywords Oxidative stress • Myocardial infarction • Inflammation • Fibrosis • Hypertrophy • Angiogenesis

23.1 Introduction

Heart failure has emerged as a major health problem during the past two decades. It appears most commonly in patients with previous MI. Myocardial remodeling, which occurs in both infarcted and non-infarcted myocardium, contributes

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significantly to the development of heart failure [1–3]. Following MI, cardiac repair is associated with angiogenesis and inflammatory reaction, which are followed by scar formation at the site of infarction. Cardiac remodeling is represented as hypertrophy with interstitial fibrosis in non-infarcted myocardium. This leads to alterations in left ventricular size, shape, and wall thickness [4–6]. Angiogenesis and fibrous tissue that form at the site of cardiomyocyte loss preserve the structural integrity and are integral to the heart's recovery, while the structural remodeling of viable myocardium impairs tissue behavior. Multiple factors may, in fact, contribute to left ventricular remodeling at different stages post-MI. There is experimental evidence to suggest that oxidative stress mediated by ROS plays a role in the pathogeneses of myocardial repair/remodeling after MI, a hypothesis that has been earning growing recognition [7–11]. Oxidative stress results from an oxidant/antioxidant imbalance: an excess of oxidants relative to the antioxidant capacity. The heart with acute MI undergoes an increased ROS production as well as antioxidant deficit, first in the infarcted myocardium, followed by the non-infarcted myocardium. Experimental studies have also demonstrated that oxidative stress can induce most, if not all, of the changes that are thought to contribute to myocardial remodeling including angiogenesis, proinflammatory cytokine release, cardiomyocyte apoptosis [12], fibrogenesis [13], cell proliferation [14], angiogenesis [15], and hypertrophy [16]. Chronic antioxidant treatment suppresses cardiac oxidative stress; attenuates ventricular remodeling, partially preserving left ventricle function; and improves survival in rats or mice with experimental MI [17–19]. In this chapter, the potential relevance of oxidative stress on cardiac repair/remodeling in the infarcted heart will be discussed. The role of antioxidants in cardiac remodeling will be also discussed.

23.2 Occurrence of Cardiac Oxidative Stress Following Myocardial Infarction

Superoxide (O_2^-), hydroxyl (OH^-), and peroxynitrite ($ONOO^-$) are simple molecules characterized by the presence of unpaired electrons. ROS can be produced intracellularly through electron leakage from mitochondria during oxidative phosphorylation and through the activation of several cellular enzymes, including NADPH oxidase, xanthine oxidase, and nitric oxide synthase [20–22]. O_2^- can rapidly react with nitric oxide (NO) to form $ONOO^-$ or convert to H_2O_2 to form OH^- [20]. ROS in low concentrations serve as signaling molecules [23]. However, these agents elicit harmful effects when produced in excess [20]. The toxicity associated with the excessive production of these compounds is prevented by antioxidant defense systems that maintain a healthy cellular environment. Living cells have both enzymatic and nonenzymatic defense mechanisms to balance the multitude of oxidative challenges presented to them. The enzymatic subgroup includes superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx) [24–26]. The dismutation of O_2^- by SOD results in the generation of H_2O_2, which catalase further metabolizes into water and oxygen. The nonenzymatic group includes a

variety of biologic molecules, such as vitamins E and C [27]. Oxidative stress occurs when ROS production is enhanced and/or antioxidant reserve is suppressed.

In the myocardium, as in other tissues, antioxidant enzymes protect cells by maintaining O_2^- and H_2O_2 at low levels. Following MI, oxidative stress is developed in both infarcted and non-infarcted myocardium. Singal and colleagues have shown the evidence of progressive decrease in SOD, catalase, and GSHPx activity as well as vitamin E levels in the rat infarcted heart, first in the infarcted myocardium and then followed by non-infarcted myocardium [7, 28]. Our study has shown reduced SOD gene and protein expression in the infarcted myocardium [29]. Cardiac glutathione levels are also decreased in patients with acute MI [10]. These observations raise the possibility that impaired antioxidant capacity contributes to oxidative stress in the infarcted heart. NADPH oxidase is a major source of O_2^- in the heart [30]. In the infarcted heart, NADPH oxidase expression ($gp22^{phox}$ and $gp91^{phox}$ subunits) is significantly increased in the infarcted myocardium [8, 29], with neutrophils and macrophages as the primary cells expressing the enzyme. These findings suggest that ROS production is also enhanced in the infarcted myocardium. Moreover, 3-nitrotyrosine, a marker of oxidative stress, is highly expressed in the inflammatory cells of the infarcted myocardium, supporting the occurrence of cardiac oxidative stress following MI [29]. Oxidative stress in non-infarcted myocardium is contributed by multiple sources. Increased mitochondrial production of ROS has been suggested in non-infarcted myocardium of mice as one of them [19]. Increased ROS levels in non-infarcted myocardium also reflect increased activity of intracellular oxidase complexes, such as NADPH oxidase, xanthine oxidase, and nitric oxide synthase [31]. In addition, reduced SOD levels were observed in the failing heart with infarction [11]. These observations indicate that the imbalance between ROS production and antioxidant defense capacity contributes to oxidative stress in non-infarcted myocardium.

23.3 Oxidative Stress and Cardiac Repair/Remodeling Following Myocardial Infarction

23.3.1 Oxidative Stress and Cardiomyocyte Apoptosis

Loss of cardiomyocytes is an important mechanism in the development of myocardial remodeling and cardiac failure [32]. Following MI, apoptotic cardiomyocyte death occurs in the infarcted myocardium as well as the surviving portions of the wall adjacent to and remote from the infarcted myocardium [33, 34]. However, the number of apoptotic cardiomyocytes is greater in the infarcted region than in the region away from infarction. The regulation of cardiomyocyte apoptosis involves multiple mechanisms. ROS have been proven to be one of the stimulators of cardiomyocyte apoptosis [35–39]. High levels of oxidative stress have been demonstrated to cause cell necrosis, while lower levels of oxidative stress can cause cell apoptosis. In vitro studies have shown that treatment with O_2^- or H_2O_2 in cardiomyocytes

induces apoptosis [40]. In vivo studies have further demonstrated that oxidative stress triggers cardiomyocyte apoptosis in several cardiovascular diseases, including MI, ischemia/reperfusion injury, cardiomyopathy, atherosclerosis, and heart failure [34, 41–46]. Long-term treatment with the antioxidants, probucol or pyrrolidine dithiocarbamate, attenuates oxidative stress and cardiomyocyte apoptosis within non-infarcted myocardium in rats [18, 36]. Another antioxidant, carvedilol, is shown to attenuate apoptosis induced by ischemia–reperfusion in the rat heart [47]. Oxidant scavengers, such as SOD and vitamin E, have been demonstrated to reduce ROS and inhibit cardiomyocyte apoptosis [48].

The mechanisms responsible for oxidative stress-mediated apoptosis in the infarcted heart are not fully understood. Multiple studies have shown that ROS can induce cardiomyocyte death by one or more mechanisms. Apoptosis is tightly controlled by a number of genes, including those primarily suppressing and those promoting apoptosis. Our previous studies identified markedly increased proapoptotic Bax expression in the infarcted heart, particularly at the site of infarction. Enhanced Bax expression coexists with oxidative stress and apoptosis in the infarcted heart [49]. Overexpression of antiapoptotic Bcl-2 decreases cardiomyocyte apoptosis [50]. Cesselli et al. have shown that in dog dilated cardiomyopathy, oxidative stress-induced cardiac apoptosis is related to increased $p66^{she}$, cytochrome c release, and activation of caspase-9 and caspase-3 [51]. Taken together, these findings suggest that oxidative stress may trigger cardiomyocyte apoptosis via regulation of apoptotic genes.

Cytokines, such as tumor necrosis factor (TNF)-α and interleukin-6, are proven to stimulate cardiomyocyte apoptosis, which can be mediated by oxidative stress [52, 53]. TNF-α-induced apoptosis is dependent on the induction of inducible nitric oxide synthase (iNOS) [54]. NO produced by iNOS appears to cause apoptosis through the formation of $ONOO^-$, activation of the death receptor Fas through upregulation of Fas ligand expression, inhibition of mitochondrial ATP synthesis, and inactivation of serial antioxidant enzymes [55, 56]. In the infarcted mouse heart, iNOS expression is elevated in both infarcted and non-infarcted myocardium, whereas in iNOS-knockout mice, cardiomyocyte apoptosis was reduced compared to wild-type mice with MI [57]. TNF-α-induced cardiomyocyte apoptosis can be inhibited by antioxidants such as thioredoxin and *N*-acetylcysteine [58]. Following acute MI, cardiac interleukin-6 expression is also significantly increased, which initiates inflammatory response, particularly at the site of infarction. Interleukin-6 is also verified to cause cardiomyocyte apoptosis, which can be suppressed by antioxidant treatment [59]. These findings suggest that oxidative stress is involved in different pathways that induce cardiomyocyte apoptosis following infarction and can be suppressed by antioxidants.

23.3.2 Oxidative Stress and Cardiac Inflammatory Response

MI is associated with an inflammatory response, ultimately leading to healing and scar formation. Inflammatory response in the infarcted myocardium is related to the

coordinated activation of a series of cytokine and adhesion molecule genes. A critical element in the regulation of these genes involves nuclear factor-kappa B (NF-κB), a redox-sensitive transcription factor. NF-κB maintains an inactive form bound to its inhibitory subunit Ikappa B under normal conditions. When tissue is injured, NF-κB can be activated by various local substances including ROS [60]. Upon activation, NF-κB stimulates inflammatory and immune responses and cellular growth by increasing the expression of specific cellular genes. NF-κB activation has been demonstrated in various models of myocardial ischemia and reperfusion [61, 62]. Activated NF-κB triggers gene expression of interstitial and vascular adhesion molecules, leading to leukocyte infiltration into the infarcted myocardium, as well as monocyte chemoattractant protein-1, and inducing recruitment of mononuclear cells. NF-κB also triggers gene expression of proinflammatory cytokines, such as TNF-α and interleukins, and initiates inflammatory response [63]. TNF-α is not constitutively expressed in the normal heart. In rodent models of MI, TNF-α expression is significantly upregulated in the infarcted myocardium as well as in the non-infarcted myocardium [64]. TNF-α can stimulate inflammatory protein synthesis, macrophage phagocytosis, and cell growth, differentiation, and apoptosis [65]. In the infarcted myocardium, elevated NADPH oxidase is spatially coincident with activated NF-κB and enhanced TNF-α expression in the inflammatory cells [61]. In addition to NF-κB pathway, recent studies suggest that H_2O_2 can directly induce cardiac TNF production via the p38 MARP pathway and, in turn, mediate myocardial inflammation. Free radical scavenger treatment has been demonstrated to diminish inflammatory response and cardiac remodeling [18]. The antioxidant, probucol, has been shown to attenuate cardiac inflammation and improve ventricular function [66]. Thus, these findings indicate that ROS serves as a proinflammatory mediator in the cardiac healing process following infarction and its role in cardiac inflammation involves several pathways.

In the infarcted heart, oxidative stress is mostly evident at the border zone, the area between the infarcted and non-infarcted myocardium [9]. ROS are known to cause oxidative damage to various cellular structures, including membranes, proteins, DNA sequences, and DNA repair enzymes and therefore have the potential to injure cardiomyocytes and vascular cells in the neighboring non-infarcted myocardium, causing additional cardiac damage/inflammation and infarct size extension. Studies have shown that antioxidant treatment reduces infarct size in rat MI model [67, 68]. The beneficial effect of antioxidant on infarct size may be associated with the prevention of ROS-induced damage/inflammation in the border zone of the non-infarcted myocardium.

23.3.3 Oxidative Stress and Cardiac Fibrosis

A growing bulk of evidence supports a causative role of oxidative stress in fibrogenesis in various tissues including liver, lung, arteries, nervous system, and heart [69, 70]. Extensive fibrosis is a major feature of myocardial remodeling in the

infarcted heart, represented as scar at the site of infarction and interstitial fibrosis in non-infarcted myocardium [71]. Cells responsible for fibrous tissue formation at the site of infarction consist principally of phenotypically transformed fibroblast-like cells termed myofibroblasts. These cells contain α-smooth muscle actin microfilaments and are responsible for collagen synthesis and scar contraction [72]. They appear early after MI, mainly located in and around the infarcted myocardium, and persist throughout healing [73]. Interstitial fibroblasts are responsible for normal collagen turnover and are considered to be a source of myofibroblasts. Myofibroblast differentiation and proliferation as well as collagen synthesis are tightly controlled by the fibrogenic cytokine transforming growth factor-beta (TGF-β). TGF-β mRNA and concentration are significantly increased in both the infarcted and non-infarcted myocardium in rats [74]. Oxidative stress is shown to upregulate the expression of TGF and type I collagen [75]. Following MI, enhanced expressions of TGF-β and NADPH oxidase are spatially coincident at the site of infarcted myocardium. Treatment with the antioxidant taurine reduces oxidative stress, suppresses TGF-β gene expression, and attenuates hepatic fibrosis [76]. Furthermore, in vitro studies have indicated that ROS promotes fibroblast proliferation and type I collagen gene expression in cardiac fibroblasts [77]. Chronic antioxidant treatment is shown to attenuate cardiac fibrosis [17]. However, Frantz and his coworkers have shown that targeted deletion of the NADPH oxidase subunit gp91phox does not affect left ventricular remodeling including myocardial fibrosis following MI and does not decrease the production of ROS [78]. It suggests that NADPH oxidases might not be the only important pathway for the production of ROS after MI. In addition to the heart, antioxidants d-a-tocopherol and butylated hydroxytoluene are confirmed to suppress type I collagen gene expression in the repairing liver [75]. After acute MI, progressive global left ventricular dilation occurs over months [2]. MMPs favor this adverse remodeling. It was shown that inhibition of MMPs decreases the severity of remodeling in the infarcted heart [79]. In vitro studies have shown that ROS activates MMPs in cardiac fibroblasts [80]. Oxidative stress may, therefore, play a role in the pathogenesis of left ventricular dilation following infarction. However, in vivo studies on the regulatory role of ROS on MMPs and cardiac dilatation are lacking and further studies are required on this concept.

23.3.4 Oxidative Stress and Cardiac Hypertrophy

Myocardial hypertrophy is often developed in non-infarcted myocardium in weeks or months after MI. Extracellular stimuli such as mechanical strain, neurohormones, or cytokines have been well recognized to promote cardiomyocyte hypertrophy. Recent studies have further demonstrated that these extracellular stimuli may mediate cardiomyocyte hypertrophy via oxidative stress. ROS released acutely in large amounts have been implicated in the cell death associated with MI. The chronic release of ROS has been, however, linked to the development of left ventricular hypertrophy and heart failure progression [20, 81]. The chronic release of ROS

appears to derive from the nonphagocytic NADPH oxidase and mitochondria [20]. The existence of nonphagocytic NADPH oxidase has been suggested in the cardiomyocytes. Experimental data suggest that abnormal activation of the nonphagocytic NADPH oxidase in response to neurohormones contributes to cardiomyocyte hypertrophy [82]. Acute MI is accompanied by systemic and local activation of numerous neurohumoral factors, including angiotensin (Ang)II, TNF-α, and norepinephrine. Increased AngII (AT1) receptors and angiotensin-converting enzyme (ACE) are colocalized with NADPH oxidase in the infarcted heart [83, 84]. Accumulating experimental evidence has demonstrated that AngII stimulates the expression of NADPH oxidase leading to the production of ROS in the repairing heart and vessels [85]. Treatment with ACE inhibitor attenuates ROS formation and prevents cardiomyocyte hypertrophy in diabetic rats to the same extent as the antioxidant *N*-acetylcysteine [86]. Moreover, in vitro studies further demonstrated that antioxidants prevent AngII-induced myocyte hypertrophy [87].

TNF-α is shown to provoke a hypertrophic cardiac phenotype [88]. In vitro studies have demonstrated that TNF-α-induced cardiomyocyte hypertrophy is mediated through NF-κB activation via the generation of ROS [89]. Norepinephrine-induced cardiac hypertrophy likewise also requires ROS. Treatment of cultured adult rat cardiomyocytes with norepinephrine increases NADPH oxidase and leads to cardiomyocyte hypertrophy.

The exact pathways of ROS-mediated hypertrophy remain to be determined. ROS can participate as second messengers in myocardial signaling events, activating stress-related nuclear transcription factors, leading to cellular hypertrophy [90]. These second messengers include MAP kinases, small GTP-binding proteins, the Src family of tyrosine kinases, and cytokine cascades. Pretreatment with a SOD mimetic inhibits both the hypertrophy and ROS production. The overexpression of GSHPx could attenuate left ventricular hypertrophy and improve cardiac function after MI [81].

23.3.5 Oxidative Stress and Cardiac Angiogenesis

Angiogenesis is the formation of new capillary blood vessels from existent microvessels. It is a tightly regulated process, which involves the degradation of the basement membrane surrounding an existing vessel, migration and proliferation of endothelial cells into the injured space, adherence of the endothelial cells to each other, and lumen formation and maturation. Angiogenesis turns on in repairing tissue. Matrix metalloproteinases (MMPs) play an important role in the degradation of the basement membranes and other extracellular matrix (ECM) components, allowing endothelial cells to detach and migrate into new tissue [91]. Angiogenic mediators such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and integrins then stimulate endothelial proliferation and tube formation, while angiopoietins further promote vascular maturation and survival [92, 93].

Following acute MI, myocytes/interstitial cells and existing blood vessels in the infarcted myocardium undergo necrosis. Angiogenesis becomes evident in the infarcted myocardium particularly during the first 2 weeks post-MI. Newly formed vessels are seen first in the border zone, the region between the infarcted and non-infarcted myocardium, and then extend into the infarcted myocardium. Angiogenesis is subsequently converted into quiescence in the late stage of MI, when cardiac repair is completed. Acute MI is accompanied by increased expression of proangiogenic mediators, such as VEGF, PDGF, and FGF, in the border zone and infarcted myocardium in the very early stage of MI [94–96]. The activity of MMP-2/MMP-9 and αvβ3 integrin is also found elevated in the infarcted myocardium in the early phase of MI [97, 98]. These changes are spatially and temporally coincident with angiogenesis in the infarcted myocardium, indicating their involvement in cardiac angiogenesis following MI.

A considerable body of evidence has indicated that ROS, including O^{-}, H_2O_2, and NO, mediate angiogenesis in certain pathological situations [99–107]. In the infarcted heart, angiogenesis is crucial for cardiac repair. Our recent study has shown that ROS promote angiogenesis in the infarcted (necrotic) myocardium, thereby facilitating cardiac repair [15]. In addition, it has been demonstrated that ROS are required for coronary collateral development in coronary artery occlusion and reperfusion in rats [108, 109]. However, mechanisms responsible for ROS-mediated angiogenesis in the infarcted heart remain uncertain. Angiogenic response in the infarcted myocardium is extremely active and short-lived, and inflammatory cells play a major role on ROS production in the infarcted myocardium. These cells also largely release various proangiogenic growth factors and other angiogenesis-related substances such as MMPs, integrins, and cytokines. Thus, ROS may mediate angiogenesis in the infarcted myocardium through multiple pathways at the same time. ROS have both beneficial and deleterious effects in the infarcted heart. ROS induces angiogenesis in the infarcted myocardium in the early stage of MI and facilitates cardiac repair. However, ROS also contribute to cardiac remodeling, such as hypertrophy and interstitial fibrosis in non-infarcted myocardium in the late stage of MI. Thus, further studies are required to provide valuable information on the timing of antioxidant treatment, which can attenuate cardiac remodeling, but not disturb cardiac angiogenesis.

23.4 Conclusion

Experimental evidence has demonstrated that cardiac repair/remodeling is subject to oxidative stress following MI. This chapter has highlighted several mechanisms by which oxidative stress is involved in cardiac repair/remodeling in the infarcted heart. Following acute MI, oxidative stress occurs in both the infarcted and non-infarcted myocardium and ROS plays a significant role in the initiation as well as the regulation of cardiac molecular and cellular changes including angiogenesis, cardiomyocyte apoptosis, inflammatory/fibrogenic responses, and hypertrophy that

contribute to myocardial repair/remodeling. These cardiac events can be significantly inhibited by antioxidants in animal models of MI. Further studies are required on the potential therapeutic interventions with antioxidants in limiting cardiac remodeling in patients with MI.

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Chapter 24
Role of SPARC in Cardiac Extracellular Matrix Remodeling After Myocardial Infarction

Davy Vanhoutte and Stephane Heymans

Abstract Research of the last decades has established that the cardiac extracellular matrix (ECM) is not a static but rather a dynamic environment in which the cellular and acellular components of the heart communicate and function. Remodeling after myocardial infarction (MI) associates with cardiac rupture or maladaptive left ventricular remodeling resulting in cardiac dilatation, dysfunction, and increased mortality. The dynamic synthesis and breakdown of the cardiac ECM-related proteins and the formation of a well-organized collagen scar play a major role in the structural and functional recovery of the infarcted heart. Secreted protein acidic and rich in cysteine (SPARC) is a prototypical collagen-binding matricellular protein and has been proven to coordinate procollagen processing and facilitate collagen fibril assembly in the pericellular environment. SPARC is vastly upregulated in response to cardiac stress, including MI. The lack of SPARC resulted in a fourfold higher incidence of mortality following MI, due to increased rates of cardiac rupture and heart failure, which was associated with a highly impaired and immature collagen scar. In contrast, adenoviral overexpression of SPARC improved collagen maturation in the infarct scar, associated with increased transforming growth factor (TGF)-β signaling, and prevented cardiac dilatation and dysfunction after MI. Therefore, this

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chapter focuses on (1) the role of SPARC during cardiac remodeling after MI and (2) the potential mechanisms involved in SPARC-mediated extracellular post-synthetic procollagen processing in the infarcted heart.

Keywords SPARC • Matricellular proteins • Myocardial infarction • Extracellular matrix • Remodeling • Transforming growth factor-β • Procollagen processing • Collagen

24.1 Introduction

24.1.1 The Cardiac Extracellular Matrix and Myocardial Infarction

The cellular components of the heart are enmeshed in a complex network of extracellular matrix (ECM) that is primarily composed out of type I collagen and to lower extent of type III, type V collagen, fibronectin, and elastin [1, 2]. In addition to its structural elements, the cardiac ECM also harbors more specialized proteins including glycoproteins (fibronectin, periostin, fibromodulin, and vitronectin), proteoglycans (such as syndecans, lumican, and biglycan), glycosaminoglycans (hyaluronic acid and dermatan sulfate), and matricellular proteins (including SPARC and others) [3–5]. On the other hand, a large reservoir of recruitable matrix metalloproteinases (MMPs) exists within the cardiac ECM representing the main extracellular proteases involved in degradation the cardiac ECM [6].

Discoveries of the last decade have drastically changed our traditional perception of the cardiac ECM. It has become clear that the ECM not only acts as a structural support but also provides a unique environment in which the embedded cells communicate and function [1–4]. By activating signaling cascades, the ECM transduces extracellular changes into cellular and acellular responses that play central roles to maintain normal chamber geometry and ventricular function during homeostasis and in response to injury. As such, dramatic changes in the composition of the cardiac ECM occur after myocardial infarction (MI) [7, 8]. In response to MI, the left ventricle (LV) undergoes a continuum of molecular, cellular, and extracellular responses that result in LV wall thinning, dilatation, and dysfunction, collectively referred to as cardiac remodeling [8]. In summary, cardiac wound healing after MI can be divided in three distinct but overlapping phases: the inflammatory, granulation, and maturation phase [6]. The inflammatory phase is characterized by massive inflammatory infiltrates, rapid protease activation, and matrix degradation. Macrophages remove the necrotic cells and matrix debris and produce growth factors, inducing fibroblast migration, proliferation, and activation. The necrotic tissue is consequently replaced by granulation tissue, consisting of inflammatory cells, fibroblasts, myofibroblasts, and new blood vessels embedded in a fibrin/fibronectin-based provisional matrix.

Next, the provisional matrix is reabsorbed, and myofibroblasts accumulate to produce large amounts of ECM proteins. Finally, a mature collagen-based stable scar is formed, characterized by tightly aligned, well-organized, and cross-linked collagen fibers that provide mechanical support to the infarcted heart [6, 7, 9]. Importantly, excessive degradation and delayed or defective formation of the newly formed cardiac ECM could lead to cardiac rupture, a fatal complication of acute MI. In later phases, this alters the mechanical properties of the heart, resulting in exaggerated maladaptive cardiac remodeling which is associated with higher incidence of arrhythmia, increased mortality, and the development of chronic heart failure [6, 7, 9].

24.1.2 Matricellular Protein, Essential Modulators of Cardiac Remodeling After MI

Recently, we have witnessed increasing interest in a class of nonstructural ECM proteins, known as "matricellular proteins" (Table 24.1). Although the term describes a family of structurally unrelated extracellular matrix macromolecules with distinct functional properties, they do exhibit several general characteristics (extensively reviewed in [3] and [4]). First, unlike collagens, matricellular proteins do not directly contribute to the structural integrity of the heart but serve as a bridge between cells and the ECM. As such, they influence a broad range of critical regulatory cell–cell and cell–matrix processes through the direct binding with cell surface receptors, growth factors, proteases, and other bioactive effectors, as well as various structural ECM proteins. Secondly, expression of matricellular proteins, including SPARC (secreted protein acidic and rich in cysteine), thrombospondins, osteopontin, tenascins, periostin, and others, is generally low during normal postnatal life but is upregulated during embryogenesis and in response to tissue injury, including pathophysiological conditions of the heart (Table 24.1) [3, 4]. Concordantly, matricellular proteins are released into the infarcted heart and are degraded as the infarct scar matures. Finally, their mechanistic roles have been—at least in part—elucidated using gene-targeted mice. Surprisingly, the majority of mice that lack one of the matricellular proteins survive embryogenesis and only produce mild abnormalities under normal physiological conditions, suggesting a limited role in tissue homeostasis (Table 24.1) [3, 4]. In contrast, when subjected to pathophysiological mouse models, such as MI, they display striking phenotypes, demonstrating that the re-expression of matricellular proteins activates key molecular signaling pathways that are essential for proper cardiac healing and remodeling after MI [3, 4]. A comprehensive overview of known phenotypes in matricellular protein-deficient mice after MI is presented in Table 24.1.

This chapter presents and discusses the current knowledge on SPARC and its role proposed after MI. More specifically, it will focus on SPARC's expression, pathophysiologic relevance, and the critical role that SPARC plays in myocardial remodeling and extracellular collagen processing after MI.

Table 24.1 Matricellular proteins and myocardial infarction

Matricellular protein	Baseline ECM organization	After Myocardial Infarction			
		Increased expression	Phenotype in gene-deficient mice	Cardiac function	Proposed mechanism
TSP-1	No change	Early phase inflammatory cells	Increased inflammation, expansion of infarct, and LV dilatation	ND	Increased inflammation, MMP-9 activity, and decreased TGF-β signaling
TSP-2	Abnormal collagen fibrillogenesis	Late phase fibroblasts	Cardiac rupture >90%	ND	Impaired maturation of infarct scar and increased MMP activity
OPN	ND	Early and late phase macrophages and fibroblasts	Increased LV dilatation, reduced collagen synthesis, and deposition in the infarcted and remote myocardium	↓	Decreased macrophage recruitment and phagocytosis, impaired fibroblast adhesion, proliferation, and myofibroblast differentiation
TN-C	ND	Early phase fibroblasts	Reduced end-diastolic pressure, LV stiffness, dilatation, and fibrosis	↑	Decreased de-adhesion, MMP production, and fibrosis
TN-X	Reduced collagen content in skin	ND	ND	ND	ND

Periostin	Aberrant collagen I fibrillogenesis in skin	Early and late phase fibroblasts	Increased incidence of cardiac rupture; reduced LV wall stiffness Decreased recruitment of myofibroblasts and impaired matrix deposition	↑	Decreased ECM integrity with smaller and less abundant collagen fibrils; and decreased adhesion-dependent FAK signaling
CCN1 (Cyr61)	ND	Cardiomyocytes and endothelial cells	Embryonic lethality associated with vascular and septal defects	ND	ND
CCN2 (CTGF)	ND	Myofibroblasts and cardiomyocytes	CCN2-null mice die within minutes after birth	ND	ND
SPARC	For an extended overview of SPARC, see Table 24.2				

The data presented in this table is more extensively reviewed by Schellings et al. [3, 46] and Frangogiannis [4]
MI myocardial infarction, *TSP* thrombospondin, *TN* tenascin, CCN (acronym for Cyr61, Connective tissue growth factor [CTGF], and Nov), *Cyr61* cysteine-rich, angiogenic inducer 61, *CTGF* connective tissue growth factor, *SPARC* secreted protein acidic and rich in cysteine, *LV* left ventricle, *TGF-β* transforming growth factor-β, *MMP* matrix metalloproteinase, *FAK* focal adhesion kinase, *ND* not determined, ↑ increased, ↓ decreased

24.2 SPARC, a Multifunctional Matricellular Protein

SPARC, secreted protein acidic and rich in cysteine (also known as osteonectin or basement membrane protein-40), is a prototypic collagen-binding, counter adhesive matricellular protein [14, 15]. It is the product of a single gene that is highly conserved in both vertebrates and invertebrates, suggesting a fundamental function for SPARC in matricellular biology [15, 16].

After the removal of a 17-amino acid signaling peptide sequence, the final mature SPARC consists out of three distinct modular domains (Fig. 24.1a) [14, 15, 17]. The 53-amino acid NH_2-terminal acidic domain contains a low-affinity, high-capacity Ca^{2+}-binding domain. The central portion includes a 24-amino acid follistatin-like domain and a 55-amino acid protease-like inhibitor region. The 151-amino acid COOH-terminal domain contains two high-affinity Ca^{2+}-binding EF hands.

Like every matricellular protein, SPARC exhibits a remarkable functional complexity that is dependent on the effectors it binds to and the cell types it associates with in the different tissues (Fig. 24.1b) [14, 16, 17]. In more than 30 years since its discovery, a number of biological activities have been associated with SPARC in vitro [16, 17]. These functions include processes that regulate (1) cell proliferation, adhesion, migration, differentiation, and survival; (2) growth factor signaling; and (3) MMP activity (Fig. 24.1b). However, phenotypic characterization of SPARC-null mice pointed towards a primary role for SPARC in collagen fibril assembly, morphology, and metabolism (reviewed in [14] and [15]). In contrast to primitive organisms where abrogation of SPARC is lethal [18], SPARC-null mice are viable and exhibit progressively milder defects [14]. The lack of SPARC resulted in significant morphological defects in fibrillar collagen and/or reduced collagen in many tissues. As such, dermis, heart, adipose tissue, and periodontal ligament of SPARC-null mice contain less fibrillar collagen compared with wild type (WT) [10, 19–21]. For example, abnormalities in the dermis were characterized by smaller collagen fibrils with a more uniform diameter and reduced tensile strength [19]. Interestingly, SPARC is able to bind fibrillar collagen I, III, and V as well as collagen type IV, the most abundant collagen in basal lamina with its COOH-terminal domain [22]. To add another level of complexity, proteolytic cleavage of SPARC into smaller fragments that produce diverse and sometimes opposing cellular responses is compared to naïve SPARC (Fig. 24.1b) [17, 23, 24]. As such, cleavage of a single peptide bond in the central module of SPARC by several MMPs (including MMP-2, MMP-3, MMP-7, and MMP-13) markedly increases its affinity for collagens [23]. MMP-3, on the other hand, generates several fragments that influence angiogenesis, whereas unprocessed SPARC plays an anti-angiogenic role (Fig. 24.1b) [24]. Nevertheless, the molecular mechanisms responsible for these functions of SPARC still remain largely unclear.

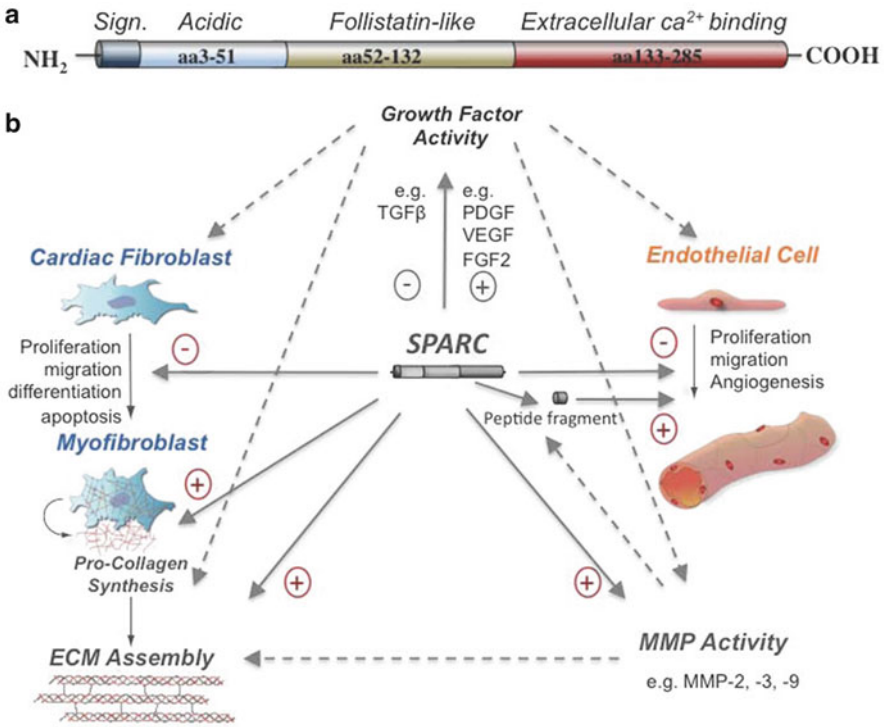

Fig. 24.1 SPARC, a pleiotropic matricellular protein in the heart. (**a**) After removal of the N-terminal signaling peptide (sign), the modular structure of SPARC contains (1) an acidic region, (2) a follistatin-like domain, and (3) a C-terminal extracellular Ca^{2+}-binding region (EC-module). (**b**) SPARC exhibits pleiotropic effects on (1) fibroblasts, (2) myofibroblasts, (3) extracellular matrix (ECM) assembly, (4) matrix metalloproteinase (MMP) activity, (5) endothelial cells, and (6) growth factor activity; all strongly involved in cardiac injury, remodeling, and repair. Moreover, proteolytic cleavage of SPARC (e.g., by MMPs) could result in peptide fragments that produce opposing biological actions. *TGF-β* transforming growth factor-β, *PDGF* platelet derived growth factor, *VEGF* vascular endothelial growth factor, *FGF2* fibroblast growth factor-2, + stimulates, – inhibits

24.3 SPARC and Myocardial Infarction

A growing body of evidence suggests a pivotal role for SPARC in a variety of cardiac pathophysiologic conditions, including aging-associated cardiomyopathy, myocardial infarction (MI), and pressure overload (Table 24.2) [10–13, 25]. Although this chapter will focus on its role during cardiac remodeling after MI, common biological features seem to be at play.

Table 24.2 SPARC in cardiac health and disease

		Phenotype in SPARC-deficient mice			
Mouse model	SPARC expression	Pathology	Cardiac function	Proposed mechanism	References
Cardiac homeostasis	High in embryonic heart; low in adult myocardium	Reduced collagen content, thinner collagen struts	Normal systolic function; decreased passive stiffness	Altered post-synthetic procollagen processing and matrix maturation	[10]
Cardiac aging	Markedly increased vs. young hearts	Reduced fibrillar collagen content and collagen cross-linking	Decreased diastolic stiffness	Impaired post-synthetic procollagen processing and decreased collagen content with aging of the heart	[10]
Myocardial infarction	Increased in early and late phase (myo)fibroblasts and leukocytes infiltrating the myocardium	Increased incidence of cardiac rupture (41% vs. 9%); increased myofibroblast infiltration, disorganized granulation tissue, and immature collagen matrix	Increased heart failure in surviving mice	Impaired collagen matrix maturation; decreased TGF-β signaling	[11]
			Attenuated early phase systolic dysfunction	Impaired fibroblast activation with differential expression of ECM and adhesion molecule genes	[12]
Pressure overload	Significantly increased 4 weeks after TAC	Decreased fibrillar collagen content and cross-linking	Decreased diastolic stiffness	Reduced post-synthetic procollagen processing and development of mature cross-linked collagen fibrils	[13]

SPARC secreted protein acidic and rich in cysteine, *TGF-β* transforming growth factor-β, *ECM* extracellular matrix, *TAC* transaortic constriction

24.3.1 Increased SPARC Expression Post-MI

Characteristic for matricellular proteins, expression of SPARC is low in the adult heart but upregulated during embryogenesis and in response to cardiac stress or injury (Table 24.2) [11, 12, 25]. Several studies using rat, canine, and mouse models of MI revealed a dramatic increase of SPARC in the infarcted LV as well as a moderate increase in the non-infarcted remote LV [11, 12, 25]. Interestingly, the spatial and temporal expression of SPARC in the infarcted heart closely paralleled that of collagens I and III deposition and ECM remodeling in those four species [11–13, 25, 26]. In mice, Schellings et al. [11] demonstrated a moderate ≈2-fold increase of SPARC protein in the infarcted and non-infarcted remote LV at 3 days after MI. At day 7 and 14 after MI, SPARC levels peaked in the infarct zone, whereas the initial increase in the non-infarcted remote LV was no longer detectable as compared to sham-operated hearts [11]. Concordantly, collagen transcription and deposition was detectable as early as day 3 and continued to rise at 7 and 14 days after MI. Further detailed analysis of infarcted mouse and canine hearts confirmed that SPARC primarily co-localized with infiltrating α-smooth muscle cell actin-positive myofibroblasts and CD45-postitive leukocytes in the infarcted LV [11, 25]. Thus, SPARC showed a localized deposition simultaneous with the formation of granulation tissue and progressive maturation of the infarct scar.

In human heart disease, a single study revealed elevated SPARC expression in cardiac tissue of patients with LV hypertrophy [27]. Based on previous animal studies, one can predict that SPARC expression will be elevated in myocardial tissue of patients with ischemic heart disease or age-related cardiomyopathy. Nevertheless, further detailed histopathological studies are mandatory and will be of great value to completely elucidate the biological and clinical relevance of SPARC expression in human heart disease.

24.3.2 Pathophysiologic Significance of Increased SPARC Expression Post-MI

The pathophysiologic relevance of increased SPARC expression in response to MI recently became clear when Schellings and colleagues (1) provided genetic evidence by subjecting SPARC-null mice to a mouse model of MI (Table 24.2) and (2) explored the effects of adenoviral-mediated overexpression of SPARC in WT mice after MI [11].

24.3.2.1 Physiologic Relevance of Increased SPARC Expression Post-MI

Surprisingly, SPARC-null mice displayed a fourfold increase in mortality post-MI, as compared to infarcted WT controls [11]. This mortality was mainly due to a

higher incidence of cardiac rupture (41% vs. 9%) and failure after MI (Table 24.2). Three days after MI, increased lung weight to body weight ratio indicated increased cardiac dysfunction in absence of SPARC. In fact, hemodynamic stress induced by dobutamine increased LV contractility and relaxation in infarcted female WT hearts but to a lesser extent in female SPARC-null hearts, 14 days post-MI. In order to translate these findings into a preclinical setting, Schellings and colleagues went on and overexpressed SPARC by systemic adenoviral delivery in WT mice [11]. Enhanced SPARC protein levels in WT mice markedly protected against cardiac dilatation and dysfunction without affecting infarct size at day 14 post-MI, as compared to control-treated mice. Thus, increased SPARC expression protects the heart against cardiac rupture and failure after MI.

In contrast, a recent study revealed that the absence of SPARC is associated with attenuated systolic dysfunction during the early stages—day 3—post-MI (Table 24.2) [12]. The authors propose that lack of SPARC might switch from a positive early to a negative late effect on cardiac remodeling, given that cardiac rupture occurred later. Nevertheless, differences in genetic background and age of the mice used in the experimental design might explain the discrepancies between these studies [11, 12]. Thus, further studies comparing the effects of SPARC deletion across species and ages are warranted.

24.3.2.2 Disorganized Granulation Tissue and Deficient Maturation of the Infarct Scar in Absence of SPARC

While inflammatory cell density and the angiogenic response were similar, increased cardiac rupture and dysfunction in SPARC-null mice post-MI appeared to coincide with disorganized granulation tissue as well as abnormal collagen maturation [11]. As discussed before, a well-organized ECM plays an important role to maintain the structural and functional integrity of the heart after MI [7, 9]. The absence of SPARC did not affect collagen synthesis. No significant differences were detected between both genotypes in the transcript levels of collagen I and III nor in Sirius red stained collagen deposited in the infarcted LV [11]. However, more in-depth analysis of the newly formed infarct scar revealed a disorganized and immature collagen matrix in absence of SPARC. Sirius red polarization and transmission electron microscopy revealed a shift to smaller, disorganized, and loosely assembled collagen fibrils in SPARC-null infarcts [11]. Adenoviral-mediated overexpression of SPARC in WT mice—on the other hand—significantly enhanced collagen quantity, and maturation in the infarct zone, was associated with increased transforming growth factor-β (TGF-β)/Smad2 signaling, without influencing the amount of leukocytes, myofibroblasts, and coronary vessels compared to control-treated mice at day 14 post-MI [11]. These findings are consistent with an array of studies indicating an essential role for SPARC in post-synthetic procollagen processing and assembly, ranging from (1) abnormalities in SPARC-deficient dermal collagen fibrils [19], (2) a decrease in mature cross-linked insoluble collagen fibrils in the heart in response to pressure overload [13], to (3) alterations in fibrillar collagen content and diastolic function in the aged myocardium in absence of SPARC (Table 24.2) [10].

24.4 SPARC and MI, Proposed Mechanisms

Although we cannot exclude the importance of other molecular mechanisms, evidence supports a protective role for SPAR—at least in part—mediated through enhanced TGF-β signaling and altered fibroblast kinetics post-MI. On the other hand, SPARC has been strongly implicated as an important regulator of extracellular post-synthetic procollagen processing in the heart, and this is independent of its effect on TGF-β signaling.

24.4.1 SPARC, Modulator of TGF-β Signaling and (Myo)fibroblast Kinetics Post-MI

TGF-β is a well-established yet complex paradigm in the regulation of cardiac remodeling post-MI (reviewed by Dobaczewski et al. [28]). SPARC had previously been indicated as a positive regulator of canonical TGF-β/Smad2 signaling in epithelial and primary kidney mesangial cells in vitro [29, 30]. Interestingly, Schellings et al. [11] were able to translate these findings to isolated cardiac fibroblasts, where binding of SPARC with the TGF-β/TGF-β receptors type II complex is hypothesized to act as a cofactor that enhances its downstream signaling. In addition, the protective in vivo effect noted upon SPARC overexpression was associated with increased canonical TGF-β/Smad2 signaling in the infarcted heart [11]. In contrast, no significant differences were observed in Smad2 activation in SPARC-deficient infarcts. Nonetheless, infusion of TGF-β in SPARC-null mice decreased rupture rates (14% vs. 67%) and improved extracellular collagen remodeling in the infarct scar [11]. Together, these findings provide strong evidence that SPARC communicates—at least in part—by influencing TGF-β signaling in the infarcted heart.

Moreover, several lines of evidence suggest that SPARC might influence TGF-β-dependent and TGF-β-independent pathways that control (myo)fibroblast kinetics post-MI, and this in a time- and space-dependent fashion. In line with the observed phenotype in SPARC-null mice, a recent study showed that early TGF-β inhibition increased mortality, adverse collagen remodeling, and exacerbated LV dilatation and dysfunction post-MI [31]. In contrast, TGF-β inhibition after the inflammatory phase decreased collagen deposition but was surprisingly associated with increased numbers of myofibroblasts in the infarct scar due to impaired apoptosis [32]. Increased myofibroblast numbers were also noted in SPARC-null infarcts 7 days post-MI, indicating that increased myofibroblast differentiation alone was not sufficient to drive increased collagen deposition [11]. On the other hand, we cannot rule out TGF-β-independent changes in cardiac fibroblasts lacking SPARC. In fact, an independent study recently revealed altered expression of 22 genes encoding ECM and adhesion molecules in cardiac fibroblasts isolated from SPARC-null hearts at baseline and from remote and infarcted LV 3 days post-MI compared to WT controls [12]. However, further detailed investigations are mandatory to fully unravel the molecular constituents of these findings.

Taken together, the above indicates that SPARC probably functions through TGF-β-dependent and TGF-β-independent pathways that control (myo)fibroblast kinetics post-MI. It will be interesting to investigate how these time-related effects potentially translate into a therapeutic setting.

24.4.2 SPARC, Major Modulator of Cardiac Collagen Assembly and Remodeling

Fibrillar collagen homeostasis is regulated by at least three regulatory control mechanisms: (1) procollagen biosynthesis, (2) post-synthetic procollagen processing, and (3) collagen degradation [15]. The balance between synthesis, processing, and degradation determines the total fibrillar collagen content of the heart, and the smallest alteration could be sufficient to influence how the heart structurally and functionally recovers after MI [7]. Interestingly, SPARC has been indicated as a potential switch for all three of these processes (reviewed by Bradshaw [15]). However, in the heart, multiple studies advocate a primary role for SPARC in post-synthetic procollagen processing and assembly in the stressed heart, including MI (Table 24.2 and Fig. 24.2) [10–12, 15, 19, 33].

24.4.2.1 Procollagen Biosynthesis

Cardiac fibroblasts produce the majority or fibrillar collagen, of which collagen I and III are the most abundant after MI [15]. The hypothesis that SPARC might be directly upstream of collagen expression emerged from the fact that SPARC expression consistently parallels that of fibrillar collagens in various adult tissues undergoing wound repair or fibrotic disease [3, 14]. In addition, a diminished capacity to produce a robust fibrotic response in various pathological mouse models had been identified in SPARC-null mice [15]. However, as previously discussed, the absence of SPARC did not appear to alter the transcription of collagen I and III nor did it affect the total amount of collagen deposition in response to MI compared with WT infarcts [11]. Furthermore, no significant differences were noted in the procollagen I production by SPARC-deficient primary isolated cardiac fibroblasts upon TGF-β stimulation [33]. In contrast, McCurdy et al. [12] did reveal reduced mRNA expression levels of collagen III and IV in cardiac fibroblasts isolated from SPARC-null hearts 3 days post-MI. However, pathway analysis indicated that collagen is probably indirectly regulated by SPARC in this setting.

Taken together, these studies do collectively indicate that SPARC is not likely to be the primary contributor of elevated procollagen biosynthesis in response to MI.

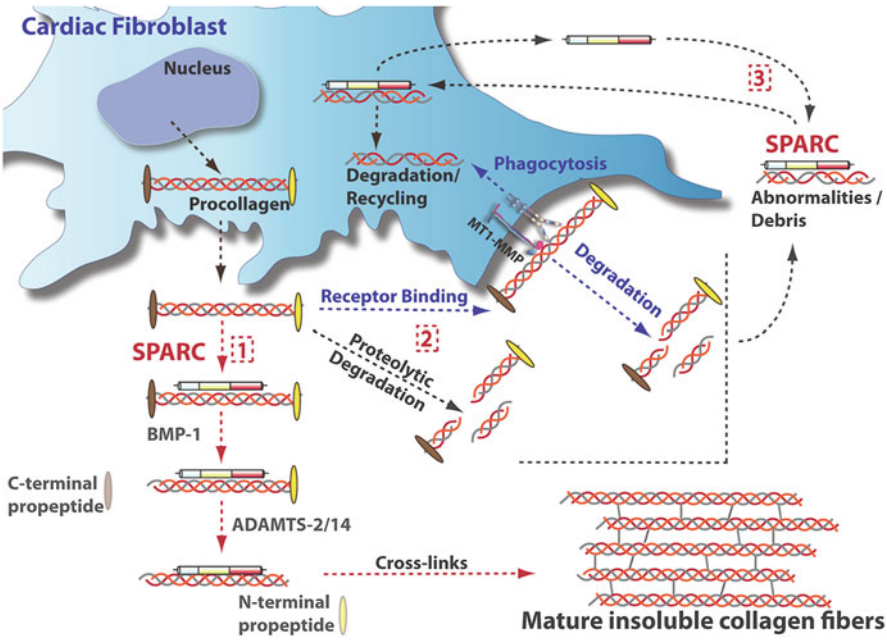

Fig. 24.2 SPARC in post-synthetic procollagen processing: "a working model" (1) SPARC binds to newly secreted procollagen and chaperones it through a series of ordered post-synthetic processing steps—including the removal of its C- and N-terminal propeptide by procollagen C- and N-terminal proteinases (e.g., BMP-1 and ADAMTS2/14, respectively)—and favors the formation of a mature cross-linked insoluble ECM. (2) SPARC thereby prevents proteolytic degradation and limits the association of procollagen with cell surface receptors that promote collagen turnover by phagocytosis or degradation by cell surface-associated collagenases (e.g., MT1-MMP). (3) In addition, SPARC might function as a scavenger chaperone that clears the extracellular space from ECM abnormalities and debris. *BMP* bone morphogenetic protein, *ADAMTS* a disintegrin-like and metalloproteinase domain with thrombospondin-type motif, *MT1-MMP* membrane type 1-matrix metalloproteinase

24.4.2.2 Post-Synthetic Procollagen Processing

After synthesis, procollagen is secreted into the extracellular space, where it must undergo a series of ordered, time- and location-sensitive post-synthetic processing steps and is predicted to have two major outcomes (Fig. 24.2) [15, 34]. Newly secreted procollagen either (1) is subjected to further processing within the extracellular space that favors the incorporation into a mature cross-linked insoluble ECM as collagen fibers or (2) associates with cell surface receptors that promote collagen turnover by phagocytosis or degradation by cell surface-associated collagenases.

The dramatic post-MI phenotype observed in absence of SPARC was in line with two recent studies that revealed impaired incorporation of cardiac fibrillar collagen

into the insoluble ECM in response to pressure overload or with cardiac aging in SPARC-deficient hearts (Table 24.2) [10, 11, 13]. On the other hand, adenoviral overexpression of SPARC in WT mice improved collagen processing and maturation in response to MI [11]. Using SPARC-deficient primary cardiac fibroblasts, Harris and colleagues revealed increased cell surface-associated collagen, in addition to enhanced processing of procollagen I to collagen I [33]. These findings are similar to those previously obtained with dermal fibroblasts, which were associated with impaired mechanical and functional properties of the dermis [35]. Moreover, addition of recombinant SPARC to SPARC-null cardiac fibroblasts reduced the association of collagen I with the cell surface demonstrating a partial rescue of the phenotype by addition of exogenous SPARC protein [33]. Notably, the SPARC-binding sites within collagen Ia1 and IIIa1 are identical to that of cardiac fibroblast specific collagen cell surface receptor discoidin domain receptor 2 (DDR2) [36–38]. Therefore, it is plausible that SPARC and DDR2 compete for collagen binding after cardiac injury. Although further research is mandatory, the latter could result in increased collagen binding to DDR2 that traps collagen at the cell surface in the absence of SPARC, thereby preventing proper post-synthetic collagen processing and ECM remodeling after MI. Enhancement of SPARC levels after MI, on the other hand, further limits collagen association with the cell surface and chaperones the collagen molecule through the time- and location-sensitive processing steps within the extracellular space. SPARC thereby enhances collagen deposition and the formation of a mature cross-linked insoluble collagen scar (Fig. 24.2).

24.4.2.3 Collagen Degradation

In the adult heart, as much as 92% of the newly synthesized collagen is immediately degraded before incorporation into the insoluble ECM [39, 40]. In addition, collagen turnover—ratio of degradation to synthesis—is a tightly regulated process and significantly increases in response to MI to insure proper scar formation. The balance between collagen-degrading MMPs and their tissue inhibitors TIMPs plays a central role in this process (reviewed by Vanhoutte et al. [6] and [41]). Interestingly, in vitro studies have suggested that SPARC acts to increase the activity of certain MMPs, notably MMP-2 and MMP-9 [42, 43], both of which are widely associated with cardiac rupture and adverse ventricular remodeling after MI [6]. In contrast, a single study recently showed that in baseline conditions, the LV of SPARC-null mice contains increased levels active MMP-3, an upstream activator of several MMPs [12, 44]. However, after MI, MMP-3 activity levels were reduced comparable to those in WT infarcts [12]. Thus, although SPARC is probably not a primary contributor to MMP activity post-MI, this matter remains of significant interest for future studies.

On the other hand, SPARC might fulfill a completely different function during extracellular collagen degradation. A recent finding suggested that SPARC might function as a novel class of matricellular scavenger chaperones that shuttles from the ECM into the cell and back (Fig. 24.2) [45]. In dermal fibroblasts, Chlenski and

colleagues demonstrated that at high extracellular Ca^{2+} concentrations, SPARC was able to bind multiple ECM targets (including collagen I and fibronectin), promotes the disassembly of solid networks, and directs the cellular internalization of their components [45]. At low intracellular Ca^{2+} concentrations, SPARC releases its target, and free SPARC is recycled outside the cell, serving as a shuttle that scavenges various structural and soluble ECM proteins. Whether SPARC functions as a scavenger chaperone in the stressed heart and whether this is directed through specific cell surface receptors remain intriguing questions. If so, this could imply that during the high rates of matrix turnover after MI, SPARC also functions as scavenger that clears the extracellular space from proteolytic ECM debris or abnormalities in the newly formed collagen matrix. The lack of SPARC would consequently result in premature termination of the growing extracellular collagen networks and an impaired ECM deposition [11]. Nevertheless, further understanding of this mechanism is necessary and may provide better understanding of matrix-associated abnormalities during cardiac homeostasis and disease.

24.4.2.4 SPARC, Collagen Assembly and Cardiac Remodeling: "A Working Model"

Putting previously described data together, increased expression of SPARC in response to MI—or cardiac stress in general—is very likely to bind with newly secreted procollagen, chaperones it through the processing steps within the extracellular space, and limits binding of procollagen to specific cell surface receptors that promote collagen degradation in the adult heart (Fig. 24.2). By diminishing procollagen engagement with these receptors, SPARC facilitates the optimal sequence and timing of post-synthetic processing steps and prevents procollagen from being prematurely degraded or improperly processed, thereby improving collagen deposition and maturation into well-organized and tightly cross-linked collagen fibers (Fig. 24.2) [15, 33, 35]. At the same time, SPARC might scavenge ECM debris and abnormalities within the collagen scar and clear them from the extracellular space (Fig. 24.2). Although further molecular and biochemical analysis of these mechanisms is mandatory, the regulation of SPARC and post-synthetic procollagen processing and degradation may provide important mechanism-based targets to improve matrix maturation and strengthening of the infarct scar within the first days after MI.

24.5 Conclusions

The cardiac ECM is a finely tuned and adaptable system that allows the heart to survive various pathological insults. Results obtained with SPARC-null mice have demonstrated that SPARC serves a critical role in post-synthetic collagen processing in the pericellular environment that facilitates efficient collagen incorporation

into the cardiac ECM. Furthermore, it underscores the necessity of well-organized extracellular collagen remodeling to preserve proper structure and function and to prevent cardiac rupture and dilatation after MI.

However, several key questions remain to be answered to push the field forward. For example, further analysis that unravels the functional basis of SPARC in the regulation of collagen assembly and remodeling may provide important mechanism-based targets to improve matrix maturation and strengthening of the infarct scar after MI. Moreover, further dissection of SPARC's multiple biological actions and identifying the distinct structural domains and pathways responsible could allow us to design peptides that either mimic a specific protective action or act as a dominant negative in the stressed heart, while limiting undesirable side effects. In this aspect, it will also be of considerable interest to fully investigate the cell-specific biological effects of SPARC, including its effects on cardiomyocyte function and the inflammatory process. Next, it will be of equal interest to identify the complete catalogue of SPARC-binding partners. To what level does proteolytic cleavage of SPARC co-determine its binding to specific cell surface receptors and other ECM-related proteins? And to what extent does this mediate the differential context-dependent functions of SPARC? In addition, it will also be exciting to see whether members of the SPARC-related proteins, including SMOC-1, SMOC-2, and Hevin [11], display similar functional roles in the infarcted heart.

Taken together, further in-depth studies will without a doubt bring novel and exciting perspectives, not only into the biology of SPARC but also to the field of matricellular proteins, collagen, and cardiac remodeling during cardiac homeostasis and disease.

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Chapter 25
Tissue Inhibitor of Matrix Metalloproteinases in the Pathogenesis of Heart Failure Syndromes

Dong Fan, Abhijit Takawale, and Zamaneh Kassiri

Abstract One of the characteristics of heart failure, regardless of its initial cause, is remodeling of the myocardium and the extracellular matrix (ECM). Disruption of the ECM results in structural instability as well as activation of a number of signaling pathways that could lead to fibrosis, hypertrophy, and apoptosis. The integrity of the ECM is maintained by a balance in the function of matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitor of metalloproteinases (TIMPs). An imbalance between the activity of MMPs and TIMPs in heart disease results in adverse outcomes. In addition to their MMP-dependent functions, TIMPs possess a number of MMP-independent functions. In this chapter, we will discuss the structure, functions, and regulation of TIMPs and their role in heart failure syndromes. We will review the knowledge that we have gained from clinical studies and animal models on the contribution of TIMPs in the development and progression of heart disease.

Keywords Heart disease • Animal models • Cardiac extracellular matrix • Matrix biomarkers • Matrix metalloproteinase • Tissue inhibitor of metalloproteinases

Abbreviations

ADAM	A disintegrin and matrix metalloproteinase
CITP	Carboxy-terminal telopeptide of collagen type I
DCM	Dilated cardiomyopathy
ECM	Extracellular matrix

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FGF	Fibroblast growth factor
HB-EGF	Heparin-bound epidermal growth factor
IL	Interleukin
LV	Left ventricle
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MT-MMP	Membrane-type MMP
PDGF	Platelet-derived growth factor
PICP	Procollagen type I carboxy-terminal propeptide
PIIINP	Procollagen type III amino-terminal propeptide
PINP	Procollagen type I amino-terminal propeptides
TGFβ	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinases
TNFα	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor
WT	Wild type

25.1 Introduction

Cardiovascular disease remains a major cause of morbidity and mortality [1]. Following an injury, the heart undergoes structural remodeling which eventually leads to maladaptive remodeling and cardiac dysfunction. The relationship among the cardiomyocytes, fibroblasts, and blood vessels is coordinated by the extracellular matrix (ECM). Cardiac ECM is connected to cardiomyocytes through a collagen–integrin–cytoskeleton–myofibril relationship (Fig. 25.1). As heart disease progresses, cardiomyocytes can undergo eccentric or concentric hypertrophy, and fibroblasts can migrate and propagate to trigger interstitial fibrosis, while the ECM can be disrupted resulting in destabilization of the vasculature and myofibrillar architecture, leading to ventricular dilation.

Matrix metalloproteinases (MMPs) are the predominant proteases responsible for degradation of the ECM proteins, the first step in ECM turnover. MMPs are Ca^{2+}- and Zn^{2+}-dependent proteases that are synthesized as inactive zymogens (pro-MMPs) and can be activated by the removal of an amino-terminal propeptide domain either by autoproteolysis or via processing by another MMP or serine protease. Activated MMPs can be inhibited by their physiological MMP inhibitors, including the tissue inhibitor of metalloproteinases (TIMPs), α2-macroglobulin, and RECK (reversion-inducing cysteine-rich protein with kazal motif) [2]. TIMPs are specific MMP inhibitors in the tissue compartment and can reversibly inhibit activated MMPs, whereas α2-macroglobulin irreversibly clears MMPs by forming a complex endocytosis only in plasma and in liquid compartments with minimal inhibitory function in the tissue [3]. Finally, RECK is a cell surface protein with MMP inhibitory capacity, which proposed a role in endothelial migration and angiogenesis since mice lacking RECK do not survive beyond day 10 of the gestation (E10.5) [4].

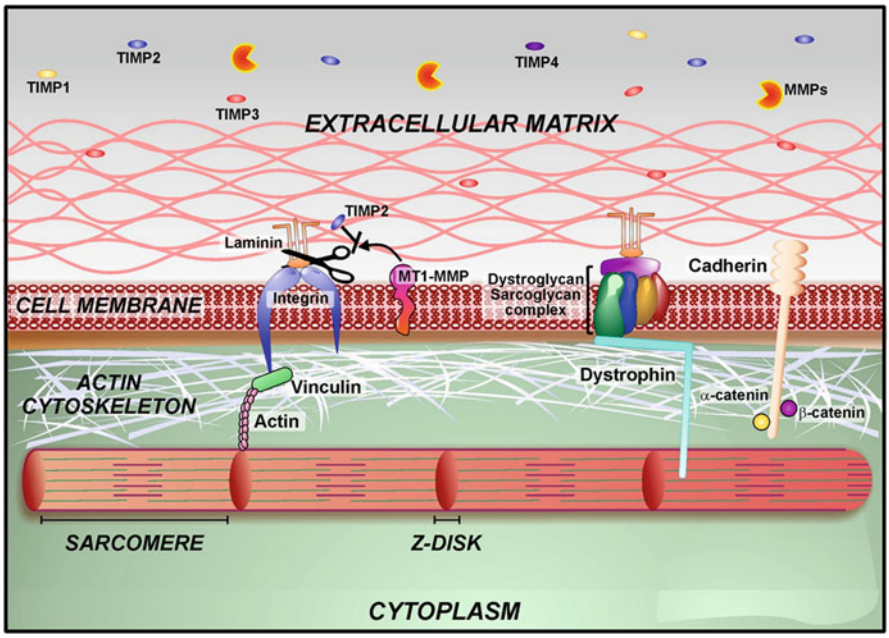

Fig. 25.1 Schematic illustration of the organization of the extracellular matrix and cardiomyocytes and localization of the TIMPs and MMPs

However, baseline expression of RECK in the heart is minimal [5], and its role in cardiac function has not yet been identified. Among these MMP inhibitors, TIMPs have been shown to be the most potent physiological inhibitors of MMPs. The role of the MMPs in heart disease has been extensively studied and reviewed [6–8]. In this chapter, we will focus on the contribution of TIMPs in development and progression of heart failure syndromes.

25.2 Cardiac Extracellular Matrix

Cardiac ECM is the supporting network that provides architectural integrity to adjoining myocytes and the neighboring vessels and capillaries and translates single myocyte contractility into a ventricular syncytium. It is comprised of highly organized protein structures, predominantly collagen type I and type III, and basement membrane components including laminin, fibronectin, collagen type IV, and fibrillin. This structural arrangement prevents myocyte slippage and overstretching and contributes to cardiac recoil during diastole.

In the myocardium, the ECM is connected to the cell membrane and to the myocytes via a number of structures. The ECM maintains alignment of myofibrils within

the myocyte through a collagen–integrin–cytoskeleton–myofibril relationship (Fig. 25.1). Integrins link the ECM to the cellular cytoskeleton [9, 10]. An integrin-mediated insertion into the basal lamina localized to the Z-band of the sarcomere forms direct connections between adjacent cardiomyocytes [11]. The dystroglycan complex also works as a transmembrane linkage between the ECM and the cytoskeleton, thereby providing structural stability for the myocardium. In the myocardium, the ECM is connected to the cell membrane and to the myocytes via a number of structures. Integrins are transmembrane heterodimers comprised of alpha (α) and beta (β) subunits that link the ECM to the cellular cytoskeleton [9]. Cadherins are a large family of single-pass transmembrane proteins principally involved in Ca^{2+}-dependent homotypic cell adhesion and bind to the cytoskeleton via α-catenin and β-catenin. Cadherin is essential for optimal cardiac function and remodeling in disease [12], while increased membrane translocation of β-catenin has been shown in patients with hypertrophic cardiomyopathy [13]. Figure 25.1 shows a schematic of the arrangement and assembly of the ECM, cell membrane, and the sarcomeres.

25.3 Biology of TIMPs

TIMPs are 21–30 kDa proteins that share substantial sequence homology and structural identity. TIMPs possess 12 conserved cysteine residues forming six disulfide bonds that fold the protein into two domains each containing three loops, an MMP-inhibiting N-terminal domain (loops 1–3) and a C-terminal domain (loops 4–6) [14]. Disulfide bonds at 12 strictly conserved Cys residues maintain the six loop structures. Four TIMPs have been identified and cloned in vertebrates, TIMP1, TIMP2, TIMP3, and TIMP4. TIMPs can be present in glycosylated and unglycosylated forms [15]. Glycosylation results in an increase in the molecular weight of TIMPs but has not been shown to alter their MMP inhibitory function [16, 17]. TIMP1, but not TIMP2, has two N-linked glycosylation sites which are important in the correct folding and enhanced stability of TIMP1 [17]. TIMP3 has also been shown to be *N*-glycosylated [15]; however, this glycosylation is not required for MMP inhibition or ECM binding of this TIMP [16]. Glycosylation of TIMP4 occurs by O-linked glycosylation [18], but no report has been made on the effect of glycosylation on the inhibitory capacity of TIMP4 [19].

Classically, TIMP1, TIMP2, and TIMP4 are known to be localized extracellularly in soluble form while TIMP3 is tightly bound to ECM via the interaction of its COOH-terminal with sulfated glycosaminoglycans [16, 20]. However, a number of recent studies have provided new insights into this dogma. An in vitro study has shown that TIMP3 could also be present in the extracellular space in soluble form [21], while TIMP4 has been reported to be localized intracellularly along the thin myofilaments of sarcomeres in rat cardiomyocytes [22]. Hence, the localization of TIMPs may not be as restricted as originally believed.

In the healthy adult murine heart, TIMP2 and TIMP3 mRNA are most abundant TIMPs followed by TIMP4 and TIMP1. Although TIMP4 expression was originally

believed to be restricted to the heart and the brain [5], it has become increasingly evident that TIMP4 is in fact expressed in a number of organs and tissues including kidney, lung, pancreas, colon, and testes [23, 24]. The robust expression of TIMP4 in healthy cardiomyocytes could have given rise to the original misconception.

TIMPs are inducible proteins. Expression of TIMP1 can be induced by growth factors (b-FGF, PDGF, EGF), phorbol esters, serum, cytokines (IL-6, IL-1, and IL-1β) [25], and erythropoietin [26]. Although TIMP2 was initially believed to be constitutively expressed [25], induction of TIMP2 mRNA and protein has been reported in myocardial tissue from heart failure patients [27–29], as well as in animal models of heart disease [30] and kidney disease [31, 32]. TIMP3 can be transcriptionally induced by TGFβ [33], IL-1β [34], TNFα [35], and reactive oxygen species [33]. TIMP1, TIMP2 [36, 37], and TIMP3 [38] have been shown to be transcriptionally upregulated by cAMP. Transcriptional regulation of TIMP4 has been less explored.

25.4 MMP-Dependent Functions of TIMPs

TIMPs reversibly inhibit the proteolytic activity of activated MMPs by forming non-covalent 1:1 stoichiometric complexes that are resistant to heat denaturation and proteolytic degradation [39]. The inhibitory function of TIMPs occurs through an interaction between the Zn^{2+} of the MMP active site and the N-terminal cysteine residues of the TIMP, particularly the cys1-cys70 disulfide bonds [17]. Although there is a degree of overlap among TIMPs in their inhibitory functions, each TIMP possesses unique properties and inhibits MMPs with different specificity and affinity. TIMP1 is a prototypic inhibitor for most MMPs with a selective preference to bind to and inhibit MMP9 [40] but a poor inhibitor of the membrane-type MMPs (MT-MMPs) and MMP19 [25]. TIMP2 is unique in that in addition to inhibiting MMPs, it mediates activation of MMP2 by selectively interacting with membrane type 1 (MT1)-MMP to facilitate the cell surface activation of pro-MMP2 [41]. One MT1-MMP acts as an anchor and binds to TIMP2, which then recruits pro-MMP2 forming a trimolecular complex: MT1-MMP/TIMP2/pro-MMP2. A second MT1-MMP molecule acts as an activator and cleaves pro-MMP2 first to its intermediate form (64 kDa) and then to its fully active form (62 kDa) [42]. While low levels of TIMP2 promote this cell surface activation of pro-MMP2, high levels of this TIMP prevent this activation process by inhibiting the second MT1-MMP. Pro-MMP2 also forms a complex with TIMP3 or TIMP4 in a similar manner, but not TIMP1. The pro-MMP2–TIMP4 complex interacts with MT1-MMP but does not result in MMP2 activation [43], while TIMP3 inhibits the pro-MMP2 activation in this process [21]. TIMP2 and TIMP4 have high potency against MMP2 and MT1-MMP [30, 44, 45], while TIMP3 can inhibit a broad spectrum of MMPs, MT-MMPs, and ADAMs [46, 47].

Among the ADAMs inhibited by TIMP3, ADAM12 [48] and ADAM17/TACE (TNF-alpha-converting enzyme) [49, 50] are of particular relevance to heart failure.

ADAM12 plays an integral role in the hypertrophic response upon stimulation of G-protein-coupled receptors (GPCRs) through cleavage of membrane-bound HB-EGF and subsequent transactivation of the epidermal growth factor receptor (EGFR) [51]. TACE is a potent sheddase, and its presence is required for shedding of TNFα [52] and TGFα [53]. TIMP3 deficiency led to enhanced TACE activity and elevated TNFα processing in pressure-overloaded cardiomyopathy [54], as well as in other diseases [32, 55]. TIMPs are stoichiometrically outnumbered by MMPs, but by targeting a broad spectrum of MMPs, they compensate for this numerical mismatch. In addition, MMP activities are also regulated at the transcription and activation steps [35]; thereby, a tight regulation of the proteolysis events is maintained in health.

25.5 MMP-Independent Functions of TIMPs

TIMPs possess a signaling capacity distinct from their MMP inhibitory activity. TIMP1 can interact with CD63, a member of the tetraspanin family of proteins [56]. TIMP2 can bind to integrin α3β1 [57] whereby it mediates anti-angiogenic effects by dephosphorylating the activated VEGF receptor and transforming differentiating endothelial cells into a quiescent state (Fig. 25.2) [58]. TIMP2 can also regulate neuromuscular junction development in fast-twitch muscles through transcriptional regulation of integrin β1 [59]. In vitro studies have shown that TIMP3 can exert anti-angiogenic effects by interrupting the interaction between VEGF and VEGF receptor 2 in endothelial cells (Fig. 25.3) [60]. TIMP3$^{-/-}$ mice subjected to myocardial

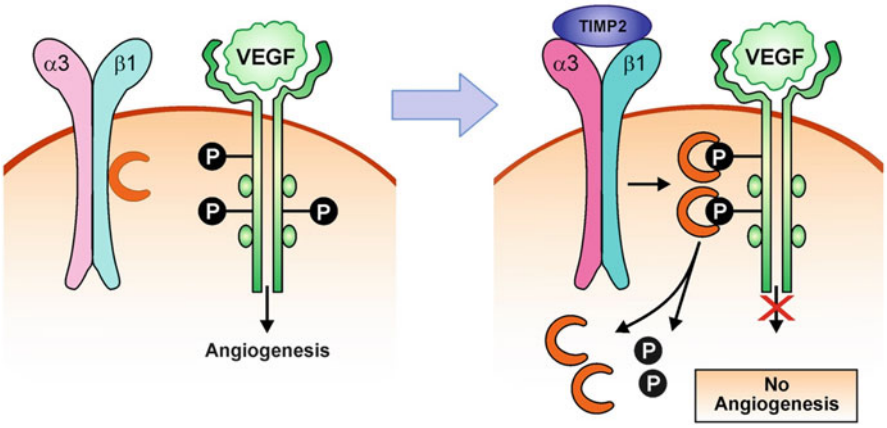

Fig. 25.2 TIMP2 and angiogenesis. TIMP2 binds to integrin α3β1 and promotes dephosphorylation of the activated VEGF receptor, thereby interrupting the VEGF-induced signaling and angiogenesis

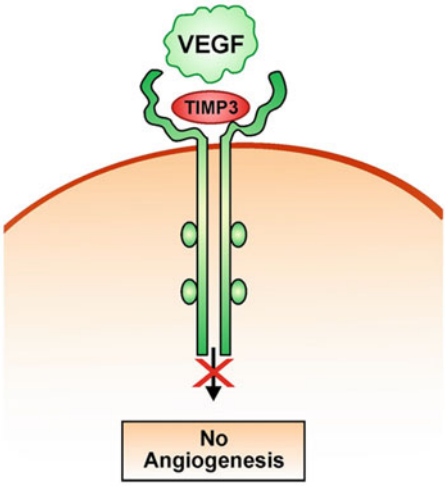

Fig. 25.3 TIMP3 and angiogenesis. TIMP3 disrupts the interaction between VEGF and VEGF receptor 2 in endothelial cells thereby inhibiting angiogenesis

infarction were reported to have enhanced angiogenesis [61], which one would predict to be beneficial to the ischemic cardiomyocytes and to limit the expansion of the infarct scar and improve cardiac function. However, TIMP3$^{-/-}$-MI mice exhibited markedly higher rates of LV rupture, greater infarct expansion, and more severe LV dilation and dysfunction [62]. In addition, TIMP3$^{-/-}$ mice generally do poorly when subjected to different diseases [32, 54, 55, 63, 64]. Hence, the role of TIMP3 in angiogenesis and the subsequent beneficial outcomes may not be simply explained by its presence or absence and certainly needs to be addressed more systematically in vivo.

A number of in vitro studies indicate that TIMPs also possess cell growth-promoting properties. Adenoviral overexpression of all TIMPs induced fibroblast proliferation, while Ad-TIMP2 additionally increased collagen synthesis, and TIMP3 triggered apoptosis [65]. TIMP1 promoted proliferation of different cell types including fibroblasts and endothelial cells [66], whereas TIMP2 promoted fibroblast growth [67] but hindered endothelial cell proliferation [68]. Growth-promoting function of TIMP1 and TIMP2 appears to be independent of their MMP-inhibiting function since reductively alkylated TIMPs, which lack MMP inhibitory activity, maintain their cell growth-promoting activity [69]. TIMP3 stimulated growth of chick embryo [70] and cardiac fibroblast [65] in addition to apoptosis in the latter group [65], whereas TIMP3 deficiency promoted proliferation of cultured neonatal mouse cardiomyocytes [71]. TIMP4 inhibited growth of tumor cells [72] and induced apoptosis in transformed fibroblasts [73]. Overall, these studies indicate that the growth-related function of TIMPs is specific to tissue and cell type and proposes potential key roles for TIMPs in cardiac repair and wound healing process which need to be further explored.

25.6 Remodeling of Myocardial ECM in Heart Failure

Maladaptive myocardial remodeling is characterized by an overall imbalance in ECM turnover, which can result in excess accumulation or disruption of the ECM structural proteins, mainly collagens. This will lead to altered spatial orientation of cells and the vasculature, diminished systolic performance, decreased compliance, and diastolic dysfunction in failing hearts. The turnover rate for collagen is about tenfold slower than non-collagen proteins [74]; thus, the myocardium is vulnerable to aberrant remodeling in conditions associated with increased ECM degradation.

Procollagen type I carboxy-terminal propeptide (PICP) and amino-terminal propeptides (PINP) and those of type III collagen peptide (PIIICP and PIIINP) are released with biosynthesis of collagen type I or type III in a stoichiometric manner and hence are considered as biomarkers of collagen synthesis. However, CITP and CIIITP, carboxy-terminal telopeptides of collagen type I and type III, are produced when collagen type I or type III are degraded and as such are considered as biomarkers of collagen degradation [75].

In a Caucasian population of hypertensive patients, the plasma levels of markers of ECM collagen turnover (CITP, PICP, PIIINP) and MMP2 were predictors of diastolic dysfunction and heart failure with preserved ejection fraction [76]. The serum PICP/CITP levels were higher in hypertensive patients [77] and in patients with dilated cardiomyopathy [78] who also exhibited increased collagen accumulation in the myocardium. Although early degradation of collagen type III has been associated with adverse LV dysfunction in later stages of the disease following MI [79], a recent study reported that in patients with acute MI, CITP was the strongest marker of outcomes among other collagen turnover markers including BNP, as a predictor of heart failure development and all cause death in these patients [80]. This is not unpredictable since MI and the subsequent "wound healing" of the myocardial tissue involve a great extent of ECM turnover. Immediately after MI, the existing ECM structure is degraded while normal collagen struts are lost, and cross-linking within the ischemic myocardium is reduced. This collagen turnover is accompanied by increased protease activity and increased probability of left ventricular rupture [81]. The degraded ECM structure allows for infiltration of inflammatory cells consistent with early elevations in MMP8 and MMP9 levels, the MMPs produced by neutrophils, in patients with MI [82]. This further leads to proliferation and maturation of fibroblasts, which synthesize new collagens and result in matrix deposition and scar formation. During the later phase of post-MI remodeling, an inefficient support of the newly formed ECM within the infarct and peri-infarct region will lead to LV wall thinning and slippage of the surviving myocytes resulting in further infarct expansion, LV dilation and dysfunction, and eventually heart failure. In addition, insufficient or impaired ECM remodeling can lead to LV rupture which has recently been shown to account for a high proportion of sudden death early post-MI [83, 84]. This is in agreement with the findings in experimental models of MI [30, 62, 85]. Hence, early prevention of adverse ECM remodeling in the infarct and the surrounding myocardium is crucial in preventing rupture and preserving cardiac function post-MI.

25.7 Alterations in TIMP Levels Have Been Linked to Different Phases of Heart Failure

A number of studies have examined the TIMP levels in plasma or the myocardium from patients with different types of heart disease (Table 25.1). Following acute myocardial infarction (AMI), plasma TIMP1 and MMP9 levels correlated directly with LV dysfunction and remodeling [86], while analysis of other TIMPs in AMI patients revealed elevated TIMP1, TIMP2, and TIMP4 levels by day 4 post-AMI which correlated positively with occurrence of major adverse cardiac events in these patients [88]. Interestingly, temporal changes in plasma MMPs and TIMP levels following myocardial infarction showed a rapid and sustained increase in MMP9 and MMP8, with a delayed increase in TIMP2 and TIMP4 levels [82]. This initial rise in proteinase activities was identified as the underlying cause of the adverse remodeling at later stages of disease progression since it was not blocked by a matching rise in TIMPs. These studies highlight the time-sensitive alterations in TIMP and MMP levels which should be considered when developing TIMP-dependent therapies for post-MI patients. In patients with ischemic or idiopathic dilated cardiomyopathy (DCM), myocardial protein levels of TIMP1, TIMP3, and TIMP4 were reduced while TIMP2 levels remained unaltered [73, 89], although another study reported a marked increase in TIMP2 levels in end-stage DCM patients [90]. Implantation of an LV assist device (LVAD) in DCM patients reduced MMP1 and MMP9 and increased TIMP1 and TIMP3 levels [91]. In patients with aortic stenosis, TIMP1 and TIMP2 levels were significantly elevated in the LV myocardium which directly correlated with the degree of fibrosis [27], while another study further reported increased TIMP3 and unaltered TIMP4 levels in similar patients [28].

Circulating levels of TIMP2 have also been correlated with systolic function in patients with hypertrophic cardiomyopathy as it was elevated in patients with systolic dysfunction but not in those with preserved systolic function, whereas TIMP1 levels were elevated in both groups [92]. Increased plasma TIMP1 levels have been correlated with myocardial hypertrophy [93], fibrosis, and diastolic dysfunction in hypertensive patients [94]. In addition to inhibiting MMPs, TIMP1 has been proposed to promote fibrosis by inducing collagen synthesis [93, 94]. Consistently, in patients with atrial fibrillation, collagen content in the atria correlated positively with elevated levels of TIMP1 [95]. TIMP1 levels are also increased in patients with cardiac amyloidosis, characterized by amyloid infiltration and ECM disruption, which correlated with diastolic dysfunction, but not with LV wall thickness [96]. TIMP1 levels were also significantly elevated in the myocardium of patients with progressive heart failure [97] and idiopathic dilated cardiomyopathy [90], although an earlier study had reported a reduction in TIMP1 levels in hearts with dilated cardiomyopathy [98]. In the Framingham heart study [99], a thorough analysis of plasma TIMP1 levels in relation to multiple cardiovascular risk factors revealed that in patients without heart failure or MI, plasma TIMP1 level was directly related to smoking, diabetes, and use of hypertensive treatment. In addition, TIMP1 was

Table 25.1 Alterations in TIMPs, MMPs, and ECM turnover markers in patients with different heart failure syndromes

Disease	Type of sample	Observations	References
Hypertensive with heart failure with preserved ejection fraction	Plasma	↑CITP, ↑PICP, ↑PIIINP ↔ TIMP1, ↔MMP1, ↑MMP2	Martos et al. [76]
Hypertensive with heart failure with preserved ejection fraction and ↑ filling pressure	Plasma	↑ TIMP1	Gonzalez et al. [100]
Hypertensive with increased myocardial collagen accumulation	Plasma	↑ PICP/CITP	Diez et al. [77]
Hypertensive with myocardial fibrosis and diastolic dysfunction	Plasma	↑ TIMP1	Lindsay et al. [94]
Hypertensive with LV hypertrophy	Plasma	↑ TIMP1, ↔ PIIINP	Timms et al. [93]
Myocardial infarction	Plasma	↑ CITP	Manhenke et al. [80]
		↑ TIMP1, ↑MMP9	Kelly et al. [86]
		↑ MMP3	Kelly et al. [87]
		↑ TIMP1, ↑ TIMP2, ↑ TIMP4	Kelly et al. [88]
Myocardial infarction	Plasma	↑TIMP1, ↑MMP8, ↑MMP2 (early) ↑TIMP1, ↑TIMP2, ↑TIMP4 (delayed)	Webb et al. [82]
Ischemic cardiomyopathy	Myocardium (protein)	↓ TIMP1, ↓TIMP3, ↓TIMP4 ↔ TIMP2	Li et al. [89]
Ischemic/idiopathic DCM	Myocardium (protein)	↓ TIMP4	Tummalapalli et al. [73]
End-stage DCM	Myocardium (protein)	↑↑ TIMP1, ↑↑TIMP2 ↓MMP1, ↔MMP2, ↑MMP3, ↑MMP9	Thomas et al. [90]
Aortic stenosis (pressure overload)	Myocardium (LV)	↑TIMP1, ↑TIMP2	Heymans et al. [27]
Aortic stenosis	Myocardium (mRNA and protein)	↓TIMP1, ↑TIMP2, ↑TIMP3, TIMP4(↓mRNA, ↑protein), ↓MMP1, ↓MMP9, ↑MMP2, ↑MMP3	Fielitz et al. [28]

Hypertrophic cardiomyopathy: 1. Systolic dysfunction 2. Preserved systolic function	Plasma	1. ↑TIMP1, ↑TIMP2, ↑MMP2,↔MMP3, ↔MMP9 2. ↔TIMP1, ↔ TIMP2, ↔MMP2	Noji et al. [92]
Cardiac amyloidosis (diastolic dysfunction, no hypertrophy)	Serum	↑ TIMP1, ↑MMP2	Biolo et al. [96]
Atrial fibrillation	Atrial tissue	↑ TIMP1, ↑ TIMP3	Mukherjee et al. [95]
Progressive heart failure	Myocardium	↑TIMP1	Barton et al. [97]
Chronic pressure overload 1. Predictor of hypertrophy 2. Predictor of diastolic HF	Plasma	1. ↑ TIMP1, ↑ MMP7, ↑ MMP9 2. ↑MMP2, ↑TIMP4, ↓ MMP8	Zile et al. [101]

positively associated with echocardiographic parameters of LV structural remodeling (LV mass, wall thickness, end-systolic and left atrial diameters) and negatively correlated with fractional shortening, a measure of LV function [99]. In hypertensive patients with heart failure with normal ejection fraction and elevated filling pressures, elevated TIMP1 levels were proposed to prevent collagen degradation, facilitating myocardial fibrosis and thereby contributing to the elevated filling pressure in these patients [100]. The rise in TIMP1 levels, irrespective of the type of heart disease, suggests that TIMP1 is likely a disease marker, and as such, targeting TIMP1 in treating heart disease may not provide significant therapeutic benefits. A gender dependence in TIMP levels has also been reported. Among patients with no heart failure or myocardial infarction, plasma TIMP1 levels were higher in men compared to women (other TIMPs were not examined) [99], whereas in AMI patients, plasma levels of TIMP1, TIMP2, and TIMP4 were elevated more in women versus men [88]. However, this difference in TIMP levels was not linked to the gender-dependent severity of heart disease.

A recent study on patients with chronic pressure overload demonstrated that increased plasma levels of TIMP1, MMP7, and MMP9 correlated with adverse ECM remodeling, while increased MMP2 and TIMP4, with decreased MMP8, best correlated with diastolic heart failure [101]. This study proposed that a combination of plasma markers of changes in ECM fibrillar collagen homeostasis as well as MMPs and TIMPs are indeed most suitable in identifying cardiac remodeling such as LV hypertrophy and diastolic heart failure [101]. While developing a multi-biomarker approach could be a more useful tool in identifying the specific aspects of cardiac remodeling and disease, identifying the alterations in TIMP levels, preferably in early stages of heart disease, could offer the opportunity to restore the imbalance in the TIMP–MMP interaction by delivering the identified TIMP(s) systemically or to the site of injury.

25.8 Animal Models Have Indicated Involvement of TIMPs in Heart Disease Syndromes

In addition to alterations in ECM regulatory proteins in patients with heart disease (discussed above), numerous studies have reported altered levels and activities of TIMPs and MMPs in different experimental models of heart disease leading to heart failure [6, 7, 74, 102]. The causal role of TIMPs in development and progression of heart disease is best demonstrated by using genetically modified mouse models with altered TIMP levels [103].

Deletion of TIMP1 gene resulted in a modest LV dilation in mice at 4 months of age, while LV systolic pressure and ejection fraction were preserved, and no myocyte hypertrophy was detected [104]. Myocardial infarction in TIMP1$^{-/-}$ mice led to greater LV dilation and increased LV end-diastolic volume compared to parallel wild-type (WT) mice [105], which were prevented with MMP inhibition [106]. This indicates that the MMP inhibitory function of TIMP1 is required for cardiac recovery from MI.

TIMP2 deficiency abolished activation of pro-MMP2 to its cleaved form in the fibroblast [107] and myocardium [30] in mice. While TIMP2$^{-/-}$ mice do not exhibit any baseline alteration in cardiac structure or function, myocardial infarction in TIMP2$^{-/-}$ mice led to greater infarct expansion but a similar rate of LV rupture incident compared to WT mice. TIMP2$^{-/-}$-MI mice exhibited exacerbated LV dilation with systolic and diastolic dysfunction, associated with elevated MT1-MMP activity [30]. Recently, we reported that biomechanical stress in mice lacking TIMP2 resulted in nonuniform ECM remodeling, integrin degradation, and impaired myocyte–ECM interaction leading to more severe LV dilation and dysfunction [108].

A recent study by Ramani et al. has reported that overexpression of TIMP2 in the peri-infarct myocardium reduced the infarct expansion and improved LV dilation and dysfunction [109]. This study is the first to examine the impact of TIMP overexpression in myocardial infarction, and it is intriguing to investigate if overexpression of individual TIMPs will impact different aspects of cardiac remodeling post-MI.

Mice lacking TIMP3 have been most extensively studied among the TIMP-deficient mice. Lack of TIMP3 led to dilated cardiomyopathy in aged mice (21-month-old) primarily due to elevated MMP9 levels [110], while young TIMP3$^{-/-}$ mice were found to be more susceptible to heart disease. Cardiac pressure overload in TIMP3$^{-/-}$ mice resulted in exacerbated LV dilation and dysfunction, excess hypertrophy, and fibrosis, with early onset of heart failure and increased mortality [54, 111]. This severe phenotype was due to an early rise in expression of tumor necrosis factor-α (TNFα) followed by elevated proteolytic activities (mostly MMP2 and MT1-MMP). Interruption of both these pathways was required for complete prevention of the disease in TIMP3$^{-/-}$ mice [54]. Pressure overload in the face of TIMP3 deficiency also disrupted an innate interaction between two key cytokines in heart disease, TNFα and TGFβ, which lead to excess interstitial and focal fibrotic lesions in the myocardium [111]. TIMP3$^{-/-}$ mice are also more susceptible to adverse complications of myocardial infarction [61, 62]. Post-MI survival was markedly compromised in TIMP3$^{-/-}$ mice primarily due to increased rate of LV rupture, between 3 days and 7 days post-MI, concomitant with an early and transient elevation in proteolytic activities [62]. Early inhibition of MMPs, during the first 2 days post-MI, was sufficient to significantly improve the post-MI outcomes in these mice at later stages [62].

TIMP4 deficiency did not impact the cardiac remodeling or dysfunction following pressure overload, while myocardial infarction leads to enhanced rate of LV rupture with unaltered LV dilation and dysfunction compared to WT-MI mice [112]. This is particularly interesting since TIMP4 was initially referred to as the "heart TIMP," and yet the impact of its absence on cardiac response to disease is quite subtle compared to other TIMPs. It is noteworthy that while TIMP4 may show a tissue-specific expression pattern in heart and brain, total levels of TIMP4 in the heart are in fact lower than TIMP2 and TIMP3 as reported in mice [5]. This could explain the lack of severe outcomes of TIMP4 deficiency in cardiac structure and function and its response to disease.

25.9 TIMP Deficiency and Cardiac Inflammation

Inflammation is one of the key characteristics of cardiac ischemic injury or infarction. High neutrophil count correlates with a high incidence of adverse cardiac events in patients with acute MI [113, 114]. In mice lacking TIMP2 [30], TIMP3 [62], or TIMP4 [112], myocardial infarction was accompanied by severe inflammation in the infarct and peri-infarct myocardium of mice. Migration of inflammatory cells into the damaged myocardium is triggered by a number of factors including the ECM degradation products [115–118] generated by the action of MMPs, which would be enhanced in the absence of the inhibitory function of TIMPs. ECM degradation products can act as chemoattractants for inflammatory cells. Fragments of fibronectin [115, 119] and elastin [116, 120] trigger migration of monocytes and neutrophils to the site of injury. Post-MI formation of laminin and fibronectin degradation products correlated with macrophage infiltrations in the infarcted myocardium [117], while mice lacking MMP2 showed less ECM degradation and less inflammation post-MI [117]. Neutrophils generate MMP8, MMP9 [121, 122], and MT6-MMP(MMP25) [123], while macrophages produce a number of MMPs, including MMP1, MMP3, MMP7, MMP8, MMP9, and MMP12 [124]. Hence, recruitment of inflammatory cells to the damaged myocardium can further exacerbate the injury. TIMP3 has also been reported to constrain neutrophil influx in the lungs by inhibiting MMPs [63] and to impact inflammation through regulating the TNFα-mediated signaling [55]. MMP-independent function of TIMPs in regulating inflammation has also been suggested, but no mechanism has been proposed [125].

Overall, mice deficient in specific TIMPs have helped us identify distinct roles for each TIMP in different types of heart disease. While TIMP3 seems to be critical in LV rupture and dysfunction following MI, TIMP2 deficiency only impacted the cardiac function while lack of TIMP4 only increased the rate of LV rupture. Further, TIMP1 and TIMP4 do not appear to play a critical role in cardiac response to pressure overload, whereas TIMP3 is required for optimal structure and function following biomechanical stress, while role of TIMP2 in this process remains undetermined.

25.10 Conclusions

TIMPs contribute to multiple aspects of cardiac remodeling and homeostasis and cardiac function. Alterations in TIMP levels in heart disease patients can be variable, depending on the type and stage of cardiomyopathy, which could pose the question if a reduction or an increase in their levels is beneficial or detrimental. TIMP-deficient mice have provided an invaluable tool in evaluating the causal role of each TIMP in different models of heart disease, clearly demonstrating that TIMPs are required for an optimal cardiac response to disease, as they each influence a different aspect of

disease outcome. In addition, the role of individual TIMPs in different cell types, such as cardiomyocytes, fibroblasts, endothelial cells, and smooth muscle cells which collectively determine the cardiovascular remodeling and function, needs to be determined. While overexpressing TIMPs in an organ or cell line of interest is a valid approach to explore their function in different settings, it is important to note that excess overexpression of any gene can exert stress on the cells (endoplasmic reticulum stress or ER stress) resulting in adverse effects and cell death. Hence, overexpression of TIMPs while maintaining a balance in the TIMP–MMP interaction is critical in developing therapies for diseases where TIMP levels are reduced. It is intriguing to explore if "TIMP therapy" to replenish the TIMP levels, individually or in combination, could offer a protective outcome in heart disease.

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Chapter 26
Intracellular Matrix Remodeling and Cardiac Function in Ischemia–Reperfusion Injury

Xiaohu Fan, Mohammad A.M. Ali, Bryan G. Hughes, Anna Laura B. Jacob-Ferreira, and Richard Schulz

Abstract The effects of ischemia on animal and human myocardium have been extensively studied during the last four decades. Myocardial ischemia followed by subsequent reperfusion can cause profound damage to cardiac myocytes through enhanced oxidative stress and intracellular Ca^{2+} overload. Ischemia–reperfusion (I/R) injury leads to structural and functional remodeling of multiple intracellular matrix components in the cardiac myocyte. The intracellular matrix of cardiac myocytes includes major cytosolic components that include the cytoskeleton, contractile myofibrils, and subcellular organelles such as mitochondria, sarcoplasmic reticulum, and the nucleus. There are several proteolytic pathways inside the cell which may participate in cell injury and/or cell repair upon reperfusion of ischemic heart muscle. These include matrix metalloproteinases, calpains, lysosomal proteases, and the proteasome system, which are a major focus of research in I/R injury. The discovery of intramyocyte matrix metalloproteinase-2 (MMP-2) and biologically relevant protein substrates of it in the intracellular matrix has shaped a new paradigm of the pathophysiological role of MMP-2 during myocardial I/R injury. Emerging evidence indicates that oxidative stress can efficiently activate intracellular MMP-2 which rapidly mediates intracellular matrix remodeling of injured myocytes. This chapter will focus on the structural and functional remodeling of the intracellular matrix including the sarcomere, cytoskeleton, mitochondria, and nucleus, by proteolytic and other processes, in the context of I/R injury, with a particular emphasis on the rapidly expanding knowledge of the biology of intracellular MMP-2.

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26.1 Sarcomeric Protein Degradation and Contractile Dysfunction

The cardiac sarcomere (Fig. 26.1) is the basic contractile unit of the heart which contains the contractile components of the thin (actin), thick (myosin), and third (titin) myofilaments essential for cardiac function. The acute contractile defect of the heart after I/R injury [1] has been shown to involve proteolysis and/or disorganization of myofilament proteins [2, 3] including troponin I, myosin, and titin [4, 5].

26.1.1 Calpain-Mediated Remodeling in Ischemic Heart Disease

Calpains are a family of non-lysosomal cysteine proteases consisting of several isoforms. The best characterized isoforms are calpain-1 (μ-calpain), calpain-2 (m-calpain), and calpain-3 (p94 calpain). The terms μ-calpain and m-calpain indicate the

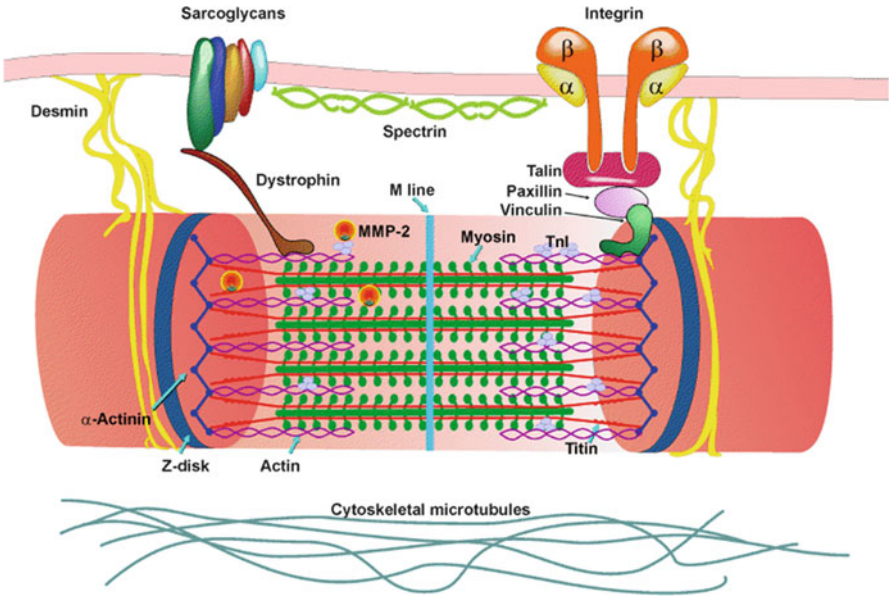

Fig. 26.1 Intracellular matrix and intracellular matrix metalloproteinase-2 (MMP-2). Schematic diagram shows the organization of sarcomeric and cytoskeletal proteins in relation to membrane anchor proteins in the cardiomyocyte. MMP-2 is localized to specific sarcomeric and cytoskeletal proteins and, upon myocardial oxidative stress injury, is able to proteolyze these substrates

required calcium concentration for in vitro activity (micromolar range for μ-calpain and millimolar range for m-calpain). Calpain-1 and -2 are considered to be ubiquitous, since they are expressed in nearly all tissues, whereas calpain-3 is expressed mainly in skeletal muscle [6]. Calpains participate in various cellular processes including remodeling of the sarcomere and cytoskeleton, signal transduction, and cell death [7]. Calpain-1, in particular, has been implicated in the pathogenesis of myocardial stunning injury, a reversible, sublethal injury of cardiac muscle which occurs upon reperfusion of the ischemic heart [8].

It has been suggested that activation of calpain-1 may lead to proteolytic degradation of sarcomeric proteins such as troponin I [9], titin, and α-actinin [10] which contributes to impaired contractile function (Fig. 26.2). In addition, calpain-1 activated during I/R injury has been shown to target various proteins involved in excitation–contraction coupling, thereby attenuating cardiac contractility. These targets include the SERCA2 pump [11], ryanodine receptor [12], and α-fodrin [13]. However, the conclusions of most of these studies rested on the use of pharmacological inhibitors of calpain, many of which were recently found to also inhibit MMP-2, another key protease in I/R injury [14]. In view of this, the relative contribution of calpain in I/R injury of the heart and other organs may need to be systemically revisited in comparison with MMP-2.

26.1.2 Intracellular Substrates of MMP-2 in Ischemic Heart Disease

Since their first description in amphibian metamorphosis [15], it has been recognized that MMPs play an active role in remodeling extracellular matrix proteins accompanying both physiological and pathological processes. Being originally described as secreted proteases, most researchers have focused on the long-term effects of MMPs on extracellular matrix remodeling following myocardial infarction (irreversible cellular injury resulting in myocyte death), hypertensive cardiac diseases, and other cardiomyopathies. In fact, MMPs have been recognized as proteases playing a pivotal role in matrix remodeling in such heart diseases (for review see [16]).

However, it has been recognized more recently that MMPs may also act on non-extracellular matrix substrates both outside [17] and inside the cell [4, 18]. This may occur within seconds to minutes rather than hours to days as occurs with so many of the extracellular matrix actions of MMPs. For example, MMP-2 was found to contribute to acute cardiac mechanical dysfunction in stunning injury before changes in extracellular matrix proteins [19]. Emerging evidence has shown that MMPs, and in particular MMP-2, are closely associated with subcellular compartments within cardiac myocytes, including the sarcomere [5, 20], cytoskeleton [21, 22], nuclei

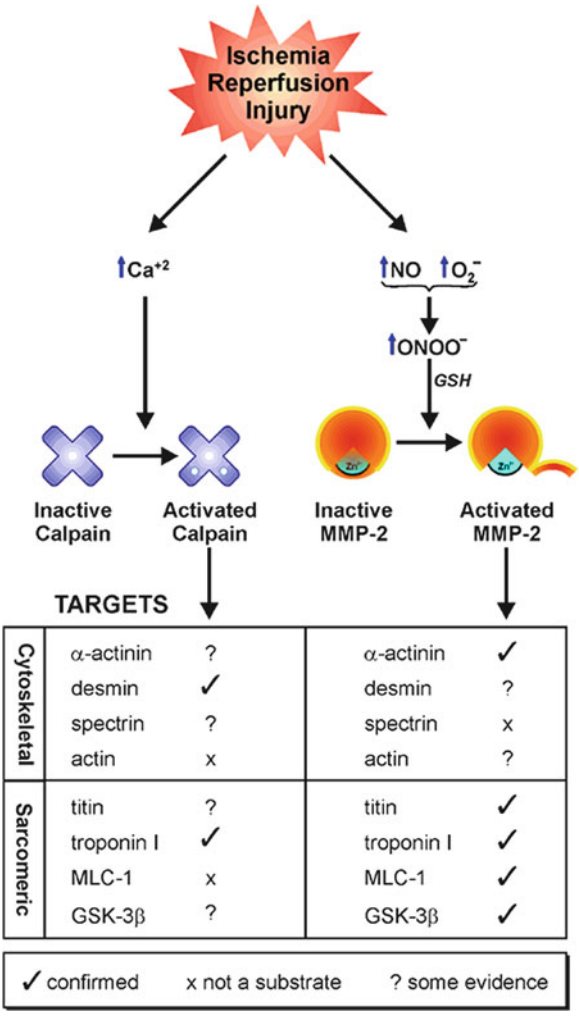

Fig. 26.2 Activation of intracellular proteases and their substrates. Mechanisms of enhanced intracellular proteolytic stress in acute myocardial ischemia/reperfusion injury include increased intracellular calcium, which leads to activation of calpains, and enhanced oxidative stress, particularly in the form of peroxynitrite biosynthesized from nitric oxide (NO) and superoxide, causing the direct activation of MMP-2. The table below summarizes potential targets for both proteases in ischemia/reperfusion injury

[23, 24], and mitochondria [20, 25]. Recently, distinct intracellular moieties of MMP-2 have been identified [25, 26], which physically confirm the concept that MMP-2 is indeed an intracellular protease [4, 18, 20].

Different mechanisms can activate and regulate MMP-2 activity, either extra- or intracellularly. MMP-2 is synthesized as a 72 kD zymogen protein which can be activated by proteolytic removal of the propeptide domain in pericellular and extracellular compartments, resulting in 64 kD MMP-2 [27]. However, 72 kD MMP-2 can also be activated without proteolysis as a direct result of oxidative stress. For example, MMP-2 is activated upon exposure to peroxynitrite, a prooxidant molecule implicated in various cardiac pathologies including I/R injury [28], via S-glutathiolation of a critical cysteine sulfhydryl moiety in the propeptide domain

[29] (Fig. 26.2). MMP-2 is also a phosphoprotein whose activity is increased by dephosphorylation [30]. MMP-2 activity can be inhibited by certain pharmacological agents, especially those which chelate the catalytic zinc found in its active site which is essential for MMP activity. Tetracyclines, especially doxycycline and minocycline, can inhibit MMPs independent of their antibacterial actions, most likely via chelation of zinc [31].

We will discuss below some cardiac sarcomeric and cytoskeletal proteins that have been shown to be targeted by MMP-2 in I/R injury (Fig. 26.2).

26.1.2.1 Troponin I

In 1999 Spinale's group showed a sarcomeric staining pattern of MMP-2 in pig heart muscle, yet did not provide an explanation of this unexpected result [21]. Our group then showed that MMP-2 is localized inside cardiac myocytes within the sarcomere and is responsible for the rapid degradation of troponin I in acute myocardial I/R injury [20]. Troponin I regulates actin–myosin interaction and is found in the thin myofilaments. Immunogold electron microscopy amongst other evidence showed that MMP-2 is an integral sarcomeric protein. Troponin I was highly susceptible to the proteolytic action of MMP-2 in vitro, and subjecting isolated rat hearts to acute I/R injury diminished myocardial troponin I content, an effect that was blocked by MMP inhibitors. In this study, we provided evidence that myocardial stunning injury is caused in part by MMP-2-mediated proteolysis of troponin I. This study was the first to recognize an intracellular biological role of a MMP as well as to identify the first intracellular target of MMP-2 in cardiac myocytes. Four years later, Lovett and Karliner's group reported that transgenic mice overexpressing a mutant, constitutively active MMP-2 in cardiomyocytes had marked derangements in the cardiac sarcomere, including troponin I degradation and reduced contractile function at the level of the myofilaments [32, 33].

26.1.2.2 Myosin Light Chain-1

Myosin light chain-1 was reported to undergo proteolytic degradation in hearts subjected to I/R injury [34]. MMP-2 activity was also found in preparations of thick myofilaments (which contain myosin light chain-1) from rat hearts, and MMP-2 was localized to the sarcomere in a pattern consistent with the known distribution of myosin light chain-1. Purified myosin light chain-1 was susceptible to proteolysis by MMP-2 in vitro. Two-dimensional gel electrophoresis followed by mass spectrometric analysis of myosin light chain-1 proteolysis products from I/R hearts identified a MMP-2 cleavage site within myosin light chain-1 at an accessible portion of the C terminus between tyrosine 189 and glutamate 190 [35].

26.1.2.3 Titin

Titin is the largest known mammalian protein (3,000–4,000 kD) and is found in cardiac and skeletal striated muscles. It spans nearly half the length of the sarcomere, from the Z-disk to the M-line region. It contains elastic segments formed by immunoglobulin-like repeats in the I band region, which allow it to act as a molecular spring, maintain the structural and functional stability of the myocyte and contribute to both active and passive stiffness of the myocyte [36].

Cardiac titin is expressed in two main isoforms: the shorter and stiffer N2B and the longer, more compliant N2BA isoforms. Hearts from adult small mammals (rats, mice, and rabbits) express predominately N2B titin, whereas large mammals including humans co-express N2BA and N2B titins at an approximate 1:1 ratio. The N2BA:N2B isoform ratio is increased in end-stage failing human hearts from chronically ischemic hearts of patients with coronary artery disease [37] and nonischemic dilated cardiomyopathy [38]. This titin remodeling decreases passive myocyte stiffness, most likely as a compensatory mechanism to counteract increased passive stiffness related to extracellular fibrosis [39]. Other forms of titin remodeling occur in heart disease, including reduced levels of titin phosphorylation, observed in dilated cardiomyopathy [40], and the formation of intramolecular disulfide bonds under oxidative stress [41], both of which could stiffen the titin spring function and contribute to impaired diastolic function following oxidative stress. The regulation of titin stiffness could affect various mechanical functions of the heart including diastolic filling, the Frank–Starling mechanism, and contractile performance in systole, the latter of which is also determined by titin [42].

In addition, earlier studies of ischemic and failing human hearts showed that titin is hydrolyzed [43] and appears highly disorganized in cardiac myocytes when analyzed by immunofluorescence microscopy [44]. We showed in rat and human myocardium that MMP-2 colocalizes with titin mainly near the Z-disk region of the cardiac sarcomere. Cleavage of titin in perfused rat hearts subjected to I/R injury, or in skinned cardiac myocytes incubated with MMP-2, was prevented by the MMP inhibitors *o*-phenanthroline or ONO-4817. Titin proteolysis in hearts was abolished in MMP-2 knockout mice subjected to I/R in vivo [5]. Thus MMP-2 appears to play an important role in titin homeostasis, which directly affects the contractile function of the heart at the sarcomeric level. Taken together, these studies reveal that MMP-2 may be a crucial protease which targets specific sarcomeric proteins as a result of oxidative stress injury to the heart.

26.1.3 MMP-2 and Calpains: A Case of Misattributed Function?

In light of the above studies, there appears to be overlap in the substrates and/or biological actions of MMP-2 and calpains in various cellular pathways (Fig. 26.2). It is now becoming evident that MMP-2 either targets a similar subset of proteins as calpain or calpain has been incorrectly identified as the protease responsible for

some intracellular proteolytic activities [1, 45]. Indeed, much of the evidence for calpain degradation of substrates in cardiac cells rests on the use of pharmacological calpain inhibitors, including ALLN and PD-150606, which we recently found to efficiently inhibit MMP-2 activity at commonly used micromolar concentrations [14]. Furthermore, the exact role of calpain in acute myocardial I/R injury (stunning) is controversial as many of the earlier studies dating back several decades did not provide evidence for the subcellular co-localization of calpain with its putative substrates [1]. In a more recent study, myocardial-specific overexpression of calpain-1 in transgenic mice showed no evidence of troponin I degradation in the heart [46], whereas as mentioned above, troponin I levels were reduced in hearts from transgenic mice with myocardial-specific overexpression of constitutively active MMP-2 [33]. It is possible that MMP-2 and calpains share similar substrates. However, given the points raised above, it would be prudent to reevaluate suggested calpain substrates in the myocardium for their susceptibility to cleavage by MMP-2 and to critically evaluate the co-localization of calpains with their putative substrates. Moreover, caution is necessary in interpreting tissue calpain activity solely on the use of peptide-based enzyme assays which may not only measure calpain but also MMP activity.

26.1.4 Proteasomal and Lysosomal Degradation of the Intracellular Matrix

Proteasomes and lysosomes play important roles in the proteolysis of cardiac proteins. Lysosomes degrade the majority of endocytosed proteins when digesting expired organelles or cell debris. However, the proteasome system removes and recycles most unneeded or damaged intracellular proteins [47]. Oxidative modification of proteins affects their secondary and tertiary structures, resulting in protein unfolding, which may lead to a loss of function and enhanced proteolysis of these proteins [48]. Damaged, oxidized, and/or misfolded proteins are removed by the ubiquitin–proteasome system (UPS). The UPS is the main non-lysosomal protease complex involved in the proteolysis of intracellular proteins and therefore plays a key role in protein quality control [49].

As ATP is required for activation of the proteasome and for proper function of the UPS, ATP depletion during ischemia could be partially responsible for decreased proteasome activity in the ischemic heart. Also, accumulation of misfolded or mutated proteins as a result of oxidative stress can inhibit the cardiac UPS and may result in cardiomyopathy [48]. On the other hand, it was also reported in animal models of myocardial I/R injury that inhibition of the proteasome system significantly reduced infarct size by more than 50% and preserved ventricular contractility suggesting a role of proteasomes in the process of I/R injury [47]. Although myosin, actin, troponin C, and tropomyosin purified from skeletal muscle can be hydrolyzed by the proteasome pathway in vitro, these proteins are much less susceptible to proteasomal degradation when present in intact myofibrils or as soluble actomyosin

complexes [47]. Thus, the rate-limiting step in their degradation seems to be their dissociation from the contractile filaments, where intracellular MMP-2 or calpains may play an important role in the scenario of I/R injury [47].

26.2 Cytoskeletal Protein Remodeling in Cardiac Ischemia and Reperfusion

The cardiac cytoskeleton (Fig. 26.1) consists of microfilaments, intermediate filaments (such as desmin), and the microtubular network. The cytoskeleton preserves cellular shape and enables cell migration and intracellular transport. It also maintains the proper localization of subcellular organelles such as the mitochondria, Golgi apparatus, nucleus, and sarcomere. The cardiac myocyte cytoskeleton is also specialized to transmit mechanical and electronic stimuli between cells. Investigation of early changes to cytoskeletal proteins in ischemic human myocardium suggest that they undergo degenerative alterations earlier than subcellular organelles [2]. Furthermore, disruption of the localization and integrity of the filamentous network of the cytoskeleton during prolonged ischemia accompanies I/R injury [50] (Fig. 26.3).

26.2.1 α-Actinin

α-Actinin connects actin filaments of adjacent sarcomeres and plays a substantial role in transmitting force generated by actin–myosin interaction. MMP-2 was found to colocalize with α-actinin in cardiac myocytes [21, 22]. We found that α-actinin is susceptible to degradation by MMP-2 in vitro and infusion of peroxynitrite into isolated, perfused rat hearts caused activation of MMP-2 with concomitant loss of myocardial α-actinin content. This was prevented by a selective MMP inhibitor, PD-166793 [22].

26.2.2 Desmin

Desmin is a cardiac-specific cytoskeletal protein with an average molecular weight of 53 kD. Desmin monomers assemble to form intermediate filaments 10 nm in diameter by polymerization which form in turn a transverse network that links the Z-bands of adjacent myofibrils. This maintains the integrity of cardiac myocytes and allows force transmission and mechanochemical signaling between cells [51]. In addition, mitochondria and T-tubules appear to be attached to the intermediate filament network [52].

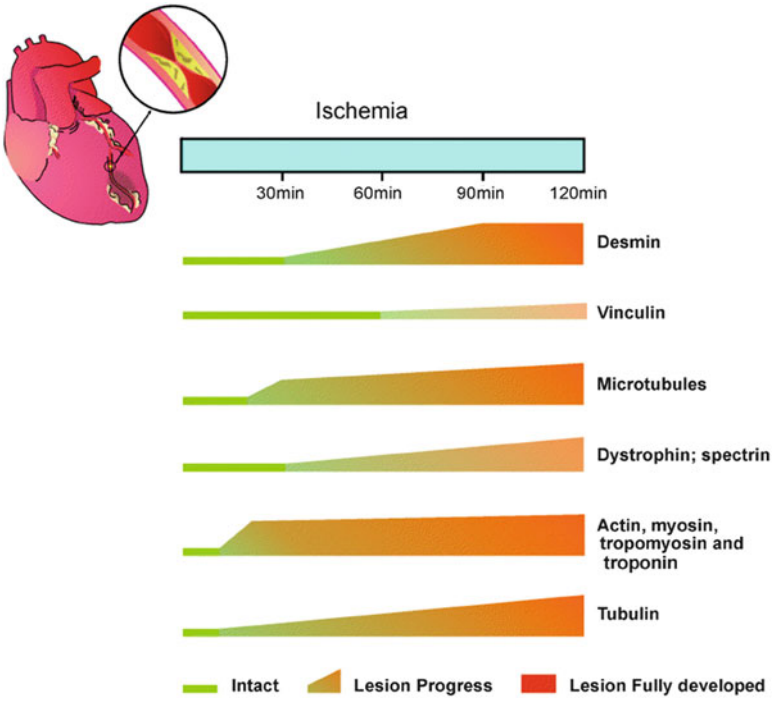

Fig. 26.3 Sarcomeric and cytoskeletal proteins have different sensitivities towards ischemic damage ("lesion"). For example, tropomyosin and troponins are among the most sensitive targets to proteolysis during myocardial ischemia, whereas vinculin resists this proteolytic damage. Reperfusion following ischemia accelerates damage to these proteins

Interestingly, although desmin is not considered to be directly involved in the generation of contractile force or the maintenance of tension, vascular smooth muscle cells from desmin knockout mice generate only 40% of the contractile force than that of wild-type controls [53]. Severe cardiac ischemia followed by reperfusion leads to intracellular Ca^{2+} overload and subsequent activation of calpain, which has the ability to proteolyze desmin [6]. Desmin content in the whole heart was shown to be decreased in myocardial I/R injury, and desmin hydrolysis by activated calpain reduces maximal force production and Ca^{2+} sensitivity in isolated cardiac myofilaments [54]. Hein et al. studied the effects of global ischemia on various cytoskeletal and contractile proteins in human left ventricles obtained from transplant recipients. Desmin was found to be affected by ischemia at later time points than the contractile filaments (Fig. 26.3). Disappearance of desmin from the cross striation pattern began after at 30 min of ischemia whereas, and the fully developed pattern of changes occurred after 90 min of ischemic injury [2]. Disruption of desmin damages the link between myofibrils and the sarcolemma and was found to

contribute to increased fragility of the myofibrils [55]. Since calpain was also found to be colocalized with desmin in the Z-band of skeletal myoblast cells [56], it was postulated that calpain-mediated degradation of desmin may contribute to the increase of cell fragility which may increase the chance for rupture of the cell during reperfusion. Note that desmin is susceptible to hydrolysis by MMP-2 in vitro [22] but whether this accompanies ischemic heart injury is unknown.

26.2.3 Vinculin

Vinculin is a membrane-associated cytoskeletal protein required for the attachment of the actin-based microfilaments to the plasma membrane (Fig. 26.1). In the cardiac myocyte, vinculin participates in the formation of the attachment complex between the plasma membrane and the Z-line of myofibrils [57]. Vinculin was also localized in the intercalated disk and in the lateral sarcolemma [2].

The effects of different durations of ischemia on cardiac vinculin were assessed by immunofluorescence, and it was found to be more resistant to the effects of ischemia than desmin, with effects not being detectable until 60 min post-ischemia [2]. Similarly, in a canine heart ischemia model, Steenbergen et al. reported an unchanged pattern of vinculin staining in longitudinal sections of myocardium subjected to 60 min of total ischemia and 60 min of reperfusion. When subject to more severe myocardial ischemia (120 min or longer), there was a progressive loss of vinculin staining and increase in inulin permeability, which likely contributes to the detachment of actin from the sarcolemma, leading to the formation of blebs and rupture of the sarcolemmal membrane [58].

26.2.4 Microtubules

Microtubules are hollow tubes 25 nm in diameter that exist as a ubiquitous filamentous structure of the cytoskeleton. Polymerized α- and β-tubulin form the microtubes which surround the nucleus and spread throughout the entire cell [59]. In myocytes, microtubules are distributed along their longitudinal axis as an irregular network.

In an in situ canine heart model [50], 15 min of cardiac ischemia caused no detectable changes in the filamentous staining pattern. After 20 min of ischemia, however, small patchy lesions appeared in some myocytes in which the immunoreactivities of microtubules began to decrease in intensity. Progressive loss of microtubular staining continued to be observed over 120 min of cardiac ischemia.

Microtubules contribute significantly to the stability of cell morphology by supporting cellular architecture, plasma membranes, myofibrils, and other cellular organelles. Protection of microtubule integrity during I/R injury could therefore be

a means of protecting against I/R-induced damage. For example, paclitaxel, a microtubule stabilizer, reduced myocardial I/R injury, myocardial infarct size, and the incidence of ischemic ventricular arrhythmias in perfused rat hearts [60].

26.2.5 Other Cytoskeleton Proteins: Talin, Dystrophin, and Spectrin

Prolonged cardiac I/R injury damages a wide array of cytoskeletal protein structures. Talin, dystrophin, and spectrin are membrane-associated cytoskeletal proteins that link the structural components of the intracellular milieu with those of the extracellular matrix via the integrins (Fig. 26.1). Dystrophin connects intracellular actin and extracellular laminin and acts as a stabilizing force and mechanotransducer for the sarcolemmal membrane [61]. Spectrin forms the backbone of the membrane skeleton providing an elastic support to the sarcolemmal membrane. Ischemia-induced loss of membrane dystrophin and spectrin was found following 30–45 min of coronary artery ligation in rabbit hearts. Loss of sarcolemmal dystrophin and spectrin seems to contribute to subsarcolemmal bleb formation and membrane fragility during the transition from reversible to irreversible ischemic myocardial injury [62]. Spectrin was not susceptible to proteolysis by MMP-2 in vitro [22].

26.2.6 Mechanisms of Cytoskeletal Damage in I/R Injury

Beside the direct degenerative effects of ischemia on the cytoskeleton, the process of restoring circulation to the ischemic heart, usually by acute intervention therapy, can also induce additional injury to the myocardium. The initial mechanism underlying reperfusion injury had been attributed to the generation of reactive oxygen/nitrogen species including peroxynitrite [22] and elevated intracellular Ca^{2+}. Beyond this, a variety of other biochemical abnormalities have been proposed to explain the myocardial contractile dysfunction that occurs after reperfusion, including excitation–contraction uncoupling due to dysfunction of the sarcoplasmic reticulum, altered fuel metabolism in mitochondria, inefficient energy use by myofibrils, altered ion channel activities, and decreased sensitivity of myofilaments to calcium. Importantly, these potential mechanisms are not mutually exclusive [1].

In I/R injury, the activity of cytosolic proteases such as calpain and MMPs is increased in response to increased Ca^{2+} and reactive oxygen/nitrogen species, respectively. The proteolysis of their cytoskeletal and sarcomeric substrates was found to be a major mechanism of intracellular pathology in I/R injury [4, 45]. These alterations to contractile and cytoskeletal proteins (Fig. 26.2), in addition to damaged subcellular organelles, play a key role in impairing cardiac function during I/R injury.

26.2.7 Cytoskeletal Injury as an Indicator of Irreversible Cell Injury

Myocardial injury is a dynamic process which, if mild, results in reversible cell injury, but if severe enough causes irreversible damage. Action to protect (or salvage) the ischemic myocardium should therefore preferably be performed before irreversible damage of myocardium occurs. Many biochemical and metabolic changes have been observed early after the onset of ischemia, but the precise cause of the transition to irreversibility is not known.

A number of hypotheses have been proposed to account for this transition to irreversible myocardial damage, including mitochondrial dysfunction, depletion of antioxidant reserves, leakage of lysosomal enzymes [63], the toxicity of metabolic end products, and lipid peroxidation caused by reactive oxygen/nitrogen species [64]. None of these postulated theories completely account for all features of the irreversible damage to cardiac myocytes. However, cytoskeletal damage can possibly explain many important biological phenomena associated with irreversible damage.

When ATP is severely depleted during ischemia, the connection between the cytoskeletal components is weakened resulting in an increased potential to dissociate under mechanical force. During the early reperfusion phase, the influx of calcium leads to calcium overload and hypercontraction [65]. Hypercontraction of the ischemia-injured myocyte will lead to cytoskeletal deformation to an extent beyond that seen under normal contraction. This will result in "cytoskeletal fracture". Since the cytoskeleton is required to maintain integrity of the cell membrane and cytosolic organelles, cytoskeletal injury is accompanied by osmotic swelling which eventually leads to rupture of the cell membrane. The membrane rupture hypothesis of irreversibility is supported by the observation that "irreversibly injured" cells can indeed recover if membrane rupture and necrosis during reperfusion are prevented [66].

26.3 Cardiac Mitochondria Remodeling in Ischemic Heart Disease

Current models of I/R injury feature mitochondria as important arbiters of cardiac myocyte survival or death [67]. The pivotal event is the opening of the mitochondrial permeability transition pore (MPTP), which results in the permeabilization of the inner mitochondrial membrane. This collapses mitochondrial membrane potential, rendering mitochondria unable to produce ATP. Responding to an osmotic gradient between the mitochondrial matrix and cytosol, water rushes into the matrix resulting in the characteristic "swollen" appearance of mitochondria in electron

micrographs of cardiac tissue subject to I/R injury. This may lead to the rupture of the outer mitochondrial membrane, releasing cytochrome c and other pro-apoptotic proteins into the cytosol.

26.3.1 A Potential Role for Intracellular Matrix Remodeling in Post-I/R Mitochondrial Dysfunction

The existence of a physical relationship between mitochondria and the cytoskeleton is well established. Mitochondria have been shown to interact with microtubules, intermediate filaments, and microfilaments, with cytoskeletal elements believed to play a role in their intracellular localization and movement and possibly morphology [52]. The possibility that intracellular matrix remodeling may play a role in I/R-induced mitochondrial dysfunction remains mostly unexplored, although there are tantalizing hints that this is the case. For instance, pharmaceutical manipulation of the cytoskeleton has been shown to affect mitochondrial functions relevant to I/R. Pharmaceutical agents that either depolymerized or stabilized microtubules prevented closure of the MPTP [68], and pharmaceutical disruption of the actin cytoskeleton impaired the effectiveness of certain drugs that decrease the vulnerability of cardiac tissue to I/R injury by manipulating mitochondria [69]. It has been suggested that the cytoskeleton plays a role in the translocation of signaling molecules to the mitochondria during ischemic preconditioning, perhaps via the endosomal system [70]. Furthermore, cardiac mitochondrial function in desmin-null mice is impaired, with mitochondria exhibiting ultrastructural changes—such as swelling—strikingly similar to those observed in I/R injury [71].

More generally, it is hypothesized that the cytoskeleton modulates mitochondrial function, although the mechanisms remain unclear [52]. One possible mechanism could be via the cytoskeletal control of the subcellular localization of mitochondria. This may be particularly relevant in adult cardiac myocytes, in which most mitochondria are arranged in highly structured linear arrays between myofibrils [72]. After I/R injury, mitochondria have been observed as appearing to be detached from myofibrils [73], suggestive of a mechanism, possibly cytoskeletal in nature, that maintains this association. Indeed, disrupting cytoskeletal structure by knocking out desmin or enzymatic digestion in vitro results in a loss of the normal mitochondrial position and neat arrangement between myofibrils [71, 74]. Mitochondria appear to be localized near sites of Ca^{2+} release from the sarcoplasmic reticulum, which may facilitate Ca^{2+} movements between the two cellular subcompartments [75, 76]; it has been proposed that the cytoskeleton may play a role in maintaining this physical orientation [77]. Likewise, the close association of mitochondria with sarcomeres and the sarcoplasmic reticulum may facilitate the channeling of ADP to mitochondria; this is supported by the finding that proteolytic digestion of cytoskeletal elements in permeabilized cardiac myocytes in vitro altered the apparent binding affinity of ADP [74].

26.3.2 *Mitochondrial Localization of Modifiers of the Intracellular Matrix*

As described above, calpains and MMP-2 have both been shown to actively remodel the intracellular matrix in response to I/R. MMP-2 and calpain-1 have both been found in cardiac mitochondria [20, 25, 78, 79]. Mitochondrial calpain-1 is activated by I/R injury and cleaves apoptosis-inducing factor, allowing it to be released into the cytosol [78]. Overexpression of constitutively active MMP-2 in the mouse heart does not affect baseline mitochondrial function; however, the response to I/R is more severe, with mitochondrial respiration and structure being affected to a greater extent than in hearts from wild-type mice [73]. Mitochondria appear to accumulate a constitutively active, N-truncated isoform of MMP-2, overexpression of which triggers the nuclear activation of several pro-inflammatory transcriptional pathways [25]. The fact that these proteases of the sarcomere and cytoskeleton also appear to be active in mitochondria suggests that the remodeling of the intracellular matrix may actually be part of a wider program of response to I/R injury.

26.4 Nuclear Matrix Remodeling in Cardiac Disease

The nuclear matrix is the network of fibers found throughout the inside of the cell nucleus. It is analogous to the cellular cytoskeleton and provides structural and organizational support for various nuclear processes. Proteolytic cleavage of the nuclear matrix occurs in processes such as apoptosis [80], regulation of the cell cycle [81], and nuclear matrix degradation [82].

Irreversible I/R injury may lead to cellular apoptosis. During apoptosis, morphological changes such as chromatin condensation, nuclear shrinkage, and the formation of apoptotic bodies occur in the nucleus [83]. These changes are associated with numerous molecular alterations, such as DNA and RNA cleavage, posttranslational modifications of nuclear proteins, and proteolysis of several polypeptides of the nuclear matrix including topoisomerase IIa, NuMA, SAF-A, lamin B1, lamins A and C, and SATB1 [83].

Nuclear MMP-2 and/or MMP-9 activities may also contribute to the I/R injury-induced apoptotic process by processing poly-ADP-ribose polymerase [18] and X-ray cross complementary factor 1, hence, interfering with the DNA repair system [84]. Indeed, MMP-2 [23] and MMP-3 [85] carry a putative nuclear localization sequence. An active, truncated fragment of MMP-3 was localized to the nucleus of several human cancer cell lines, and it is associated with the onset of apoptosis [85]. MMP-2 and MMP-9 were found in the nucleus of human cardiac myocytes, and although MMP-2 was able to proteolyze the nuclear DNA repair enzyme, poly (ADP-ribosyl) polymerase in vitro, its precise role in the nucleus remains to be discovered [23].

26.5 Future Prospects: The Intracellular Matrix as Therapeutic Target

Ischemic heart disease is the most common cause of death in developed and, ever more so, in developing countries. Studies of the pathogenesis of myocardial I/R injury reveal structural and functional remodeling of intracellular matrix components of the cardiac myocyte. Protection of the intracellular matrix may represent a novel strategy to prevent or reduce the impact of ischemic heart disease. Many cytoskeletal and sarcomeric proteins were found to be susceptible to proteolysis by intracellularly localized MMP-2; indeed, there is substantial evidence that the cleavage of these intracellular targets by this crucial protease mediates several important pathogenic processes in myocardial I/R injury. Oxidative stress generated in I/R injury can efficiently activate intracellular MMP-2 which then mediates intracellular matrix remodeling of injured myocytes. Given that MMP-2 is readily activated by prooxidant stress, such as peroxynitrite generated in I/R injury [29], MMP-2 may represent one of the earliest mediators of the detrimental actions of oxidative stress to the heart.

Doxycycline, one of the tetracycline antibiotics, has been shown to act as an MMP inhibitor at a plasma concentration below that required for its antimicrobial action [86]. Indeed, a retrospective epidemiological study found a significant reduction in the risk of first-time acute myocardial infarction for patients who had taken tetracycline class antibiotics for prior infection. This effect was not observed in patients who had received any other classes of antibiotics [87]. Furthermore, doxycycline was also found to protect against streptozotocin-induced diabetic cardiomyopathy [88] and cardiac mechanical dysfunction triggered by endotoxic shock in rats [89]. Increased activity of cardiac matrix MMP-2 and MMP-9 was found during the acute phase of Chagasic cardiomyopathy in mice, an inflammatory heart disease triggered by infection with *Trypanosoma cruzi*. This increased MMP activity was associated with mortality following infection, and doxycycline treatment significantly improved survival [90]. Based on our current understanding of MMP-2 as an intracellular protease, the cardiovascular benefits associated with tetracycline use may be reasonably attributed to the inhibition of pathological MMP activity. These findings suggest that doxycycline may be useful as a possible therapeutic regimen for ischemic heart disease. Of note, doxycycline has already been approved by the Food and Drug Administration and Health Canada in the therapeutic treatment of periodontitis. Doxycycline may therefore emerge as a promising drug in the near future for the treatment or prevention of cardiac disease.

Increasing evidence suggests that blocking MMP-2 activity can alleviate I/R-mediated cardiac injury in animal models. It is important to consider the fact that MMP-2 plays very diverse roles in various pathological and physiological circumstances other than cell injury, such as cell cycle control, cell death, inflammation, cancer, development, and tissue remodeling. Universal MMP inhibitors could therefore have detrimental or undesirable side effects due to a lack of selectivity and/or specificity [91]. Development of pathway-specific or subcellular location “selective”

MMP-2 inhibitors, based on the advancing knowledge in this field, may help to alleviate acute myocardial I/R injury or detrimental chronic cardiac remodeling, while spare physiological MMP-2 activities inside and outside the cell.

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Chapter 27
Aging and Markers of Adverse Remodeling After Myocardial Infarction

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Abstract Acute myocardial infarction (MI) is a catastrophic event in patients aged 18 years and above. While improved coronary reperfusion strategies and medical therapies over the last 2 decades have increased survival, hearts of survivors undergo progressive remodeling with changes in structure, size, shape, and systolic function that lead to heart failure. Morbidity from heart failure in post-MI survivors remains high, especially after ST-segment-elevation MI (STEMI) and in older compared to younger patients. Cumulative knowledge of the biology of cardiovascular aging suggests that the aging process is progressive and results in physiological, cellular, and molecular changes that can negatively impact post-MI remodeling. Emerging evidence suggests that aging may adversely influence key factors in post-MI remodeling, including the extent of damage to the cardiac muscle and extracellular matrix (ECM) on the one hand and adequacy of the healing/repair process (with inflammation, ECM remodeling, fibrosis, hypertrophy, and angiogenesis) on the other. These effects may result in differential outcomes of therapy between younger and older survivors of STEMI. Furthermore, reperfusion therapy that is delayed beyond several minutes of acute STEMI results in significant reperfusion damage and adverse remodeling that may be amplified with aging. Biomarkers, including emerging healing-specific proteins, can be used not only to noninvasively monitor the remodeling process and responses to therapy but also to predict adverse post-STEMI remodeling and clinical outcome. Novel biomarkers of adverse remodeling hold great promise for developing novel therapeutic strategies to limit adverse remodeling and improve outcome after STEMI in patients of different ages.

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27.1 Introduction: Statement of the Problem

Myocardial infarction (MI) is a major killer worldwide, and adverse remodeling is a major cause of morbidity and mortality in survivors of ST-segment-elevation MI (STEMI) [1–4]. An acute STEMI is one of the most catastrophic events in the patient with coronary artery disease. While STEMI is seen in adults aged 18 years and above, it is most prevalent in elderly (age ≥65 years) patients [5–8]. What follows after an acute STEMI, during the healing/repair phase in survivors, remains a major problem that has not been fully addressed despite many advances in molecular/subcellular mechanisms that lead to adverse remodeling during that phase ([8] for review).

27.2 Definition of STEMI

For purposes of translational research and discovery, it is important to appreciate that the commonest culprit lesion of an MI is a coronary atherosclerotic plaque with thrombosis, resulting in coronary artery occlusion [9–13]. This puts myocardium in the distal occluded bed at risk of ischemic damage [14], and persistent occlusion usually results in pathologically transmural (≥50% of the transmural extent) and electrocardiographic Q wave MI or STEMI [15–18]. Angographically, coronary obstruction is considered critical if the stenosis is ≥50% of the luminal diameter, but ischemic symptoms may occur with stenosis <50% of the luminal diameter or area depending on pathophysiological conditions. Occlusions <50% of the lumen and depending on several factors (such as collateral blood supply, hemodynamic load and oxygen demand, metabolism) usually result in subendocardial, non-Q wave or non-STEMI (NSTEMI). Acute coronary artery syndromes can result in either STEMI or NSTEMI that have different clinical outcomes needing different management strategies [15–18]. All evidences indicate that STEMI has grave prognosis and outcome. This chapter will focus on STEMI.

27.3 Morbidity and Mortality After STEMI

Effective medical therapies that reduce myocardial oxygen demand and/or increase myocardial oxygen supply result in myocardial salvage [19]. Successful early coronary reperfusion, by limiting the transmural march to necrosis [20],

results in non-transmural, subendocardial, or NSTEMI that are less prone to adverse remodeling [1–4, 15, 16, 21–24]. These early concepts have withstood the test of time. However, while improved coronary reperfusion strategies and medical therapies since the 1990s have increased the number of survivors of acute STEMI, the hearts of the survivors undergo progressive remodeling with negative changes in structure, size, shape, and systolic function that lead to heart failure [21–25]. Increased survival after acute STEMI has expanded the pool of patients at risk of complications of adverse left ventricular (LV) remodeling and heart failure. Simply put, decreased early mortality has resulted in increasing numbers of post-STEMI survivors who experience prolonged morbidity. In fact, morbidity from heart failure in post-STEMI survivors remains high, especially in old compared to young patients [6]. The reasons for these discrepancies need urgent investigation [8].

27.4 Aging and Reperfusion Damage

One explanation for the discrepancies may relate to aging and the healing/repair process. Increasing knowledge of the biology of cardiovascular aging suggests that the aging process is progressive [26] and results in physiological, cellular, and molecular changes that can negatively impact post-STEMI remodeling. Emerging evidence suggests that aging may adversely influence key factors in post-STEMI remodeling, including the extent of damage to the cardiac muscle and extracellular matrix (ECM) on the one hand and adequacy of the healing/repair process (with inflammation, ECM remodeling, fibrosis, hypertrophy, and angiogenesis) on the other [8, 27]. Elderly post-STEMI survivors do poorly, with increased morbidity and greater risk of adverse LV remodeling and heart failure, compared to younger patients [5, 8]. These effects may explain differential outcomes of long-term therapy between younger and older survivors of STEMI [8].

A second explanation relates to reperfusion injury [28–36]. Cumulative evidence shows that reperfusion therapy that is delayed beyond several minutes of an acute STEMI results in significant reperfusion damage of cardiac muscle, matrix and blood vessels [28–36], and adverse LV remodeling that may be amplified with aging [8, 27]. Work in our laboratory and others indicates that the healing/repair process after STEMI is highly complex, involving multiple factors, and is associated with significant adverse LV remodeling [8]. While very early reperfusion with primary coronary intervention (PCI) and thrombolytic therapy can be very effective [5, 37], delayed reperfusion of STEMI by only a few hours is still associated with adverse outcome, persistent LV remodeling, and heart failure despite medical therapy during the healing/repair phase [38], and older patients do poorly despite prolonged optimal medical therapy recommended in guidelines [8, 39–41].

27.5 Aging and Post-Infarct Remodeling

The elderly population is increasing worldwide [5, 8, 26, 42 for review]. Concurrently, morbidity and mortality after STEMI in the elderly and related healthcare costs have been increasing [5, 8, 26]. Not only is STEMI most prevalent in the elderly [6, 7], but clinical studies show that age is a strong predictor of adverse events after STEMI [7]. Despite improved coronary reperfusion and medical therapies for acute and subsequent subacute and chronic phases after STEMI, post-STEMI mortality remains high in old patients [6]. Nearly 85% of cardiovascular deaths occur in that group [6]. As mentioned, older adults and elderly post-STEMI survivors do poorly, with increased morbidity and greater risk of adverse LV remodeling and heart failure compared to younger patients [5, 8]. While reperfusion is widely used in acute STEMI, data on healing and remodeling post-reperfused STEMI in the elderly are lacking [8].

While reperfusion with restoration of antegrade flow has undisputed benefits especially when achieved very early [8], late reperfusion is associated with significant reperfusion damage [29] with flow/function mismatch. This is mediated by microvascular damage and persistent no-reflow [29, 35, 36], excess free radicals [29], apoptosis [32, 33], necrosis [27, 34], enhanced ECM degradation [23, 30, 31, 43–48], increased MMP activity [47, 48], decreased collagen cross-links in scars [49], decreased tensile strength of scars [49, 50], enhanced inflammation [23, 51], altered healing [8], and persistent LV remodeling and dysfunction [27, 29, 38]. Post-STEMI survivors who develop heart failure on therapy have a tenfold greater risk of dying [25], and the elderly are at even greater risk [5, 39, 40]. The survivors with persistent post-ischemic LV dysfunction after reperfused STEMI remain at risk for progressive remodeling and its consequences including heart failure [22–24, 52]. In the old, impaired healing/repair mechanisms and adverse remodeling may further aggravate outcome [8, 41]. It is therefore important to study healing/repair in both non-reperfused and reperfused STEMI groups and in both young and old animals for easier translation of basic research findings to the bedside.

27.6 Aging and Healing/Repair Post-STEMI

Evidence suggests that aging-related impaired or defective healing/repair may be a major culprit resulting in adverse remodeling after STEMI [8, 41]. Physiological, cellular, and molecular changes that occur with cardiovascular aging can negatively impact the response to injury including reperfused STEMI [8]. Before 2008, most research on MI was done in young animals [8, 53]. Previous studies showed that MI triggers the healing process, which through a timed sequence of biochemical, molecular, and cellular reactions over weeks results in a firm fibrotic scar that replaces the dead tissue [8, 22, 53], at least in young animals (Fig. 27.1a, b). During healing, timed release of several factors modulate healing and repair [8, 23, 24, 53].

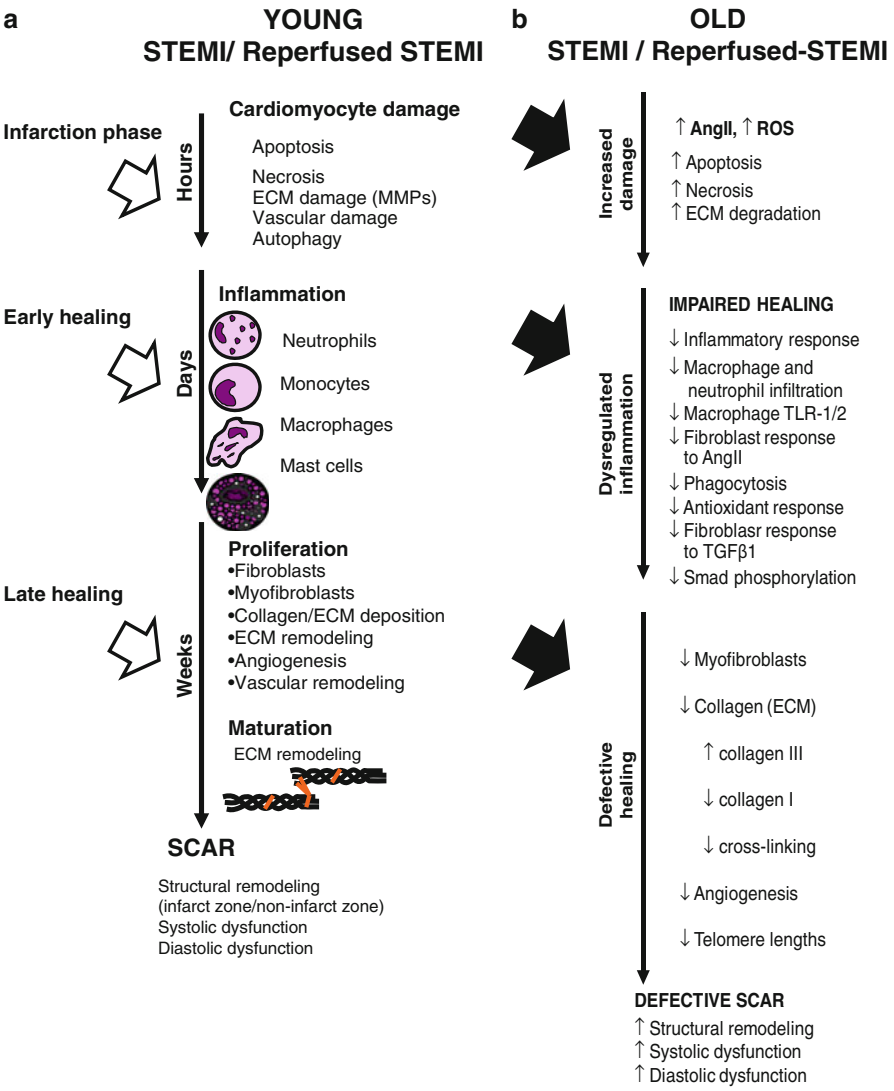

Fig. 27.1 Cellular and molecular changes in healing/repair after STEMI (**a**). The traditional concept is that an acute STEMI triggers healing; this process involves an orchestrated sequence of two highly dynamic phases: an early phase is dominated by inflammation, while a late phase is dominated by repair. The end result is formation of a fibrotic scar and fibrosis in remote myocardium, with variable dilative remodeling and dysfunction. This was based on data mostly in the young (**b**). In the twentieth century, it is recognized that in old hearts, there is amplification of early damage in the infarction phase, dysregulation of inflammation in the early healing phase, and defective healing/repair in the late phase. The end result is a defective scar and augmented adverse dilative remodeling and dysfunction. *AngII* angiotensin II, *ECM* extracellular matrix, *MMP* matrix metalloproteinase, *ROS* reactive oxygen species, *STEMI* ST-segment-elevation myocardial infarction, *TLR* toll-free receptor

These include chemokines, cytokines, growth factors, matrix metalloproteinases (MMPs), and other matrix proteins including the so-called healing-specific matrix and matricellular proteins (HSMPs) including secretory leucocyte protease inhibitor (SLPI), secreted protein acidic and rich in cysteine (SPARC), and osteopontin (OPN) that orchestrate inflammation, remodeling of the ECM, and fibrosis, under the modulatory influence of angiotensin II and other factors such as oxidative stress (Fig. 27.2). Increased MMPs relative to tissue inhibitors of MMPs (TIMPs) is one proposed major pathway leading to ECM and adverse LV remodeling during early healing post-STEMI [22–24].

In 2005, Ertl et al. proposed that SLPI, SPARC, and OPN might improve post-MI healing and remodeling on the basis of data in various transgenic/knockout young mouse models and named them healing-specific proteins [54]. This triggered more research in those proteins as potential targets and markers. SLPI is a potent inhibitor of serine proteases that upregulates production of anti-inflammatory cytokines in monocytes [55], macrophages [56], and skin wounds [57]. Ashcroft et al. reported that skin wound healing is impaired in young C57/BL6 mice lacking SLPI [57], but did not study healing after STEMI. OPN is an ECM protein that interacts with integrins and collagen and promotes collagen synthesis [58, 59], and Trueblood et al. reported decreased collagen, infarct expansion, and increased LV dilation post-MI in young B6.129S6 mice lacking OPN [60]. SPARC is an ECM protein that modulates cell-matrix interactions and regulates fibroblast migration [61–63]. Schellings et al. reported increased immature collagen, LV dilation, and cardiac rupture post-MI in young C57/BL6 mice lacking SPARC [64]. Recently, McCurdy et al. confirmed these findings [65].

In 2008, Bujak et al. first reported defective infarct healing (based on findings of suppressed inflammation and reduced collagen) and increased adverse remodeling after reperfused STEMI in old C57/BL6 mice aged >2 years versus young mice aged 8–12 weeks [66]. They also found that OPN is reduced in 1- and 3-day-old infarcts [66]. Ashcroft et al. had reported decreased collagen in skin wounds of aging mice [67] and delayed healing in skin wounds of aging humans [68], but they did not study MI or reperfused MI in aging animal models.

Since therapy for optimizing healing is lacking for both young and old age groups [8, 69], we considered the hypothesis that in aging hearts, increased myocardial angiotensin II, through its pro-inflammatory, prooxidant, and pro-remodeling effects, may amplify increases in pro-inflammatory cytokines, MMPs, and oxidative markers and contribute to impaired healing/repair and adverse LV remodeling [8] (Fig. 27.3). We postulated that aging may result in a dysregulation of the response to the novel HSMPs, leading to dysregulated inflammation and fibrosis pathways, impaired healing, and adverse LV remodeling post-reperfused STEMI (Fig. 27.3). Thus, aging-related impaired or defective healing after STEMI may be the major culprit leading to defective infarct fibrosis that in turn might result in amplified adverse maladaptive LV remodeling, increased progressive LV enlargement, and increased disability and/or death in older patients. Recent evidence in our laboratory supports the idea that aging-related adverse remodeling may be due in part to impaired healing and repair mechanisms after reperfused STEMI [8, 27, 70], although further research is needed.

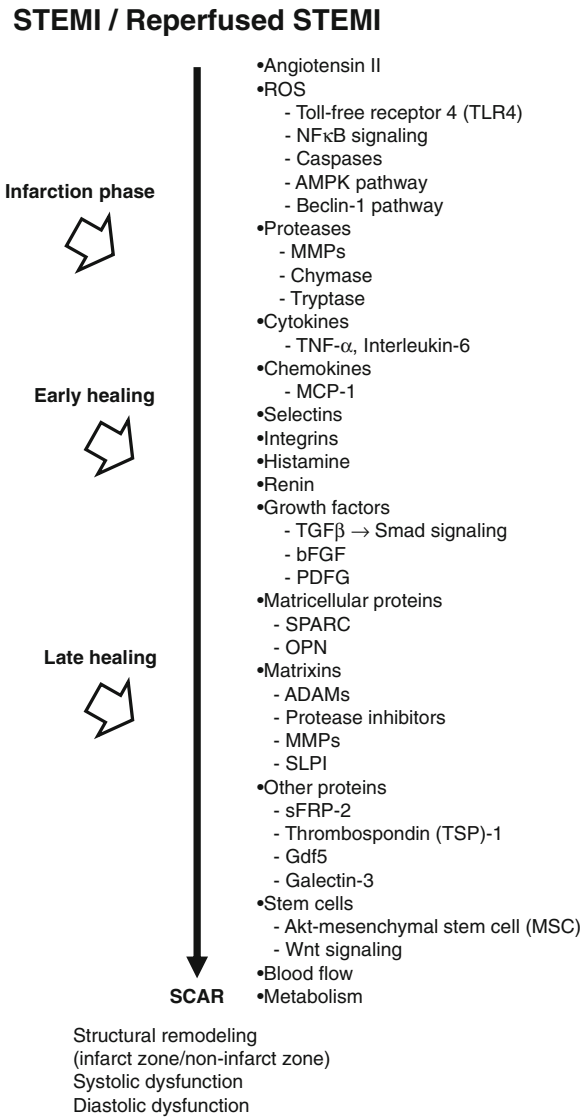

Fig. 27.2 Modulators of healing/repair after STEMI. Cellular and molecular changes during stages of post-STEMI healing and known modulators. *ADAM* a-disintegrin metalloproteinase, *AMPK* AMP-activated protein kinase, *AngII* angiotensin II, *bFGF* basic fibroblast growth factor, *ECM* extracellular matrix, *Gdf5* growth differentiation factor-5, *MCP-1* monocyte chemoattractant protein-1, *MMPs* matrix metalloproteinases, *OPN* osteopontin, *PDGF* platelet-derived growth factor, *ROS* reactive oxygen species, *sFRP-2* secreted frizzled-related protein-2, *SLPI* secretory leucocyte protease inhibitor, *SPARC* secreted protein acidic and rich in cysteine, *STEMI* ST-segment-elevation myocardial infarction, *TGF-β* transforming growth factor-β, *TNF-α* tumor necrosis factor-α

27.7 Changes in HSMPs After Reperfused STEMI

Our preliminary data in dogs, rats, and mice suggest that the 3 HSMPs (SLPI, SPARC, and OPN) are concurrently upregulated post-reperfused STEMI; may interact with concurrently upregulated ECM-proteolytic, inflammation, and fibrosis pathways; and contribute to remodeling in the young animals [27, 70]. Importantly, this upregulation of the HSMPs and proteolytic, inflammation, and fibrosis pathways was amplified in the old animals that developed more severe LV remodeling and dysfunction [27, 70, 71].

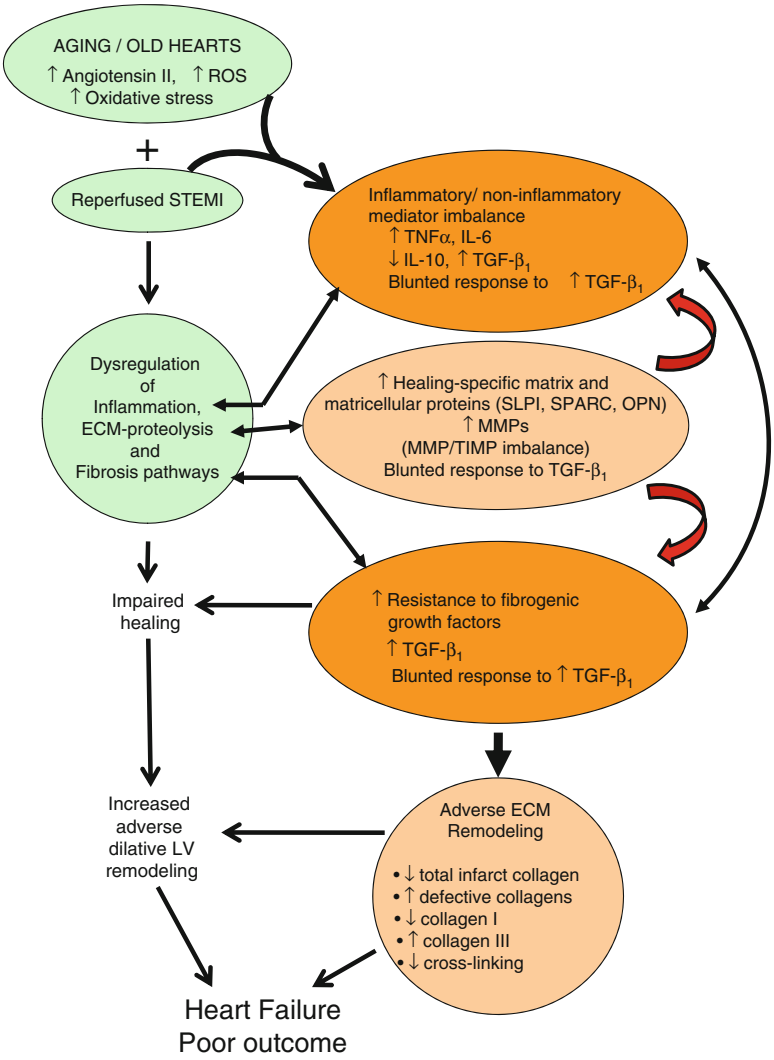

Fig. 27.3 Proposed mechanism of augmented adverse remodeling during healing/repair after reperfused STEMI in old hearts. Increased AngII and ROS augment cardiac muscle, matrix, and vascular damage. Dysregulation of the inflammatory response results from pro-inflammatory, and anti-inflammatory mediator imbalance results in blunting of inflammatory cell recruitment. Macrophages secrete MMPs, reactive oxygen species (ROS) or oxygen free radicals, and cytokines during healing. Concurrent increase in healing-specific matrix and matricellular protein (HSMP) expression (SLPI, SPARC, and OPN), resistance to fibrogenic growth factors, and an imbalance between MMPs and their endogenous tissue inhibitors (TIMPs) contribute to impaired healing with defective collagen synthesis in myofibroblasts and result in adverse ECM remodeling. The end result is augmented adverse dilative LV remodeling. ↑ increase, ↓ decrease, + increase/augment, – inhibit. Other abbreviations as in Fig. 27.2

27.8 Early Changes in the HSMPs

On day 1 in the dog model, reperfused STEMI induced early regional increases in infarct zones of young dogs in several markers of ECM remodeling including SLPI, SPARC, and OPN, MMP-9 and MMP-2, and a-disintegrin and metalloproteinase (ADAM)-10 and -17. Concurrently, markers of inflammation also increased, including pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6, the anti-inflammatory and fibrogenic cytokine transforming growth factor (TGF)-β_1 and its signaling molecule Smad-2, and inducible nitric oxide (NO) synthase (iNOS) [27]. Importantly, aging augmented these changes as well as markers of early LV structural remodeling and dysfunction [27]. More importantly, early angiotensin II type 1 receptor (AT_1R) blockade with candesartan attenuated these changes across the old groups albeit with a trend towards lesser benefit in the oldest [27], implicating angiotensin II and dysregulation of inflammatory and ECM-proteolytic pathways in the augmented remodeling during early hours after reperfused STEMI in aging hearts. Of note, old sham dogs showed the typical aging phenotype (increased LV mass, wall thickness, and fibrosis). Interestingly, levels of TGF-β_1 (fibrogenic and anti-inflammatory) were higher (suggesting enhanced upregulation), and levels of IL-10 (anti-inflammatory cytokine) were lower (suggesting impaired upregulation) in infarct zones of the senescent hearts—even during the early phase or reperfused STEMI and before substantial healing occurs [27]. In addition, there was an age-related increase in infarct size and apoptosis and evidence of progressive necrosis at day 3. These findings underscore the importance of assessing effects of aging on these markers and intersecting pathways and in vivo remodeling during both the infarction and subsequent healing phase after reperfused STEMI [8], especially since infarct size may impact healing [20, 41].

Taken together, our data suggest that the 3 HSMPs SLPI, SPARC, and OPN that are increased post-reperfused STEMI may interact with inflammation and fibrosis pathways and improve healing and LV remodeling in the young, but the pathways become dysregulated in the old [27]. Thus, aging amplifies responses in the critical cellular signaling and ECM-proteolytic pathways (MMPs, HSMPs, bioactive molecules) and interaction with the angiotensin II/AT_1R pathway after acute reperfused STEMI in the clinically relevant dog model [70].

27.9 Changes in HSMPs During Healing

Our preliminary unpublished data during healing after reperfused STEMI in rats [70, 71] also suggest that aging amplifies the increased expression of HSMPs, MMPs, inflammatory, and fibrogenic cytokines in infarct zones during healing after reperfused STEMI. We showed increased regional expression of SLPI, SPARC, OPN protein, and mRNA in infarct zones at 3 weeks that were augmented with aging. In that study [70, 71], SLPI, SPARC, and OPN were colocalized in macrophages

and monocytes. We also confirmed increased HSMPs at day 2. Importantly, AT_1R blockade with candesartan over day 2 to day 23 suppressed these changes in the HSMPs and remodeling in young and old rats, implying regulation by angiotensin II with aging. Candesartan also attenuated increases in MMPs, inflammatory and fibrogenic cytokines, and iNOS in the young and old rats, implying regulation by angiotensin II during healing. The data also showed age-related increase in tissue myeloperoxidase (MPO, oxidant activity marker) and MPO-positive granulocytes and CD68 and MAC387-positive macrophages at day 25, implying persistent inflammation and granulation tissue with impaired healing in the late phase after reperfused STEMI. Of note, old sham rats showed the typical aging phenotype. Importantly, attenuation of the molecular changes and LV remodeling in young was less in the old than young rats, implying impaired inhibition of effects of angiotensin II with aging.

Interestingly, Bradshaw et al. reported increased SPARC expression and collagens in old (18–24 months) versus young (3 months) wild-type (C57.BL6/SV129) mice without MI and attenuation of increased collagen in old SPARC null mice without MI [72]. In our other studies of chronic reperfused STEMI HSMPs in the young rat model, we showed that reperfused STEMI consistently induces LV dysfunction and adverse LV remodeling that persist over 3 weeks and correlate temporally with marked increase in MMP-9 activity and MMP-9/TIMP-3 protein ratio, and less increase in MMP-2. Interestingly, the persistent increase in MMP-9 paralleled that in iNOS. Importantly, candesartan improved LV function and attenuated LV remodeling during healing, and these benefits correlated temporally with improvement in MMP-9, TIMP-3, and iNOS levels and the MMP-9/TIMP-3 ratio [72]. We looked for neutrophils, a known rich source of MMP-9 [73], in infarct or border zones between 7 and 21 days and found very few. MPO levels correlated with MMP-9, and both MPO and MMP-9 colocalized in border zone myocardium. Interestingly, immunohistochemistry showed weak staining for SLPI and strong staining for SPARC and OPN at 3 weeks in the same hearts. Candesartan normalized increases in TNF-α and TGF-β_1 as well as increases in MMP-9 and MMP-2 mRNAs [71]. Compared to reperfused STEMI, healing after non-reperfused STEMI was not associated with persistent increase in MMP-9 or LV remodeling and dysfunction at 3 weeks.

Together, the data suggest that aging upregulates two critical pathways: (1) increased ROS → iNOS-NO → peroxynitrite → MMP and HSMP activation → adverse remodeling and dysfunction and (2) increased inflammatory cytokines → MMP and HSMP activation → adverse remodeling and dysfunction (Fig. 27.3). We propose that the differential responses in the old versus young may be due to an aging-induced dysregulation of the expected response to SLPI and the other HSMPs (found in young mice), leading to amplified inflammatory, ECM-proteolytic, and pro-fibrotic pathways, blunting of expected responses to the increased HSMPs, and/or resistance in the TGF-β_1 pathway. Aging-related increase in angiotensin II results in enhanced interactions among ECM-proteolytic, inflammation, and fibrosis pathways and resistance in the TGF-β_1 signaling pathway, leading to defective healing and a cascade to amplified maladaptive LV remodeling and poor outcome in older patients.

Bujak already documented blunted fibroblast response to TGF-β_1 in old mice [66]. Simply put, depending on the stage of the healing/repair process after reperfused STEMI, a little increase in the HSMPs may be good, but too much may be harmful.

27.10 Interactions Among Different Pathways

Evidence suggests that interactions among HSMPs, inflammatory, ECM-proteolytic, and pro-fibrotic pathways occur in early and late phases of the healing process. Evidence in young animals suggests that SLPI suppresses MMP-9 and inflammation [55] and ischemia/reperfusion injury [74]. SPARC increases TGF-β_1 and MMP activity in acute MI [8, 63]. OPN interacts with integrins, activates inflammation, and increases iNOS, MMP-2, and MMP-9, thereby increasing ECM proteolysis in acute reperfused STEMI [8, 27]. After MI, OPN may decrease fibroblast response, impair healing, and decrease ECM [75].

Other evidence suggests that interactions among HSMPs, inflammatory cytokines, and NOS can aggravate the imbalance between MMPs and TIMPs, thereby leading to enhanced ECM damage and remodeling in acute stages [8, 56, 74, 75]. Angiotensin II increases inflammation and modulates damage via inflammatory cytokines that in turn regulate HSMPs and NOSs [8, 76]. By contrast in old animals, enhanced upregulation of HSMPs may contribute to changes in the ECM-proteolytic pathway either directly via MMPs/TIMPs or indirectly via interactions with pro-inflammatory cytokines and iNOS [27]. Enhanced upregulation of inflammatory cytokines leads to increased MMPs, reactive oxygen species (ROS), iNOS-derived NO, and peroxynitrite which in turn increases ECM damage and LV remodeling [27, 77].

We postulate that aging-related increases in LV angiotensin II [78], a known regulator of ECM expression and remodeling [23], and increased ROS and therefore oxidative stress [79], via dysregulation of inflammatory cell, ECM-proteolytic, and fibrotic pathways, may play major roles in aging-induced impaired healing (Fig. 27.3). More research is needed to identify potential novel therapeutic strategies, proteins, and/or pathways that may be targeted to optimize healing and limit maladaptive fibrosis, adverse LV remodeling, and dysfunction in patients post-reperfused STEMI [8].

27.11 The TGF-β_1/Smad Signaling Pathway and HSMPs

Evidence suggests that interactions between HSMPs and inflammation, ECM-proteolytic, and pro-fibrotic pathways may converge on the TGF-β_1 pathway and Smad signaling. As mentioned, we hypothesize that aging enhances these interactions and leads to dysregulation with increased resistance in this critical TGF-β_1

pathway, resulting in defective healing and ECM/LV remodeling (Fig. 27.3). Increased resistance to TGF-β_1 in old hearts may represent a "protective brake" against high TGF-β_1 levels or exaggeration of a brake gone "haywire." Data on HSMPs and healing after reperfused STEMI in old versus young wild-type mice or mice with absence of the HSMPs are lacking.

27.12 Aging and Events During Healing

We and others [23, 24, 53] have shown that post-MI healing in young animals involves 3 sets of timed molecular and cellular events over 2 highly dynamic phases: (1) an early phase dominated by inflammation—with ECM degradation and inhibition of tissue proliferation, (2) a late phase dominated by tissue repair—with fibroblast proliferation, ECM deposition by fibroblasts and myofibroblasts (containing α-actin), vascular and ECM remodeling with maturation, scar formation, and fibrosis, and (3) structural and functional remodeling spanning early and late phases, through dilation, hypertrophy, and angiogenesis. Emerging evidence suggests that aging can profoundly alter these cellular responses [27, 70, 71].

27.13 Aging and Rate of Healing

It is important to remember that healing takes time; how long depends on age, infarct size, the species and other molecular, cellular, and subcellular factors [21, 22]. Infarct size modulates the rate of healing, reparative and remodeling responses, and functional outcome irrespective of the age or species [21, 23]. Large infarcts heal slower, with more severe LV remodeling [21, 23]. Reperfusion may alter infarct healing in the young [80] and impair healing in the old [55, 66]. However, aging-related adverse remodeling occurs despite small infarct size [41, 66]. It is therefore important to assess both infarct size and LV topography during healing in research studies and in clinics.

27.14 Aging and Regional Differences in Collagen and Fibrosis

The regional distribution of fibrosis and the quality and quantity of collagen are important aspects of remodeling during healing after reperfused STEMI. Normally, the quantity of collagen and fibrosis by the end of the healing phase is several fold greater in infarct than non-infarct zones [23, 24]. Collagen type III deposited during healing is immature, weak, and distensible, and the healing area is more susceptible to dilation. As it matures to stronger and less distensible type I towards the end of the healing phase, the scar becomes less prone to dilation from increased cardiac load. Evidence suggests that aging may amplify these changes and prime the heart for adverse remodeling [27]. It is therefore important to assess regional collagens,

quantity and quality, types I and III, and fibrosis in infarct and non-infarct regions in research studies.

27.15 Aging and the Window of Vulnerability

Decrease in ECM during infarct healing favors adverse LV remodeling, dysfunction, and rupture [23, 24]. Rapid ECM degradation post-STEMI or reperfused STEMI followed by slow synthesis, deposition of type III collagen, and slow maturation to type I results in a window of vulnerability for adverse remodeling. This window provides opportunity for therapeutic interventions [23, 24]. Our recent data suggests that aging may widen this window. It is important to assess remodeling at critical time points before and after collagen plateaus during healing [23, 24].

27.16 Aging and Risk of Adverse Post-MI Remodeling and Rupture

Although age is a strong predictor of adverse events post-STEMI and elderly cohorts are at high risk for heart failure and adverse remodeling [39–41], current therapies do not target these issues [8]. Studies show that the age-related increase in post-reperfused STEMI mortality involves cardiac rupture [40]. Post-MI rupture is common in young mice [81], and ECM damage by MMPs mediates rupture [82]. In humans, thrombolytics have been implicated in post-reperfused STEMI RMI rupture [83], and involves MMP activation [24, 84]. Infarct size is a key factor in rupture and LV remodeling [85, 86]. Rupture induced by high afterload in healed STEMI is linked to decreased and defective collagen [87, 88]. LV remodeling and dysfunction after reperfused STEMI [38] can be aggravated by aging-related enhanced ECM damage and defective collagen and healing. Old mice show defective healing post-reperfused STEMI (impaired phagocytosis of damaged cardiomyocytes, fibroblast resistance to TGF-β_1, decreased myofibroblasts, and infarct collagen) and develop adverse remodeling and rupture [66]. Old rats develop more remodeling and infarct-scar expansion [89], but rupture is rare. Elderly subgroups in clinical trials not only show higher post-STEMI morbidity and mortality from dilative remodeling and heart failure [39, 40] but may develop catastrophic rupture post-reperfused STEMI [83].

27.17 Aging and Impaired Post-MI Healing

As discussed, multiple defects associated with aging can impair post-STEMI healing [8, 69]. Aging affects most factors that modulate post-STEMI healing, including infarct size, neurohormones such as angiotensin II, oxidative stress via oxygen free radicals, NADPH oxidase and nuclear factor kappa B (NFκB) signaling,

inflammation, ECM, HSMPs, and other matrix proteins [8]. Besides dysregulation of repair mechanisms, increased matrix degradation and decreased ECM deposition [90], increased angiotensin II and ROS, increased oxidative damage, dysregulated inflammation, and decreased endothelial NO availability may be involved (Fig. 27.3). It is important to consider effects on HSMPs as well as the inflammation, fibrosis, and ECM-proteolysis pathways when developing therapeutic strategies.

27.18 Aging and Dysregulation of Inflammation and Fibrosis Pathways

Analysis of data from us and others in mice, rats, and dogs [20, 27, 66, 70, 71, 82, 86, 87, 91–103] suggests that impaired post-STEMI healing in older hearts is associated with a healing triad that should be addressed in research studies. The triad consists of the following: (1) impaired, decreased, and/or dysregulated inflammatory cell response [66, 71], with altered expression of mediators of inflammation (increased pro-inflammatory cytokines TNF-α and IL-6), decreased anti-inflammatory cytokine IL-10, increased anti-inflammatory cytokine TGF-β_1, and blunting of inflammatory cell recruitment with decreased inflammatory cells [71]; (2) dysregulation in the fibrosis pathway (with enhanced increase in expression of HSMPs and other matrix proteins and imbalance between MMPs and TIMPs), increased ECM remodeling, delayed granulation tissue formation, decreased myofibroblast density, increased resistance of myofibroblasts (key cellular mediator of fibrosis) to TGF-β_1, and defective collagen in the infarct scar [66, 71]; and (3) adverse LV remodeling and deaths [8, 66, 71].

27.19 Aging and the ECM Post-STEMI

It is important to consider healing and remodeling after STEMI as processes that progress in parallel [22–24]. The current concept is that remodeling is a major mechanism of LV enlargement [23]. ECM disruption promotes dilation and remodeling [23, 104]. Angiotensin II is a major driver of ECM and LV remodeling [23]. ECM degradation after MI is mainly due to MMPs, and TIMPs provide post-translational control of MMP activity [24]. MMPs such as MMP-2 and MMP-9 play a major role in remodeling [8, 24]. A sharp rise in MMPs, with a high MMP/TIMP ratio, induces rapid ECM proteolysis, decreased collagen, adverse ECM and LV remodeling, and dysfunction [23, 24, 48]. Chronically high MMP/TIMP ratios may promote continued ECM degradation and contribute to progressive LV dilation during healing, whereas a low MMP/TIMP ratio may contribute to increased ECM and fibrosis and diastolic dysfunction. Increased MMPs with aging may result in increased LV remodeling and dysfunction during healing after reperfused STEMI [27, 70, 71]. It is important to assess MMP/TIMP balance.

27.20 Aging, Inflammatory Mediators, and TGF-β Signaling

Fibrogenic growth factors such as TGF-β_1 and pro-inflammatory cytokines such as angiotensin II and TNFα modulate MMPs and TIMPs, ECM degradation, fibrosis, and remodeling during healing post-MI [23, 24, 102]. Inflammatory cytokines may have favorable or unfavorable effects on remodeling [105, 106]. TGF-β signaling plays a critical role in healing and remodeling [107], under the regulatory influence of angiotensin II [108]. TGF-β null mice show excessive inflammation and early death [109]. In young animals, TGF-β exerts anti-inflammatory effects during early healing (such as repression of inflammation, macrophage deactivation, resolution of inflammatory infiltrate) and activates pro-fibrotic pathways during late healing (such as fibroblast activation, ECM deposition, myocyte hypertrophy) [107]. Early TGF-β activation protects against early myocardial damage, whereas sustained activation leads to adverse LV remodeling and failure in later stages post-MI in young mice [110]. Attention to timing and differential temporal responses is critical. In young animals, increased TGF-β_1 post-MI can promote ECM preservation during early healing through increased protease inhibitors (e.g., TIMPs, SLPI) and suppression of inflammatory cytokines (e.g., TNF-α and IL-6) and ECM degradation, and promote ECM deposition during later healing by suppressing proteases (e.g., TIMPs, SLPI) [107], and thereby lead to maladaptive remodeling [110]. Our recent data [27, 70, 71] suggest that with aging, increased inflammatory mediator expression together with increased ECM-proteolytic markers and reduced cellular responsiveness to growth factors such as TGF-β_1 may contribute to impaired healing and adverse remodeling after reperfused STEMI. These data suggest that too little or too much inflammation can be bad depending on the stage of healing (early vs. late). This important concept is becoming recognized [111, 112], underscoring the need to assess inflammatory mediators and TGF-β_1 in the infarct and non-infarct zones during both early and late phases of healing.

27.21 Cardiac Drugs and Fibrosis During Healing Post-STEMI

Most therapeutic cardiac drugs used for the management of patients after STEMI have pleiotropic effects that are pertinent during healing [81, 86, 113] and to which senescent STEMI show increased vulnerability [8, 69]. While ACE inhibitors, AT_1R blockers, statins, and beta-blockers are recommended post-STEMI therapies [5], they all decrease collagen synthesis, cytokines, MMP activation, and inflammation in infarct regions of young hearts in experimental studies [8, 54]. However, data with aging is lacking. Angiotensin II is well known to elicit several deleterious effects. ACE inhibitors reduce angiotensin II formation and AT_1R stimulation and limit remodeling post-MI [114, 115], but they do not block angiotensin II from alternative pathways [116]. AT_1R blockers inhibit

AT_1R-mediated effects of angiotensin II [117] and are beneficial for post-MI remodeling and HF [23, 113]. In old patients, both ACE inhibitors and AT_1R blockers are considered beneficial [5]. We have previously reported their beneficial effects in young dogs [88, 93, 95, 103, 118, 119]. Both attenuate TGF-β_1 [88, 105]. It is important to consider that in the real world, ACE inhibitors and AT_1R blockers as well as statins and beta-blockers are recommended therapies in post-STEMI patients, so that new therapies will need to be tested on top of those background therapies.

27.22 Biomarkers of Cardiovascular Risk

Kannel was one of the early pioneers to suggest that biomarkers may be useful for cardiovascular (CV) risk stratification and assessing benefits of therapy, and CV risk assessment may be improved by use of biomarkers, genetic markers, and vascular imaging [120]. Many studies have suggested that biomarkers [121–129] and vascular imaging [130–133] can stratify subgroups at risk of CV disease [129–133] and heart failure [124–128] and guide heart failure management [126]. Several biomarkers [121–129] including hs-CRP [121], N-terminal pro-B-type natriuretic peptide (NT-proBNP) [124–126], cardiac troponin I (cnI) [127], and cardiac troponin T (cnT) [124] can be used to predict heart failure [124–128], adverse remodeling [126, 134], and cardiovascular death in older adults and the elderly [124, 127, 128]. Elderly patients with chronic systolic heart failure (mean ages 66 and 67 years), high serum levels of cortisol, and aldosterone were shown to be independent predictors of increased mortality risk [135]. A strategy to suppress NT-proBNP levels in older adults (mean age 63 years) with chronic systolic heart failure reduced adverse events compared to the standard approach [126]. Another study showed that elderly patients (mean age 70 years) with diastolic heart failure had similar although less severe pathophysiological characteristics than those with systolic heart failure, including BNP levels [136] suggesting that BNP levels may not distinguish patients with systolic and diastolic heart failure. In predominantly elderly patients (mean age 70 years; range 18–104) presenting to the Emergency Department with congestive heart failure, BNP levels were lower in those with non-systolic heart failure than those with systolic heart failure but provided only modest discrimination of the subgroups compared to traditional parameters and felt to be best for distinguishing patients with or without congestive heart failure [137]. In another report of older adults presenting to the Emergency Department with congestive heart failure (mean age 64 years) in REDHOT, BNP levels predicted 90-day outcomes and aided stratification and triage [138]. Thus, biomarkers can be used to guide efforts to reduce CV risk on an individual basis during aging, and biomarkers such as NT-proBNP can guide measures to reduce hospitalization in elderly patients with heart failure. Whereas several markers of CV risk have been studied [139–144], the recent trend is moving towards multimarker testing for prediction of CV disease, including CRP, LpPLa2, MPO, etc., for inflammation and immune system activation; markers of adverse remodeling have not been studied in detail. Markers of collagen

turnover are being evaluated for adverse remodeling in heart failure [145] and post-reperfused STEMI [146]. Other studies suggest that circulating plasma levels of the 3 HSMPs could be used as potential markers in the clinic [147–152], but more studies are needed. The requirements for biomarkers have been reviewed [153].

27.23 Future Directions

In the context of markers of adverse remodeling after STEMI, it is important to remember five pertinent points. First, although LV remodeling during healing post-reperfused STEMI may involve many mechanisms [8, 23, 66], based on recent data [27, 66, 70, 71], it is important to focus on key markers of the healing/repair process and hone in on dysregulation of the inflammatory, ECM-proteolytic, and fibrosis pathways in the infarct zone of aging myocardium and its impact on adverse LV remodeling. Second, since organized collagen deposition and scar formation in the infarct zone, and hypertrophy in the non-infarct zone are important for preserving structural and functional integrity after reperfused STEMI, it is important to focus on key markers of both global LV and differential regional infarct and non-infarct zone remodeling in aging cohorts at the end of the healing/repair process. Third, since remodeling spans phases of necrosis, early and late healing, as shown in our longitudinal studies using two-dimensional echocardiographic (2D-Echo) imaging and three-dimensional (3D) reconstruction to measure detailed in vivo geometric and functional changes [20, 51], and infarcting myocardium undergoes early expansion [4, 15, 16, 92, 94, 95], it is important to make repeated measurements of key markers of remodeling and function throughout (Fig. 27.4), from the onset of reperfusion, during the infarction process, and the healing/repair process and beyond in different aging cohorts. As remodeling extends beyond the healing/repair phase [154], it is important to extend measurements to remote STEMI. Fourth, since healing to scar formation after MI takes ~1 week in young mice [21, 22, 66] and longer in larger animals and humans [155], and may be delayed after reperfused STEMI with aging, it is important to measure markers of in vivo structural/functional remodeling through the entire time interval using noninvasive imaging and correlate these with changes in markers of collagen turnover and other key pathways in the healing/repair process. This is crucial because dynamic changes in remodeling occur during early healing before the collagen plateau and later healing after the collagen plateau [91, 97, 154], and concurrent dynamic changes also occur in inflammation, ECM-proteolytic and fibrotic pathways, and in the intersecting pathways during early versus late phases of healing/repair. Fifth, since our preliminary recent data [27, 70, 71] suggested lesser benefit from angiotensin II inhibition and downregulation of AT_2 and ACE-2 pathways in the oldest cohorts, implying differential regulation with aging by angiotensin II, it will be important to measure markers in aging cohorts with the view of formulating therapeutic strategies for the clinic.

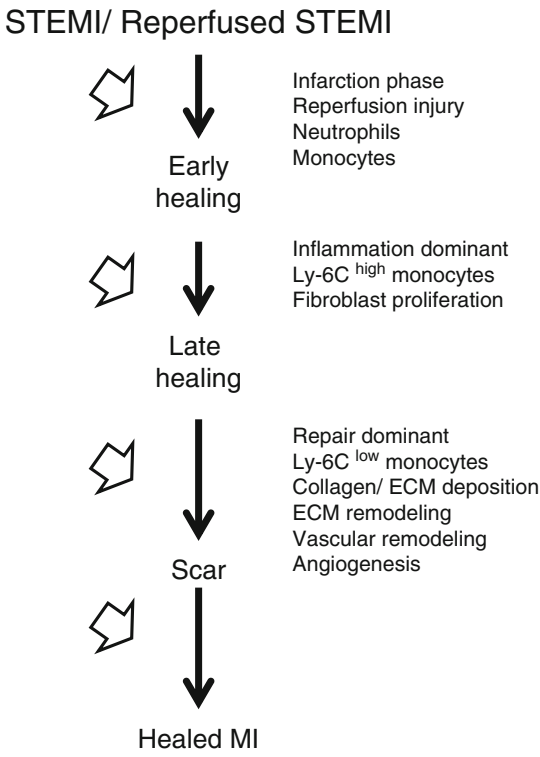

Fig. 27.4 Proposed paradigm for anti-remodeling therapy during healing/repair after STEMI The algorithm in the last 3 decades has been to use early and prolonged therapy with few pharmaceutical agents proven to improve outcome in evidence-based randomized clinical trials (RCTs). Emerging evidence suggests that there are multiple potential targets for different stages of the healing process (early, late, and remote). Pulses of therapy tailored for the different stages and novel targets may more effectively improve outcome in patients with STEMI. Multiple markers, different for the different stages, may prove more effective for monitoring pathophysiologic changes, progression and response to therapy

27.24 Conclusion

Based on emerging concepts and the recent evidence presented in this chapter, it is clear that what happens during a STEMI and the subsequent healing/repair process impacts outcome and what happens beyond. There is therefore an urgent need for more focused translational research to develop new therapeutic strategies for optimizing the healing/repair process and thereby limit adverse LV remodeling after STEMI and reperfused STEMI. In that context, emerging biomarkers may provide tools to noninvasively monitor the remodeling process, predict adverse remodeling and clinical outcome, and monitor responses to therapy in patients of different ages and at different stages after the STEMI.

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Chapter 28
Optimizing Stem Cell Therapy for Cardiac Repair Following a Myocardial Infarction

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Abstract An extensive myocardial infarction (MI) frequently results in post-infarct remodelling, which can lead to heart failure. Emergency percutaneous coronary intervention (PCI) has reduced the mortality of ST-elevation MI, and the use of beta-blockers, angiotensin-converting enzyme (ACE) inhibitors, and anticoagulants has improved the morbidity. However, progressive ventricular dysfunction is increasingly common. New treatments are urgently needed for MI survivors to prevent pathological remodelling and functional loss. Replacing the cells lost during the infarction might limit detrimental remodelling, but none of the proposed treatments have been shown to completely restore beating cardiomyocytes. Stem cell transplantation was originally proposed to replace these lost cells, but extensive animal and human studies have suggested that the benefits of cell implantation were the result of paracrine effects. Several stem cell populations that improved ventricular function in preclinical studies have also been beneficial in clinical trials to

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treat post-MI remodelling. However, most of these trials had mixed results, highlighting the need for further research into the mechanisms responsible for improved cardiac function and the need to develop new treatment strategies to augment the beneficial effects of stem cell transplantation.

Keywords Myocardial infarction • Cell transplantation • Heart failure • Stem cells

28.1 Introduction

28.1.1 Post–Myocardial Infarction, Cardiac Remodelling, and Heart Failure

A myocardial infarction (MI) results from coronary artery occlusion and prolonged cessation of blood flow to the myocardium. The extended period of ischemia leads to myocardial damage as a result of extensive cardiac cell death, which initiates ventricular remodelling and eventually heart failure.

Heart failure is the inability of the heart to provide sufficient cardiac output to meet the metabolic demands of the body. This is a disabling and deadly condition that arises from various forms of heart disease, including acute coronary occlusion. An extensive MI frequently remodels the myocardium as a result of cardiomyocyte death and the resulting extracellular matrix (ECM) turnover and inflammation. The extent of damage determines the severity of the remodelling, including scar thinning and eventual ventricular dilation and dysfunction.

Inflammation early after coronary occlusion initiates a cascade of events that result in further cell death and more widespread tissue remodelling [1]. Inflammation in the infarct area results in not only the removal of the necrotic cells but also apoptosis in surrounding tissues. After the acute inflammatory response has resolved, additional pathophysiological remodelling begins. Following the removal of the necrotic material, fibroblasts produce ECM components, and remodelling begins with fibrosis and the loss of elasticity of the noncontractile infarcted region, which then becomes noncompliant and stiff. The loss of beating cardiomyocytes increases the physiological load on the remaining cells and promotes pathophysiological hypertrophy, including altered energy metabolism, contractile proteins, and excitation–contraction coupling. In response to high intraventricular pressures and insufficient contractility, this stiff scar thins and begins to dilate. Over time, this pathological remodelling may lead to heart failure and eventual death [2–5].

28.1.2 Current Treatment Options

Coronary artery disease (CAD) is the narrowing (stenosis) of the coronary arteries by plaques [6], which restricts blood flow to the myocardium and increases the susceptibility of rupture, thrombosis, and vessel occlusion [7]. To prevent coronary

occlusion, patients can undergo percutaneous coronary intervention (PCI), which opens the narrowing by introducing a catheter into the coronary artery and inflating a balloon that crushes the plaque into the walls of the artery. During PCI, it is common to implant either a bare metal or drug-eluting stent (a wire mesh that maintains the lumen) to prevent the artery from becoming narrowed again (restenosis). An alternative to PCI is coronary artery bypass graft (CABG) surgery, in which arteries or veins from the patient's own body are employed to redirect blood around the narrowed artery. These treatments prevent the progression of CAD and reduce the risk of MI in the future.

Current therapies following an MI attempt to prevent heart failure. Beta-blockers are a class of beta-adrenoreceptor antagonists that diminish the effects of sympathetic stimulation. ACE inhibitors act on the renin–angiotensin system and also reduce the risk of heart failure and maintain the function of the blood vessels. Finally, aldosterone blockers prevent the action of aldosterone in the kidneys, which might potentiate heart failure. Both PCI and CABG surgery are very effective in relieving angina and allowing patients to return to full activity without chest pain.

There is no cure for heart failure other than heart transplantation, which is limited by donor availability. Ventricular assist devices (VADs) [8] were developed to augment the left, right, or both sides of the heart and can reverse remodelling and restore normal ventricular function and metabolism [9, 10]. Unfortunately, attempts to remove the VAD after the heart has returned to normal size and function have been associated with recurrent remodelling, resulting in the need for reinsertion of the VAD or transplantation. Given that current therapies can only prevent the progression of heart failure and do little to permanently reverse the damage caused by MI, development of new treatments is required to regenerate the heart following an extensive infarction.

28.1.3 Cell Therapy for Cardiac Repair

Although medication and interventional and surgical procedures reduce mortality and morbidity post-MI, many patients are susceptible to progressive ventricular dysfunction and become severely limited by heart failure. New treatments to prevent pathological remodelling and functional loss after surviving an MI are needed. Cell transplantation approaches that replace the lost myocardial tissue with muscle cells may reduce scar thinning and dilatation and prevent heart failure.

28.1.3.1 Fetal Cardiomyocytes

Initial studies evaluated several somatic cell types transplanted into the damaged myocardium. Adult cardiomyocytes have a limited proliferative capacity and do not reproduce in vivo to replace lost myocardium after injury. However, fetal cardiomyocytes are capable of replication. Fetal cardiomyocytes were implanted into the injured heart of rodents [11, 12], and these cells engrafted in the heart, proliferated, and integrated with the recipient myocardium. More importantly, these cells formed muscle tissue, prevented ventricular dilation, and improved cardiac function.

Although these encouraging results demonstrated the feasibility of using muscle cells to repair the heart and restore cardiac function, ethical concerns, evidence of uncontrolled propagation, and the allogeneic nature of fetal cardiomyocytes limited the enthusiasm of this cell type for clinical applications.

28.1.3.2 Skeletal Myoblasts

Skeletal myoblasts are a source of highly proliferative autologous cells that have the potential to form beating muscle in the heart. To evaluate their potential, Chiu et al. [13] implanted skeletal muscle satellite cells into the injured myocardium of dogs and provided histologic evidence of new striated muscles with intercalated discs and centrally located nuclei. Their data were consistent with the hypothesis of milieu-influenced differentiation of satellite cells into cardiac-like muscle cells. Menasche et al. [14] were the first to successfully implant skeletal myoblasts into the post-infarct scar of patients undergoing CABG. Five months after the combination of CABG and cell implantation, ventricular function improved. Subsequent studies demonstrated that transplanted myoblasts engrafted in the infarct scar, induced angiogenesis, prevented adverse matrix remodelling, and improved cardiac function [15, 16]. However, the phase II/III clinical trial (MAGIC trial) failed to demonstrate better global or regional ventricular function in the myoblast-implanted patients compared to a placebo-injected control group [17]. Although the cell transplant group had smaller ventricular volumes and improved symptomatic recovery, the trial was considered negative, and attempts to commercialize this therapy were abandoned. An additional concern related to myoblasts was the increased incidence of ventricular arrhythmias, possibly due to electrical coupling between the transplanted cells and the recipient tissue. These early clinical trials demonstrated the feasibility of implanting somatic cells into the damaged hearts of patients to restore cardiac function.

28.2 Stem Cell Therapy for Cardiac Regeneration

Stem cells hold the promise to repair the damaged myocardium and regenerate the heart. This capability has been attributed to their multipotent nature. They can form terminally differentiated cells based on their microenvironment and contribute to tissue repair. A number of stem cells have been suggested to induce cardiac repair and regeneration in both basic and clinical research studies [18].

28.2.1 Bone Marrow–Derived Stem Cells

Bone marrow (BM) transplantation has been performed following irradiation to replace hematopoietic cells. The BM houses stem cells and progenitors that are

capable of regenerating cells in different tissues. The major goal of injecting BM cells into the heart was to prevent progressive pathological remodelling and heart failure by stimulating repair of the damaged tissue [19]. Initially, investigators hoped that the transplanted cells would differentiate into cardiomyocytes to replace the cells lost during the infarction. In 2004, studies were published which demonstrated that these stem cells did not differentiate into functional cardiomyocytes. Instead, the benefits of the transplanted stem cells were attributed to the release of growth factors in the heart, which promoted angiogenesis, prevented adverse matrix remodelling, and stimulated resident cardiac stem cells to initiate repair programs [20].

As the limitations of somatic cell implantation for cardiac repair became apparent (such as age and pre-existing risk factors), other types of multipotent stem cells were evaluated for regenerating the damaged myocardium. These cells are not only capable of differentiating into new cardiomyoctes but can also form endothelial cells and smooth muscle cells, which are required to form new blood vessels to regenerate the vasculature of the infarcted area. These multipotent stem cells are required to proliferate and differentiate into the resident cell types needed to constitute viable myocardial tissue. Several candidate stem cell populations have been examined for their therapeutic potential and are examined below. Recent studies have also shed light on the importance of not only the implanted cells but also the cellular microenvironment that the cells are injected into. Stem cell function is critically influenced by extrinsic signals derived from the niche or the microenvironment that the stem cells reside in. This niche plays a key role in the therapeutic modulation of stem cell behavior [21]. Therefore, in addition to providing the necessary cells for regeneration, the optimal extracellular milieu for those cells has emerged as a critical factor in stem cell therapy for cardiac regeneration.

BM has been suggested to regenerate the heart [22], as evidenced from studies showing that transplanted cells are capable of participating in post-MI repair. However, it was not established which cell source was contributing to the cardiac repair; thus, several groups have attempted to characterize the BM-derived cells that participate in post-MI repair. Although the initial reports suggested that BM cells regenerated cardiomyocytes, endothelial cells, and smooth muscle cells in the infarcted ventricle [22], subsequent studies determined that BM cells did not transdifferentiate to form new cardiomyocytes [17, 23]. Despite the controversy over cellular differentiation, the evidence is convincing that BM cells improve cardiac function and prevent remodelling after an extensive MI.

The encouraging results from preclinical animal studies prompted several clinical trials of bone marrow mononuclear cells (BMMNCs). The initial clinical trials demonstrated improved cardiac function after an extensive MI [24, 25]. BM is a rich source of stem cells, including hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), and mesenchymal stem cells (MSCs). The following stem and progenitor cells isolated from BM have been studied for their capacity to repair the heart post-MI.

28.2.1.1 Hematopoietic Stem Cells

HSCs exist as long-term and short-term regenerating cells that cycle in the BM stem cell niche. Coculturing cardiomyocytes with HSCs has been shown to protect the cardiomyocytes from cell death via paracrine factors [26]. The differentiation of BM-derived cells to a myogenic phenotype is dependent on HSCs with intact c-kit function [27]. Furthermore, the endogenous repair of the myocardium is dependent on the function of the c-kit receptor, which is expressed on HSCs [28].

28.2.1.2 Mesenchymal Stem Cells

MSCs are multipotent stromal cells that are able to differentiate into cell types of several lineages [29]. They can be isolated from the BM or adipose tissue and can be stored long term for later implantation. In addition, MSCs suppress allo-responses and may be immuno-tolerant [30]. Therefore, they might be an ideal "off-the-shelf" product to treat MI [31, 32]. The original evaluations of allogeneic MSC transplantation demonstrated beneficial restoration of ventricular function comparable to the effects of syngeneic or autologous MSC implantation. However, the long-term effect of allogeneic MSCs was recently demonstrated to be limited by rejection [32]. Huang and colleagues demonstrated that between 3 weeks and 3 months after implantation, MSCs that differentiated became immunogenic and were rejected, resulting in deterioration of ventricular restoration and function [33].

28.2.1.3 Endothelial Progenitor Cells

EPCs are circulating stem cells involved in repair and regeneration of blood vessels. EPCs have been used for repairing the heart after an infarct in animal experiments as well as clinical trials [34]. The underlying mechanisms of cardiac regeneration are mediated by augmenting neovascularization post-MI. Enhancing revascularization reduces the extent of apoptosis in and around the infarcted region. The increase in survival of cardiomyocytes precludes the expansion of fibrosis, thus negating scar dilation.

28.2.2 Cardiac Progenitor Cells

Aside from the benefits derived from implanting noncardiac stem and progenitor cells to repair the heart, recent studies have pointed to cardiac-resident stem cell populations as also participating in cardiac repair. The benefits of cell implantation result in part from the stimulation of cardiac-resident stem cells [35]. Recently, these cardiac stem cells (CSCs) have been isolated and employed for cell transplantation. Many different types of CSCs have been described, and two have been employed in recent clinical trials. The first cell type includes the c-kit$^+$

cell populations that are believed to reside in niches within the myocardium. They were expanded from atrial appendages harvested during CABG surgery and injected into the infarct-related artery in the SCIPIO trial [36]. The other progenitor population is cardiosphere-derived cells (CDCs) produced from human endomyocardial biopsies that were expanded into spheroids in suspension cultures [37]. These cells are believed to improve heart function after an MI by paracrine mechanisms [38]. The recent CADUCEUS trial demonstrated that the intracoronary infusion of autologous CDCs after MI was safe and may be efficacious [39]. Continuing work in basic and clinical science will determine whether these cells can produce sufficient recovery in selected patient groups.

28.3 Optimizing Stem Cell Therapy to Regenerate the Infarcted Myocardium

28.3.1 Challenges of Translating Preclinical Research into Clinical Trials

Preclinical studies demonstrated profound beneficial effects of a variety of stem cells injected into the heart. However, the clinical trials have shown less robust results [40, 41]. There are several factors that contribute to the discrepancies observed between animal and human studies. Preclinical trials often do not take into consideration the comorbidities of the patients receiving this therapy. Diabetes, hypertension, chronic obstructive pulmonary disorder (COPD), dyslipidemia, and kidney disease are all strongly associated with aging, a factor that is most likely a critical determinant in the success of cell therapy for cardiac repair.

The age of the cells being injected as well as the age of the patient receiving the cells are both important determinants of the benefit achieved. The key difference between the preclinical and clinical studies is the age of the individuals treated. Preclinical studies were initially performed only in young healthy animals, but the clinical trials were performed in older patients with diffuse CAD. To improve the clinical benefits of cell therapy to the extent achieved in the preclinical studies, additional interventions will be required. These interventions include using young (allogeneic) or rejuvenated autologous stem cells, restoring the response of the recipient to stem cell injection, enhancing engraftment of the cells, and augmenting the paracrine benefits of the implanted cells.

Recent studies have demonstrated that stem cells from aged individuals have a limited capability for proliferation and differentiation, and aged recipients have a diminished capability to generate a reparative response to cell implantation [35, 42–44]. Therefore, restoration of ventricular function in aged patients by stem cell therapy will require rejuvenation of both the implanted stem cells and the response to cell implantation [35]. The methods to rejuvenate stem cells from aged individuals include cytokine therapies and BM transplantation [45]. The other approach is to use allogeneic cells from young, healthy individuals.

28.3.2 Allogeneic Stem Cells

To avoid the problems associated with autologous cells from aged individuals, allogeneic stem cells from young donors can be transplanted into the infarcted myocardium. Allogeneic cells have been demonstrated to improve cardiac recovery after MI, and they are not initially rejected early after implantation [46, 47]. Allogeneic MSCs are currently undergoing clinical trials [48]. However, allogeneic cells likely will lose their immunoprivileged state when they differentiate after implantation. A number of strategies have been proposed to prevent the rejection of allogeneic MSCs. Establishing a chimeric BM with donor BM cells may prevent rejection [49]. Induction of tolerance has also been proposed by transfection with interleukin 10 (IL-10) [50]. New approaches to immune modulation may produce dramatic and long-lasting improvements in ventricular function with allogeneic MSCs.

28.3.3 Enhancing Engraftment and Paracrine Effects of Stem Cell Therapy

Previously, the strategy for stem cell therapy for cardiac repair was one of replacing the damaged myocardium with new cells. However, with advancing research, it has become apparent that the tissue needs to be repaired with a combination of cell therapy and factors to enhance the engraftment and repair by these cells. Strategies to boost the repair capacity of implanted cells include the use of cytokines to support cell survival as well as biomaterials designed to aid in cell engraftment. The recent availability of new biodegradable biomaterials offers the opportunity to augment the survival and engraftment of cells implanted into the infarcted heart. Temperature-sensitive hydrogels that are liquid at room temperature and a gel at body temperature can enhance cell engraftment and provide prolonged cytokine delivery [51]. Biodegradable biomaterial meshes can also be employed to provide sustained cytokine release to augment stem cell survival and engraftment, and the improvement of ventricular function [52]. These biomaterials can provide sustained cytokine release to enhance the paracrine benefits of stem cell therapy to aged individuals and restore ventricular function.

28.3.4 Strategies for Improving Stem Cells for Transplantation

Additional strategies to enhance cell survival and engraftment include optimizing the time of delivery and preconditioning the cells prior to their implantation. The optimal time for cardiomyocyte transplantation after myocardial injury was found to be after the initial inflammatory reaction but before the onset of scar expansion [53]. However, the optimal time to implant non-cardiomyocytes, such as stem and

progenitor cells, has not been conclusively determined but may be similar. Ex vivo pretreatment with growth factors or hypoxic environments may confer cytoprotection to increase the survival of implanted cells in the harsh environment of the infarct region. A variety of factors have been evaluated, including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) [54], insulin-like growth factor-1 (IGF-1), and transforming growth factor-β (TGF-β), to augment the efficacy of cells for myocardial repair. Alternatively, preconditioning the stem cells by genetically modifying them to express pro-survival factors may similarly increase their survival and functionality.

28.4 Perspectives

Despite the limited benefit of stem cell therapy in the initial clinical trials, the improvements in ventricular function achieved after an extensive MI were as great as those reported with PCI, beta-blockers, or ACE inhibitors [55, 56]. However, standardization of procedures for patients and further improvements in techniques are needed to reach the benefits demonstrated in preclinical animal studies. The optimal stem cell will need to be established in comparative studies. The ideal stem cell will rapidly engraft and stimulate the cardiac stem cell niche to recruit the endogenous regenerative capacity of the heart.

Further comparative preclinical studies will permit the comparison of alternate stem cell candidates, but comparative clinical trials are also necessary to design a therapy for old, debilitated patients who are most in need of cardiac regeneration. Finally, long-term studies are needed to adequately determine the efficacy of stem cell therapy. Now, more than ever, regenerative therapies to prevent heart failure will require multidisciplinary efforts from clinicians and scientists to apply the latest advances in stem cell and regenerative biology to the patients most in need of this therapy.

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Chapter 29
Regulation of Fibrosis After Myocardial Infarction: Implications for Ventricular Remodeling

Bodh I. Jugdutt

Abstract Fibrosis plays a critical role in ventricular remodeling after myocardial infarction (MI). The normal physiologic response to tissue injury in mammals involves activation of the healing process that leads to repair of the injured area with a fibrotic scar over time. In the heart, healing of the damaged area of the left ventricular (LV) chamber after MI with a firm fibrous scar is essential for the chamber to continue to pump blood effectively into the tissues. Optimal healing and repair of the wounded heart with appropriate fibrosis is critical for restoration of chamber size, shape, integrity, and systolic function and survival with a favorable outcome. Dysregulated healing and repair mechanisms may lead to adverse remodeling and poor outcome. While reparative fibrosis in the infarct zone is desirable, reactive interstitial fibrosis in the non-infarct zone contributes to chamber stiffness and diastolic dysfunction. Evidence suggests that the regulation and temporal progression of fibrosis may differ in the two zones. In addition, fibrosis in the infarct and non-infarct zones may become dysregulated and contribute to mixed dilative and hypertrophic remodeling and mixed LV systolic and diastolic dysfunction. Besides reparative and reactive fibrosis in the infarcted LV, fibrosis that develops at remote sites such as the atrial and right ventricular chambers and the kidneys can influence outcome after MI. Understanding the differential mechanisms of fibrosis in the infarct, non-infarct, and remote zones may allow the development of new and improved therapeutic strategies for controlling fibrosis and improve outcome in survivors of MI.

Keywords Fibrosis • Healing • Inflammation • Matrix remodeling • Monocyte populations • Myocardial infarction • Reperfusion injury • Repair • Ventricular remodeling

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Fig. 29.1 The march from injury to fibrosis in noncardiac tissue

29.1 Introduction

Fibrosis plays a critical role in ventricular remodeling after myocardial infarction (MI). An important aspect of the normal physiologic response to tissue injury in mammals involves activation of the healing process that leads to reparative fibrosis and scar formation in the injured area over time (Fig. 29.1). While there are common features in the healing/repair process after skin and heart wounds, there are also important differences as the heart is a highly dynamic organ that contracts and relaxes many times per minute throughout life. For example, in the wounded heart, healing of the damaged area of the left ventricular (LV) chamber after an MI with a firm fibrous scar is essential for the chamber to continue to pump blood effectively into the tissues (Fig. 29.2). Optimal healing and repair of the wounded heart with appropriate fibrosis is therefore critical for restoration of chamber size, shape, integrity, and systolic function and survival with a favorable outcome [1–10]. Emerging evidence suggests that dysregulation of healing and repair mechanisms may lead to adverse ventricular remodeling and poor outcome [11–13]. Adverse ventricular remodeling during the healing/repair phase after MI continues to be an important problem in adult cardiology despite improved coronary reperfusion and medical therapies [14–18]. Despite the search for specific molecular targets that may lead to discovery of therapeutic strategies to optimize post-MI healing and repair, optimize quality and quantity of fibrosis, prevent adverse remodeling, and improve outcome since the 1980s, specific therapy to realize this goal is still lacking in the twenty-first century [11–13, 19]. This chapter focuses on the regulation of fibrosis after MI and its implications for ventricular remodeling. Understanding the differential mechanisms of fibrosis in the infarct, non-infarct, and remote zones may allow the development of new and improved therapeutic strategies for controlling fibrosis and improve outcome in survivors of MI.

29.2 Definitions and Caveats

Five important points must be considered in translating findings from the bench to the bedside. First, a coronary artery occlusion puts myocardium in the occluded coronary bed at risk of ischemic damage [20], and persistent occlusion usually results in two types of MI depending on net collateral blood supply [21–25]. Transmural MI correlates with pathological necrosis ≥50% of the transmural extent, or evidence of Q-wave MI or ST-segment elevation MI (STEMI) on electrocardiograms, whereas subendocardial MI correlates with pathological necrosis <50% of the transmural extent, or evidence of non-Q-wave or non-STEMI (NSTEMI) on

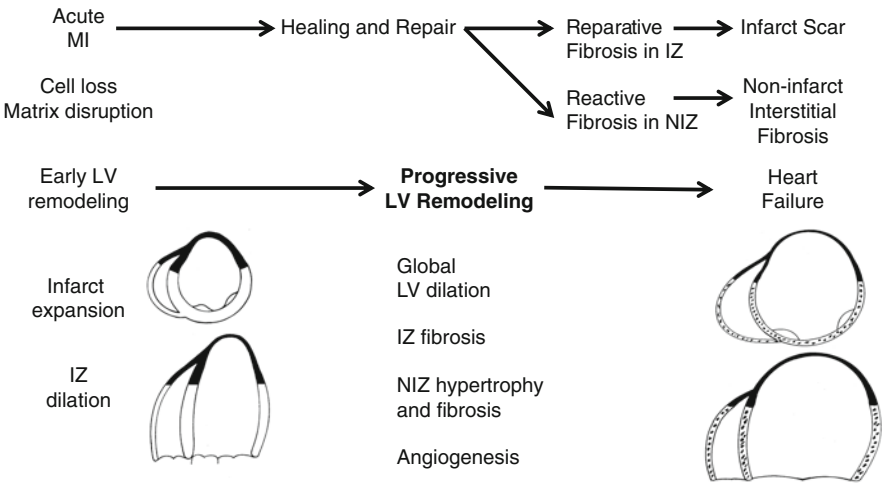

Fig. 29.2 The march from injury to fibrosis in the heart. *Upper panel*: This cartoon depicts the concept of reparative fibrosis in the infarct zone and reactive fibrosis in the non-infarct zone over time. *Lower panel*: This cartoon depicts early regional remodeling in the infarction phase and further global dilative remodeling in the healing/repair phase and beyond scar formation. Data based on anterior infarcts in the canine model. *IZ* ischemic zone, *LV* left ventricular, *MI* myocardial infarction, *NIZ* non-infarct zone

electrocardiograms [21, 22]. Clinically, an acute coronary syndrome can lead to either STEMI or NSTEMI that has different clinical outcomes and needs different management strategies [22–25]. All evidences indicate that STEMI has grave prognosis and outcome in the survivors [3, 7, 16, 18, 26]. This chapter will focus on fibrosis and remodeling after STEMI.

Second, most human MIs are the result of coronary artery occlusion caused by inflammation of an atherosclerotic plaque and thrombosis [27–30], whereas most animal models of MI lack this inflamed atherosclerotic component. In that context, Panizzi et al. recently studied MI in the hypercholesterolemic ApoE−/− mice with atherosclerosis and systemic monocytosis and showed close recapitulation of the inflammatory and cellular responses found during healing after MI in humans [31].

Third, as there are species-specific differences in the inflammatory and cellular responses during healing and repair after MI in mice and dogs [32], caution is advisable when extrapolating findings from animal studies to humans. Fourth, whereas MIs and heart failure are dominant in elderly humans [11, 33], the majority of studies of MI and fibrosis at the bench have been conducted in very young animals; caution is therefore needed in extrapolating findings from animal studies to older humans [33–35]. Bujak et al. recently showed that aging results in impaired healing/repair and adverse remodeling after reperfused MI in mice [34]. In our laboratory, studies in the dog [36, 37] and rat [38] models of reperfused STEMI showed that aging enhances adverse remodeling and the inflammatory responses [36–38].

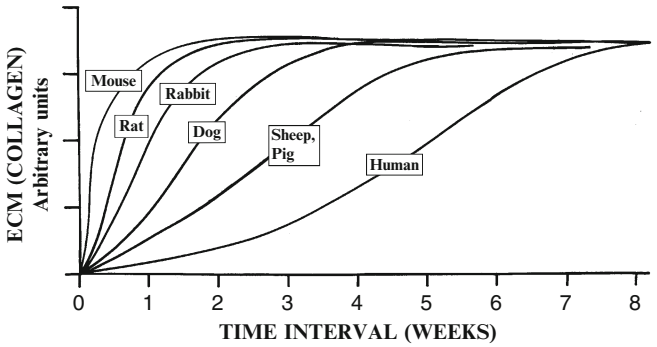

Fig. 29.3 Species and time to collagen plateau during post-MI healing and repair. Cartoon showing that healing takes time that varies between weeks and months depending on the species and infarct characteristics (size, reperfusion); large infarcts in humans may take 3–6 months to heal after MI depending on infarct size. The duration of healing is measured by the time interval to reach the collagen plateau for moderate myocardial infarct size. The duration of healing is shorter in smaller animals. *ECM* extracellular matrix, *MI* myocardial infarction

Fifth, the rate of healing and repair is influenced by several factors in any one species. These factors include infarct size, whether reperfused or not, other molecular, cellular, and subcellular factors [3–8]. Old age not only is associated with impaired healing as noted above [11, 17, 34, 35, 38] but also results in increased infarct size [36, 37]. More important, the rate of healing and repair differs among species. Thus, the march to the collagen plateau, assessed by changes in hydroxyproline concentration in the infarct zone as a marker of fibrosis, is more prolonged in larger animals (Fig. 29.3). In Fig. 29.3, the interval to the collagen plateau is between 1–2 weeks for mice, 2–3 weeks for rats, 4–6 weeks for dogs, and 8–12 weeks for humans. Accordingly, it is important to remember the time factor when interpreting data from different animal species, or extrapolating data on temporal changes from small animals to humans, or planning therapeutic interventions.

29.3 Pathophysiology of Healing, Repair, Fibrosis and Remodeling After MI

Early post-mortem and clinicopathological studies of healing/repair after myocardial infarction in the first half of the twentieth century (reviewed in [2, 6–9]) extended the simple algorithm from *injury to fibrosis of damaged tissue*, as found in the skin and other noncardiac tissues (Fig. 29.1), to the more complex one of *reparative fibrosis in the infarct zone and reactive fibrosis in the non-infarct zone* accompanied by dilation and differential structural remodeling in both zones (Fig. 29.2). Cumulative evidence from histopathological, morphological, and biochemical studies in humans and experimental animals between the late 1970s and mid-1990s expanded the algorithm further.

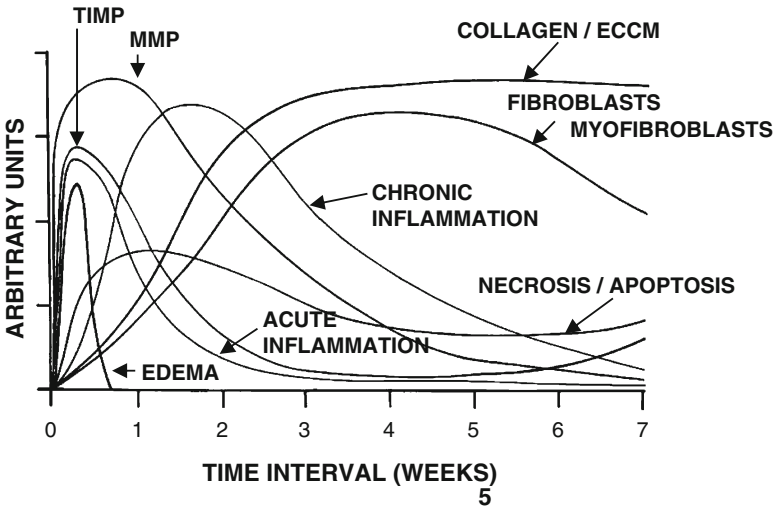

Fig. 29.4 Histopathological and extracellular matrix changes in the infarct tissue during healing and repair after MI. Cartoon depicting temporal changes in necrosis and apoptosis, edema, acute and chronic inflammation, fibroblast proliferation, myofibroblast formation, collagen, and extracellular collagen matrix (ECCM) in the infarct zone of the dog model of anterior myocardial infarction (MI). Collagen content is based on the hydroxyproline assay. The data is based on changes in the infarct zone from 194 canine hearts from previous studies. *MMP* matrix metalloproteinase, *TIMP* tissue inhibitor of MMP

In the new construct, acute MI results in early myocardial necrosis with cell loss and damage to the extracellular matrix (ECM); ECM damage drives the early dilative remodeling and progressive dilative remodeling; necrosis triggers the healing/repair process that leads to fibrosis and scar in the infarct area and maladaptive remodeling with fibrosis and hypertrophy in the non-infarct area [3–9, 11, 39–41]. Many studies attributed the ECM degradation to the release and activation of matrix metalloproteinases (MMP), resulting in an imbalance between levels of MMPs and endogenous tissue inhibitors of MMPs (TIMPs) in the infarct zone (Fig. 29.4). This elevated MMP/TIMP ratio persists during the healing/repair phase (Fig. 29.4) and is a major factor in progressive dilative remodeling [7, 8].

A host of studies between the 1990s and 2010 have addressed the molecular and cellular changes and extended the algorithm further, and identified inflammation and ECM remodeling as two key components of healing/repair, fibrosis, and remodeling after MI. The collective evidence from those studies suggests that the healing/repair process is finely orchestrated and involves tandem reactions over two highly dynamic phases: an early phase that is dominated by inflammation (with release of inflammatory cytokines, ECM proteases, and bioactive molecules, leading to ECM degradation and damping of proliferation) and a late phase dominated by repair (with release of fibrogenic cytokines, fibroblast proliferation, ECM deposition and maturation, ECM and vascular remodeling, fibrous scar formation, and remote fibrosis) [3–9, 11, 39–41].

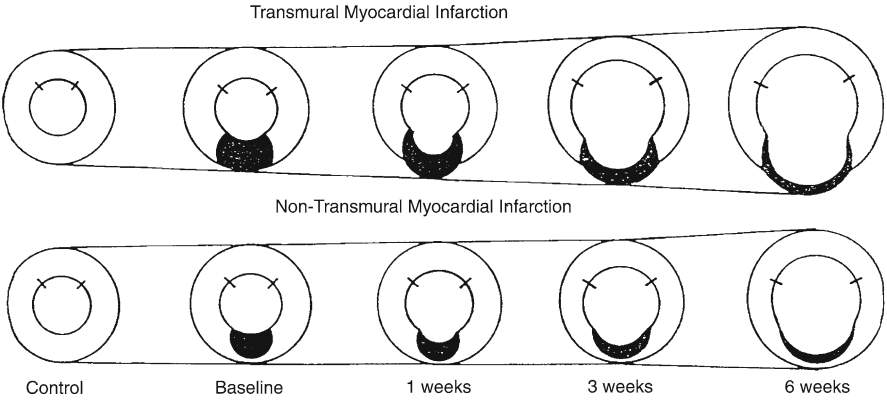

Fig. 29.5 Topographical changes during healing and repair after myocardial infarction. Cartoon depicting regional dilative remodeling and global dilative remodeling over time after anterior myocardial infarction (MI) in the dog model. Data based on systematic mapping of changes in geometry of mid-papillary transverse sections of 194 canine hearts with transmural and non-transmural infarcts at five selected time intervals using computerized morphometry. Adverse remodeling of infarct and non-infarct zones is less with subendocardial than transmural infarcts. Infarct area in black

Between the 1980s and 2010, a series of studies using repeated cardiac imaging using detailed two-dimensional echocardiography with three-dimensional reconstruction after acute MI has documented significant dilative LV remodeling after MI that spans the infarction, healing/repair phases, and beyond scar formation in the dog model and humans [42–53]. Clinical and pathological correlations were made in most of these studies [54]. Morphometric data from hearts removed at different intervals during the healing and repair phase in the dog model allowed characterization of the progressive regional infarct and global LV structural remodeling that is more severe with transmural than subendocardial MI (Fig. 29.5) and correlates with simultaneous echocardiographic imaging data [3–9].

In a longitudinal study of 70 patients with chronic heart failure after MI who were assessed prospectively, Gaudron et al. showed that progression of LV dilation and dysfunction may continue for up to 3 years [55]. This suggests that factors operating during the healing/repair phase and beyond can negatively impact LV remodeling and outcome. Importantly, they found that the high-risk subset could be predicted by initial estimates of infarct size, ejection fraction, coronary perfusion grade, as well as infarct location at 4 days [54]. These findings are in agreement with our previously reported findings in a prospective study of 244 patients with a first Q-wave MI, indicating that the degree of initial regional infarct dilation or infarct expansion (that correlated with infarct size, anterior infarct location, and low ejection fraction) predicted a high-risk subset for poor outcome [48].

Clearly large transmural infarcts would be expected to be associated with slower healing and repair, more fibrosis in both infarct and non-infarct zones, and more progressive adverse remodeling [7, 8]. Pertinent to the theme of progressive adverse

remodeling at 3 years in Gaudron's study [55], infarct scars contain specialized myofibroblasts formed by phenotypic conversion of fibroblasts and containing sarcomeric α-smooth muscle actin, rendering the scar tissue capable of contraction ([9] for review; [56–64]). This emphasizes that scars should be considered live and dynamic rather than dead tissue [60], and myofibroblasts were shown to persist for up to 17 years in the infarct scars [61]. In contrast to infarct scars, myofibroblasts are transient in skin wounds [9]. This finding has important implications for therapeutic interventions to attenuate fibrosis and use of therapeutic cardiac drugs with pleiotropic effects that impact fibrosis pathways ([65, 66] for review).

29.4 Advances in Monocyte and Macrophage Biology During Healing/Repair Post-MI

It follows from the above discussions that, besides infarct size and altered composition and integrity of the supporting ECM, the adequacy or quality of healing/repair after MI is another major determinant of infarct fibrosis and ventricular remodeling. Emerging evidence over the last decade has significantly enhanced our understanding of the cellular aspects of the march of healing and repair to fibrosis after MI and further expanded the previous algorithms (Fig. 29.6). The inflammatory reaction after MI has two main functions: (1) to clear the infarct area of dead cells and matrix debris and (2) to activate the pathways that are necessary for optimal scar formation. These functions are largely modulated by recently identified different monocyte and macrophage phenotypes ([41, 67, 68] for review) and are highly relevant clinically [69–71].

In that construct, dysregulation of the inflammatory response, with prolongation or expansion of the response, results in impaired collagen deposition and weak scars, thereby leading to an augmentation of LV remodeling and dysfunction [7–9, 41]. As noted before, inflammation and ECM remodeling are closely linked. Excessive early inflammation can augment ECM degradation and promote severe early infarct expansion and precipitate cardiac rupture [72]. Increased expression of pro-inflammatory mediators, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), can activate pro-apoptotic pathways and thereby contribute to more cardiomyocyte loss.

In a recent study, early reperfused STEMI in the dog model was associated with increased pro-inflammatory markers such as inducible nitric oxide synthase (iNOS), cytokines IL-6 and TNF-α, anti-inflammatory markers such as transforming growth factor-β_1 (TGF-β_1) and IL-10, and evidence of cardiomyocyte damage (ischemic injury, infarct size, apoptosis, blood flow impairment, and no-reflow), adverse LV remodeling (LV dilation and dysfunction), and ECM remodeling with increased expression of *s*ecretory *l*eukocyte *p*rotease *i*nhibitor (SLPI), *s*ecreted *p*rotein *a*cidic and *r*ich in *c*ysteine (SPARC), osteopontin (OPN), and *a d*isintegrin *a*nd *m*etalloproteinase (ADAM)-10 and (ADAM)-17, as well as MMP-9 and MMP-2 [37]. Recent studies in the rat model also documented increase in all these markers in the later healing phase after reperfused STEMI [38].

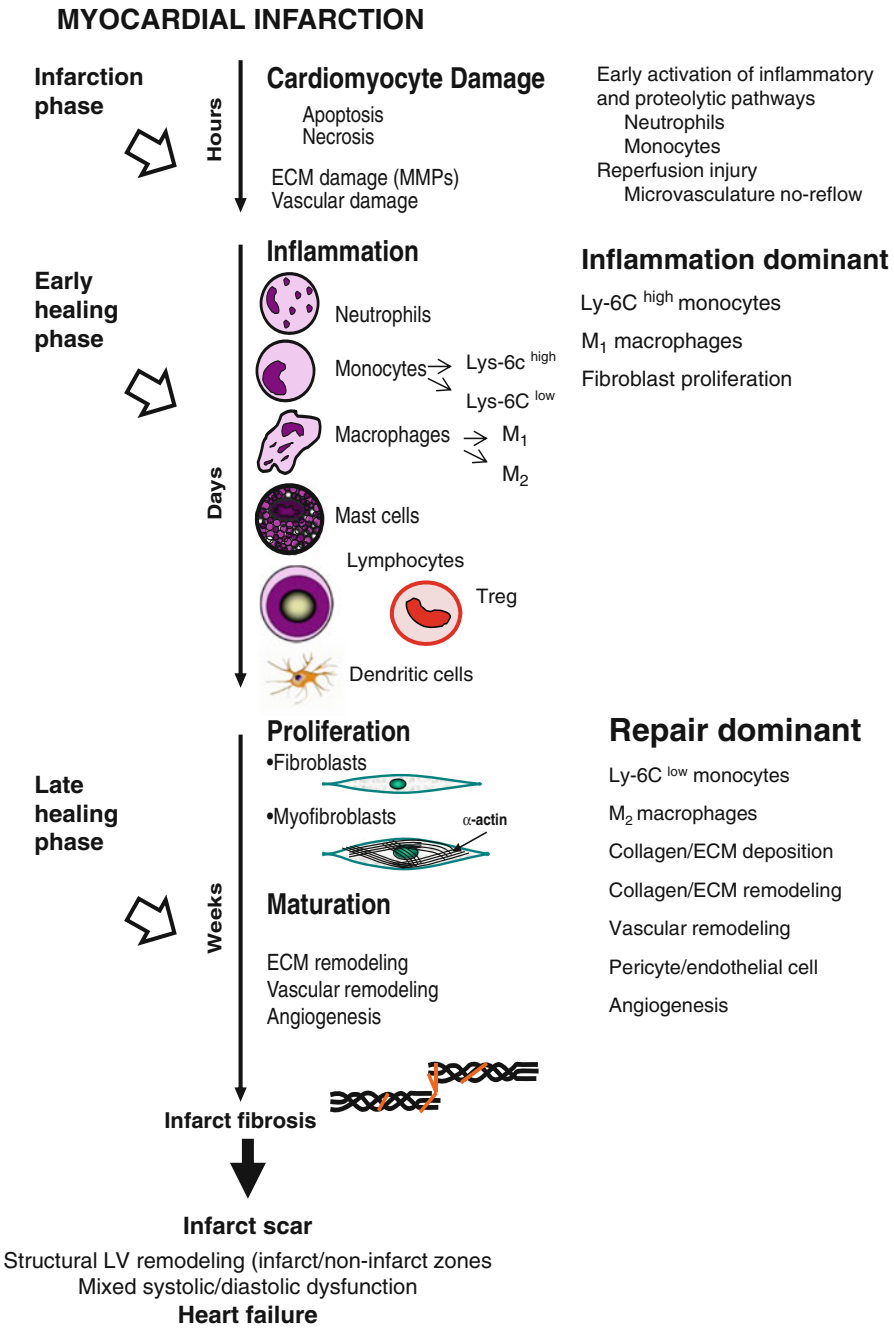

Fig. 29.6 Cellular and molecular changes during the infarction and healing/repair phases. Cartoon depicting expansion of the traditional algorithm. Acute myocardial infarction with cell loss triggers the inflammatory cascade and the healing and repair process; this process involves an orches

During the later phase of healing, resolution of the inflammatory response is critical [41] and involves three components: (1) activation followed by inhibition (negative regulation), (2) containment to the infarct zone, and (3) resolution [41]. These steps appear to involve different cellular effectors and molecular mediators that need further study [41]. The defective containment of the inflammatory reaction can lead to extension of the infiltrate into the bordering non-infarcted myocardium and thereby enhance fibrosis and worsen diastolic function. This underscores the importance of considering both the spatial and temporal aspects of regulation of the inflammatory response.

The molecular reactions after MI involve a large number of modulators and mediators [13] whose tandem release and activation are finely orchestrated [41] and converge on fibrosis and infarct formation (Fig. 29.7). Initially in the infarction phase after MI (Fig. 29.6), release of intracellular contents by necrotic cardiomyocytes and release of ECM degradation products by damaged ECM trigger activation of membrane-bound toll-like receptors (TLRs) that modulate post-ischemic inflammation ([73] for review). Concurrently, increased generation of reactive oxygen species (ROS) and reduced antioxidant reserve result in activation of the nuclear factor (NF)-κB that stimulates production and release of pro-inflammatory cytokines (such as IL-1β) and chemokines ([74] for review). Depending on the CC and CCX chemokine profile, neutrophils are attracted early and pro-inflammatory monocytes and lymphocytes later. Multiple inhibitory pathways together with coordinated actions of different cell types (such as neutrophils, mononuclears, endothelial cells, and pericytes) and interaction with the ECM lead to suppression, resolution, and containment of the inflammation [41]. At least five mechanisms have been implicated: (1) clearance of apoptotic neutrophils, (2) recruitment of inhibitory monocyte subsets, (3) macrophage differentiation, (4) recruitment of regulatory T cells, and (5) pericyte/endothelial cell interaction [41].

First, neutrophils accumulate very early after MI and peak within 1 day in the infarct zone in response to chemokines (such as IL-8), adhesion molecules (such as L- and P-selectins), and intercellular adhesion molecule 1 (ICAM-1). While they are abundant in the acute phase of MI, they undergo apoptosis (at ~3 days in mice and ~7 days in dogs), thereby releasing inhibitors of inflammation (such as annexin 1 and lactoferrin) which reduce recruitment of neutrophils and attract macrophages (through "find me" and "eat me" signals) that phagocytose the apoptotic neutrophils. This in turn activates anti-inflammatory mediators (such as IL-10 and TGF-β) and pro-resolution mediators (such as lipoxins and resolvins) ([75] for review).

Fig. 29.6 (continued) trated sequence of two highly dynamic phases: an early phase that is dominated by inflammation and a late phase that is dominated by repair. Significant remodeling of the extracellular matrix occurs in all phases. The end-result is the formation of a fibrotic infarct scar that undergoes further ventricular remodeling. Later, reactive fibrosis develops in remote myocardium. Ultimate outcome depends on the degree of dilative global remodeling and mixed systolic and diastolic dysfunction. This concept was based on data mostly in the young. *ECM* extracellular matrix, *LV* left ventricular, *MMP* matrix metalloproteinase

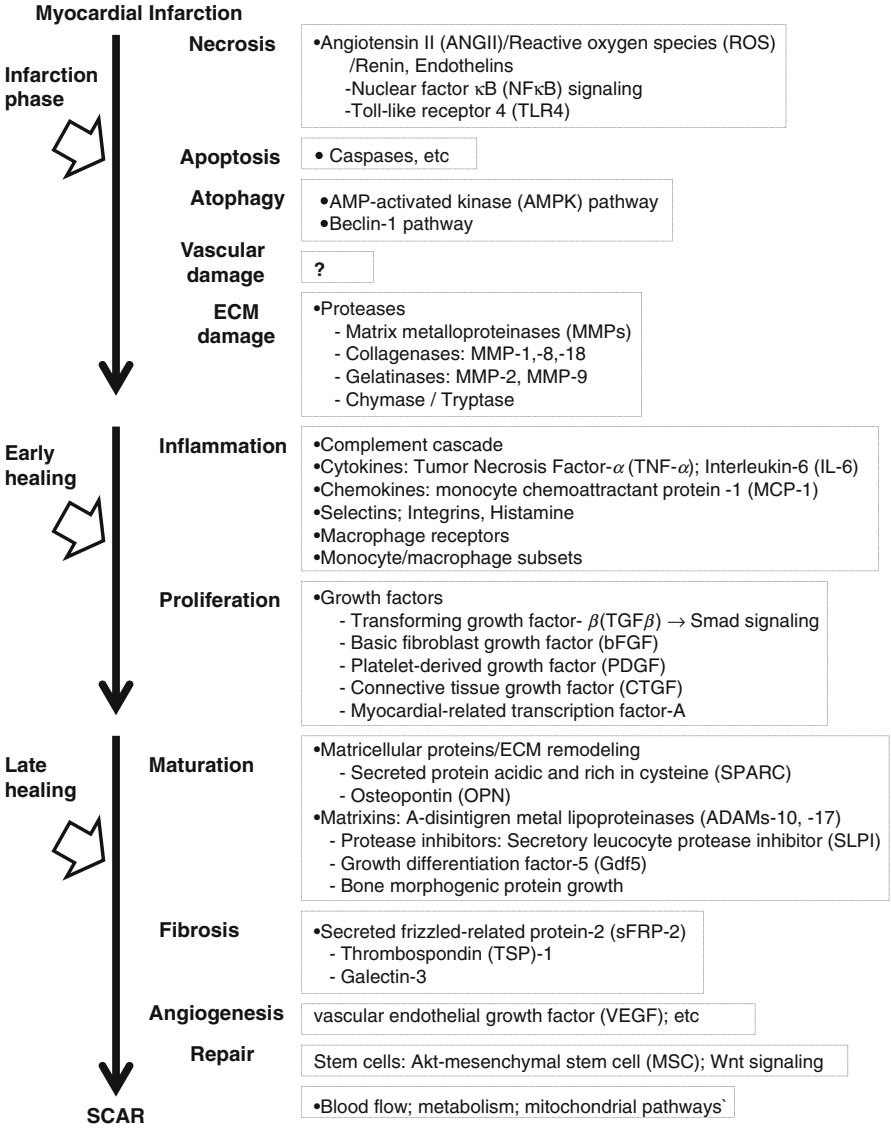

Fig. 29.7 Schematic of modulators and mediators in the healing, repair, and fibrosis processes after myocardial infarction

Second, monocytes and macrophages are present in increased numbers in the early and late phases of healing/repair and act as key regulators of the process (Fig. 29.6). Both neutrophils and monocytes belong to the myeloid lineage of cells of the innate immune system. Depending on cues from the tissue environment, monocytes differentiate into macrophages or dendritic cells. Monocytes/macrophages

dominate the repair/healing phases and have at least six functions: (1) release of inflammatory mediators (such as iNOS, ROS, interferon-γ, IL-1, IL-6, TNF-α, TGF-β_1, and macrophage inflammatory protein [MIP]-1α); (2) phagocytosis of apoptotic and necrotic myocytes and neutrophils and other debris; (3) release of proteases such as MMPs, urokinase-type plasminogen activator (uPA), and cathepsins that digest collagen matrix and facilitate cell transit; (4) secretion of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) to promote angiogenesis; (5) transport of reparative enzymes and pro-survival factors such as transglutaminases; and (6) and release of TGF-β and FGF to stimulate myofibroblasts to synthesize and deposit collagen [68].

Evidence suggests that these functions are executed in a temporally and spatially organized manner through a biphasic response of two subsets of monocytes with distinct effects on the inflammatory response during the early and late phases ([68, 76] for review). In humans, a dominant subset in the monocyte pool expresses high levels of CD14 and low or no CD16, and a minor subset expresses low CD14 and high CD16. The $CD14^+$ monocytes also express high levels of the CC chemokine receptor (CCR2) for the inflammatory chemokine monocyte chemoattractant protein 1 (MCP-1) and can release myeloperoxidase (MPO) while $CD16^+$ monocytes produce TNF-α. In mice, the Ly-$6C^{high}$ monocytes with high levels of CCR2 and low levels of the fractalkine receptor CX3CR1 resemble the human $CD16^-$ monocytes, whereas the Ly-$6C^{low}$ ($CCR2^+$ $CX3CR1^{high}$) monocytes resemble human $CD16^+$ monocytes. The pro-inflammatory Ly-$6C^{high}$ monocytes dominate the early phase of healing, whereas the pro-resolution and pro-repair Ly-$6C^{low}$ monocytes dominate the late phase of healing (Fig. 29.6). Ly-$6C^{high}$ monocytes remove necrotic debris and secrete inflammatory cytokines, OFRs, and matrix-degrading proteases. Ly-$6C^{low}$ monocytes trigger angiogenesis, collagen/ECM synthesis by myofibroblasts, and healthy infarct scar formation. In summary, monocytes/macrophages are necessary in both phases, but their actions have to be balanced so as not to delay resolution of inflammation, impair healing, ECM remodeling, and weaken the scar. Too little or too much inflammation can therefore be harmful. Several studies have suggested that insufficient or excessive monocyte numbers can impair healing [31, 65, 68–70, 76–78].

Third, macrophages also play key roles in healing/repair and polarize to different phenotypes depending on spatially and temporally regulated upregulation of cytokines, chemokines, and growth factors in early and late phases. During the early phase of healing after MI, upregulation of macrophage colony-stimulating factor (MCSF) induces monocyte differentiation [41], with polarization to classical pro-inflammatory M1 macrophages that secrete T1 helper cell Th1 cytokines (interferon-γ, TNF-α); activated M1 macrophages in turn secrete large amounts of inflammatory mediators and release ROS and nitrogen intermediates; boost inflammatory response; promote ECM degradation; and remove debris [79]. Of note, suppression of M1 macrophages by the Class A scavenger receptor may attenuate cardiomyocyte necrosis [80]. During the late phase of healing, macrophages polarize to alternatively activated M2 macrophages (akin to Th2 cells) that show enhanced phagocytic activity and high anti-inflammatory IL-10 activity (suppressor of inflammation), the decoy type 2 IL-1 receptor and IL-1 receptor antagonist

(IL-1Ra), exerting pro-resolution effects with removal of inflammatory leukocytes [81]. Pertinent here, persistent M2 activation stimulates excessive fibroblast proliferation, ECM deposition, and fibrosis [82].

Fourth, injury sites recruit pro-inflammatory effector T cells as well as regulatory T cells called Tregs, a $CD4^+/CD25^+$ subset of T lymphocytes with suppressor properties [83]. CCR5 null mice with reperfused MI have decreased $CD4^+/CD25^+$ Tregs, enhanced inflammation and MMP activity [84] that may lead to adverse remodeling, and impaired healing/repair.

Fifth, vascular maturation through acquisition of a mural cell coat may contribute to inhibition of inflammation and suppression of granulation tissue formation [41]. During the early inflammatory phase of infarct healing, vascular endothelial cells synthesize chemokines and express surface adhesion molecules, leading to adhesive leukocyte/endothelial cell interactions that recruit inflammatory cells. Subsequent replacement of the inflammatory infiltrate by granulation tissue is associated with angiogenesis (Figs. 29.6 and 29.7). Early upregulation of VEGF after MI leads to the formation of neovessels that lack a pericyte coat and are hyperpermeable and pro-inflammatory [84]. Vascular maturation that coincides with scar maturation involves coating of endothelial cells of neovessels with pericytes and smooth muscle cells under the modulatory effect of platelet-derived growth factor (PDGF)-BB/PDGFR-β signaling; this results in decreased vascular permeability and the inflammatory and angiogenic activity of endothelial cells, thereby stabilizing the scar. Inhibition of PDGFR-β resulted in defective vascular maturation and decreased infarct collagen [85].

Besides the recognized roles of MMP in ECM degradation and adverse dilative remodeling after MI and increased TIMPs in diffuse interstitial fibrosis in chronic heart failure [8, 9], dynamic changes in the ECM during healing/repair can modulate the function and gene expression in leukocytes, endothelial cells, and fibroblasts [47, 86]. Matrix and matricellular proteins such as fibronectin and thrombospondin (TSP)-1, respectively, can regulate the inflammatory responses and contain the infiltrate to the infarct zone. TSP-1 participates in suppression and containment of the inflammatory infiltrate [41]. The roles of other molecular pathways in repression and resolution of the inflammatory response post-MI, such as toll-like receptor (TLR) signaling, lipid mediators, leukocyte adhesion cascades, have been reviewed [41].

29.5 Fibrosis Post-myocardial Infarction

It is clear from the foregoing discussion that a timed sequence of multiple molecular and cellular events during the healing/repair process modulate the march to infarct zone fibrosis and ideal formation of a mature and strong fibrotic scar (Figs. 29.6 and 29.7). The temporal and spatial organization and containment of the process in the infarct zone have been emphasized in many studies and reviews [7–9, 68, 78, 85]. The regulators and suppressors of the process have also been reviewed [41], although

more research is needed to identify the "braking" and "stop" signals in the pathways. Fibrosis in the non-infarct zone has received less attention, and the mechanisms of fibrosis in the non-infarct zone remain to be fully defined. The traditional concept is that stimulation of fibroblasts by TGFβ, after the infarction phase and subsequent early healing, leads to differential deposition of ECM, collagens, and fibrosis, with greater amounts in infarct than non-infarct zones [1–22, 40–46, 49, 50, 87, 88], and increasing ratio of collagen fibril type I to type III during the later maturation process [52, 53, 89]. Concurrently during healing, differential remodeling of infarct and non-infarct zones occurs, in a bid to preserve systolic function—but this ideal scenario does not always happen [7, 8]. During early healing, net ECM degradation dominates, while later, net ECM deposition, collagen synthesis, and maturation dominate, resulting in differential ECM and LV remodeling during the two stages [7, 8].

29.6 Aging and Fibrosis

Importantly, aging can lead to dysregulation of healing and repair mechanisms and result in defective collagen deposition and excessive fibrosis [33–35]. Excessive collagen deposition, fibrosis, and diastolic dysfunction are a hallmark of aging, and post-MI remodeling is more severe in old patients and animals [11]. Defective fibrosis may be due to reduced collagen deposition or a relative paucity of mature collagen type I (which resists distension) and an abundance of immature collagen type III (which is more distensible) [11, 33–35, 90]. A key step in collagen deposition is the processing of procollagens by procollagen proteinase (pCP), under stimulation by a pCP enhancer, to form mature collagen type 1 fibrils [91]. Excessive fibrosis can lead to more diastolic and systolic dysfunction at any age. Antifibrotic therapy post-MI may decrease early collagen deposition and impair later maturation during infarct healing and thus may come at a cost [7–9, 11, 90]. New and safe therapeutic targets and strategies, above existing therapies, and aimed at differentially modulating fibrosis during healing are therefore needed, especially in the elderly.

29.7 Secreted Frizzled-Related Protein 2

One candidate is secreted frizzled-related protein 2 (sFRP-2) [90]. Wnt is linked to fibrosis with aging [92], and Wnt inhibition by sFRP-2 was suggested to limit post-MI fibrosis [93]. Recently, sFRP-2 was claimed to enhance pCP activity of bone morphogenetic protein 1 (BMP1)/tolloid (TLD)-like metalloproteinase independent of Wnt, and absence of sFRP-2 to reduce post-MI fibrosis and improve systolic function in young null mice compared to wild-type (WT) mice [94]. BMP1 can activate lysyl oxidase (the enzyme essential for collagen cross-linking leading to

formation of the mature collagen type I) by removing the N-terminal peptide from the pre-enzyme of lysyl oxidase.

Preliminary data from our laboratory showed that sFRP-2 expression increases during early healing after reperfused MI in young rats and its decrease by inhibition of angiotensin II is beneficial [95]. In contrast, others claimed that enhancing rather than inhibiting sFRP-2 may be beneficial in MI [96–99], and injection into MI early at 2 days limits remodeling, systolic dysfunction, and fibrosis in young rats [100]. The controversy between these studies targeting sFRP-2 in young mice [96] and rats [100] is unexplained and needs to be resolved before clinical testing. Neither group [96, 100] considered possible differential effects of sFRP-2 during early and late healing in the mouse or rat models, nor did they study sFRP-2 after reperfused MI (which is the common clinical scenario) or sFRP-2 post-reperfused MI in aging animals (although HF and MI are more common and post-MI healing is impaired in the elderly).

Since overexpression of FrzA/sFP1 [100], a Wnt/fz inhibitor, exerted differential effects on the cellular infiltrate, MMP-2 and -9, apoptosis, infarct size, myofibroblast density, angiogenesis, total collagen, and types I and III during post-MI healing in mice, sFRP-2 may exert similar differential effects. In human skin scar, sFRP-2 increased type III collagen, which could be harmful infarct scars. Since the amounts of collagen and fibrosis are severalfold greater in the infarct than non-infarct zones, and the biology of fibrosis differs in the two zones during healing, differential regional remodeling occurs. It follows that antifibrotic agents may have differential effects on the two zones.

29.8 Other Factors in Fibrosis Post-MI

Other local factors can affect collagen and fibrosis [8, 11]. Our recent data suggest that several intersecting pathways may converge during post-MI healing [37, 38]. Besides sFRP-2, and BMP1/TLD-like metalloproteinases [94–100], pertinent factors include angiotensin II and the TGF-β/Smad pathway [7–9, 11, 41, 102, 103], the MMP/TIMP pathway [104, 105], novel healing-specific proteins [10, 11, 36–38], and BMP14 or Gdf5 [106]. Of note, Gdf5 was also linked to repair and infarct scar expansion during the later phase of healing [106]. The TLD-like proteinase activates the TGF-β superfamily members including mycostatin, Gdf8, Gdf11, and TGF-β [100]. Angiotensin II, the primary mediator of the renin–angiotensin–aldosterone system (RAAS), drives both ECM and LV remodeling [7, 8] and regulates fibrosis throughout healing post-MI and beyond [102, 103].

The so-called healing-specific proteins [10, 11, 36–38] that modulate ECM and remodeling during post-MI healing include the following: SLPI which upregulates anti-inflammatory cytokines and can improve skin wound [107–109] and post-MI [37, 38] healing; OPN, a matricellular matrix protein that promotes collagen synthesis and can improve post-MI healing [37, 38, 110–112]; and SPARC, another matricellular protein that modulates cell–matrix interactions and regulates fibroblast

migration, may also improve post-MI healing [37, 38, 113–115]. Other matrix proteins such as ADAM (a disintegrin metalloproteinase) may also participate [37, 38]. In the chronic rat model of reperfused STEMI, we found that SLPI, SPARC, and OPN were upregulated in the infarct scars at 3 weeks, and the increases were attenuated by an angiotensin type 1 receptor (AT_1R) blocker [38]. In the dog model, these proteins were also upregulated after acute reperfused STEMI, and an AT_1R blocker attenuated the increases [36, 37].

We know that $TGF\beta_1$ modulates fibroblast phenotype and its conversion to myofibroblasts, thereby promoting ECM deposition [89, 116]. TGFβ is secreted as a large latent complex comprising of a mature TGFβ, TGFβ prodomain, and latent TGFβ-binding protein [89]. The large latent complex is bound to ECM via latent TGFβ-binding protein, preventing access to MMPs required for activating TGFβ through cleavage of TGFβ prodomains. By cleaving the latent TGFβ-binding protein, BMP1 releases the large latent complex from the ECM, thereby rendering prodomains susceptible to cleavage by MMPs [117]. As mentioned, $TGF\beta_1$ can upregulate BMP1 expression, thereby accelerating the LV remodeling. It follows that inhibition of BMP1 by sFRP-2 may interrupt this positive feedback loop and block the remodeling process. The possibility that inhibition of BMP-1 by sFRP-2 (via inhibition of canonical Wnt pathway) can potentially inhibit TGFβ activation and collagen cross-linking needs study.

Recent studies have shown that galectin-3 plays a major role in inflammation and fibrosis [118]. The mechanisms involve macrophage activation, chemotaxis, and activation of TGF-β–Smad-3 pathways [41, 118]. Galectin-3 is emerging as a promising marker of fibrosis in post-MI heart failure [119].

It should be noted that all four major currently recommended post-MI therapies, namely, angiotensin-converting enzyme (ACE) inhibitors, AT_1R blockers, beta blockers, and statins, can decrease collagen synthesis, cytokines, MMP activation, and inflammation and thereby modify post-infarct healing, fibrosis, and remodeling [10, 11, 68]. Aging may also result in remodeling of the RAAS [120]. Background therapy and comorbidities associated with older patients need to be considered in translational research.

29.9 Summary

While reparative fibrosis in the infarct zone is desirable, reactive interstitial fibrosis in the non-infarct zone contributes to chamber stiffness and diastolic dysfunction. In addition, fibrosis in the infarct and non-infarct zones may become dysregulated and contribute to mixed dilative and hypertrophic remodeling and mixed LV systolic and diastolic dysfunction. Besides reparative and reactive fibrosis in the infarcted LV, fibrosis that develops at remote sites such as the atrial and right ventricular chambers and the kidneys can influence outcome after MI. Evidence suggests that the regulation and temporal progression of fibrosis may differ in the two zones. Understanding the differential mechanisms of fibrosis in the infarct, non-infarct,

and remote zones may allow the development of new and improved therapeutic strategies for controlling fibrosis and improve outcome in survivors of MI.

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Chapter 30
The ACE2/Ang-(1–7) Pathway in Cardiac Fibroblasts as a Potential Target for Cardiac Remodeling

Randy T. Cowling and Barry H. Greenberg

Abstract Despite advances in its treatment, heart failure prevalence continues to increase and this condition remains a major contributor to mortality and morbidity in both industrialized and developing nations throughout the world. Cardiac remodeling that is activated by injury to the myocardium and/or increased wall stress adversely affects cardiac function over time and is known to play a critical role in the progression of heart failure. Excessive accumulation and stiffening of collagen (i.e., fibrosis) is a well-recognized component of the remodeling process, and it contributes to progression of disease and worsening heart failure by perturbing cardiac contractility, relaxation, and electrical conduction. Cardiac fibroblasts are interstitial cells that are responsible for synthesis and turnover of collagen in the myocardium. Current experimental and clinical evidence suggests that cardiac fibroblasts are an important target when inhibitors of the renin–angiotensin system (RAS) are used to treat heart failure. An alternative pathway of the RAS, involving angiotensin-converting enzyme 2 (ACE2), angiotensin-(1–7) (Ang-(1–7)), and the Mas receptor, has been found recently to be cardioprotective. Evidence suggests that Ang-(1–7), acting predominantly through the Mas receptor, can regulate maladaptive growth-promoting effects of angiotensin ll and inhibit both cardiomyocyte hypertrophy and cardiac fibrosis. This chapter highlights the potential regulatory role of the ACE2/Ang-(1–7)/Mas pathway and how it relates to cardiac fibroblasts during the remodeling process.

Keywords Angiotensin-(1–7) • Fibroblast • Angiotensin-converting enzyme 2 • Fibrosis • Heart • Collagen

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30.1 Introduction

30.1.1 The Cardiac Fibroblast and Cardiac Remodeling

Almost 6 million people suffer from heart failure in the United States today [1]. Similar high rates of heart failure prevalence are seen in other industrialized countries, and there is evidence that heart failure is growing rapidly as a public health problem in developing nations [2–5]. Current medical and device therapies have reduced morbidity and mortality in patients with heart failure. However, the effects of available treatment strategies have plateaued, and novel targets are needed if improvements in outcome are to continue in the future. In patients with chronic heart failure, the condition develops as a consequence of progressive changes in the structure of the heart that impair its normal function [6]. Hypertrophy of cardiomyocytes is a hallmark of the remodeling process, and there is evidence that the structural changes in these cells are intimately associated with abnormalities in their function. Another change in the structure of the heart that occurs as part of the remodeling process and which plays a critical role in the progression to heart failure is the deposition of collagen within the heart (i.e., cardiac fibrosis). Cardiac fibrosis stiffens the heart wall and alters electrical contractility, leading to diminished diastolic and/or systolic function and increasing the probability of debilitating or even fatal arrhythmias. Activation of the mechanisms that cause fibrosis as well as the extent of fibrous tissue that has been deposited within the failing heart has been associated with worse outcomes in heart failure patients. Thus, minimizing the development of fibrosis is expected to improve cardiac function, slow the development of heart failure, and help reduce morbidity and mortality in the heart failure population.

30.1.2 ACE2/Ang-(1–7)/Mas Pathway

There are several current treatment strategies for heart failure that target the renin–angiotensin system (RAS). These involve inhibition of the generation of angiotensin II (Ang II) with angiotensin-converting enzyme (ACE) inhibitors or blocking activation of Ang II receptors with angiotensin receptor blockers (ARBs). The importance of the RAS in the progression of heart failure is apparent by the beneficial effects of both ACE inhibitors and ARBs in large-scale, well-designed clinical trials [7–11]. These agents have been shown to inhibit the remodeling process as well as reduce morbidity and mortality across a broad spectrum of patients ranging from those with asymptomatic left ventricular (LV) dysfunction, to MI survivors, to patients with clinically manifested heart failure including those with advanced disease.

Recently, an alternative axis of the RAS, the ACE2/Ang-(1–7)/Mas pathway, has been receiving much attention as it is believed to function as a means of modulating the effects of Ang II and other growth-promoting factors during remodeling. Angiotensin-converting enzyme 2 (ACE2) was identified in 2000 by two independent

groups [12, 13]. ACE2 functions as a carboxymonopeptidase and has approximately 40% sequence similarity when compared to ACE. ACE2 can hydrolyze the carboxy-terminal phenylalanine from Ang II to form the heptapeptide angiotensin-(1–7) (Ang-(1–7)), or it can generate angiotensin-(1–9) from angiotensin I, at which point other peptidases can convert angiotensin-(1–9) to Ang-(1–7) [14]. Other substrates for ACE2 include des-Arg^9-bradykinin, apelin-13, dynorphin A (1–13), and β-casomorphin [14]. When considering only the angiotensin peptides, ACE2 has two, potentially beneficial functions. (1) It can degrade Ang II and (2) it can generate Ang-(1–7). As opposed to Ang II, which is considered to activate cardiomyocyte hypertrophy and fibrosis and, thus, to be deleterious by promoting remodeling, Ang-(1–7) is considered to have potentially beneficial antigrowth and antifibrotic effects that would act to inhibit the remodeling process. In human myocardium, Ang II has been shown to be the preferred substrate for ACE2. Moreover, ACE2 effects appear to be impervious to the ACE inhibitors that are used in clinical practice.

Some investigators have reported that Ang-(1–7) can act through well-characterized angiotensin receptors such as AT_1 or AT_2 [15–17]. However, Ang-(1–7) more frequently acts via its own dedicated receptor, which is widely considered to be the Mas receptor [18]. Mas is a G protein-coupled receptor that was originally described as a protooncogene [19, 20]. Mas becomes detectable after birth in the rat, increases its expression, and then plateaus at 4–6 months of age [21]. Mas exhibits a broad tissue expression profile, including the heart, kidney, testis, brain, retinal pigment epithelium, skeletal muscle, liver, and adipose tissue [22–24]. Within the heart, Mas is localized to the vasculature [25], cardiomyocytes [26, 27], and fibroblasts (detected by qRT-PCR, data not shown). Despite the passage of ten years since the initial characterization of Mas as the Ang-(1–7) receptor [18], the signaling pathways involved have not yet been fully delineated.

30.2 Responses of the Cardiac Fibroblast to Ang-(1–7)

30.2.1 Direct Responses to the Peptide

Direct activation of signaling pathways by Ang-(1–7) treatment of cardiac fibroblasts isolated from normal hearts has been reported. We have observed weak, transient phosphorylation of ERK1/ERK2 upon stimulation of adult rat cardiac fibroblasts with Ang-(1–7) (unpublished observations). We have also observed reduced endothelin-1 (ET-1) and leukemia inhibitory factor (LIF) mRNA levels between 1 and 2 h after stimulation of adult rat cardiac fibroblasts with Ang-(1–7), presumably via activation of an undetermined signaling pathway [28]. We have observed modest inhibition of Ang II-stimulated increases in phosphorylation of MAPKs (e.g., ERK, JNK, and p38) in fibroblasts which had been pretreated with Ang-(1–7). McCollum et al. also observed that treatment of cardiac fibroblasts with Ang-(1–7) reduced the Ang II- or ET-1-stimulated increase in phospho-ERK1 and phospho-ERK2 [29]. They observed that Ang-(1–7) increased dual-specificity phosphatase DUSP1 immunoreactivity

and mRNA, suggesting that the heptapeptide hormone increases DUSP1 to reduce MAP kinase phosphorylation and activity. The mechanism through which Ang-(1–7) increased DUSP1, however, was not elucidated. Increased generation of second messengers by Ang-(1–7) stimulation has been reported, including nitric oxide in cardiomyocytes [26] and aortic endothelial cells [30], cAMP in glomerular mesangial cells [31], and arachidonic acid from Mas-transfected CHO cells [18]. However, we have failed to observe activation of these second messengers in response to Ang-(1–7) in cultured cardiac fibroblasts (data not shown).

30.2.2 Indirect Responses to the Peptide

As opposed to the direct stimulatory effects of Ang-(1–7) on cardiac fibroblasts, which tend to be mild, indirect effects of the peptide can produce a much greater response. These indirect responses include alteration of other signaling pathways and the influence of second messengers that have been generated by cardiac fibroblasts, their precursors, or other nearby cell types.

30.2.2.1 Attenuation/Augmentation of Other Receptor Signaling Pathways

Stimulation of cells with Ang-(1–7) can affect the subsequent activation of other signaling pathways. Stimulation of nitric oxide production by bradykinin in endothelial cells can be augmented by prior exposure of the cells to Ang-(1–7) [32]. There is also evidence that Ang-(1–7) stimulation can prove inhibitory to signaling pathways, such as those activated by Ang II [29, 33, 34], glucose [35], and ET-1 [29]. As noted in the previous section, preincubation with Ang-(1–7) has been reported to reduce subsequent activation of MAP kinases by Ang II and other ligands [29, 33–36]. The mechanism of these effects is not known, although activation of tyrosine phosphatases in proximal tubular cells [35] and upregulation of DUSP1 in neonatal rat cardiac fibroblasts [29] have been implicated. Although DUSP1 upregulation has been implicated in the inhibitory effect of Ang-(1–7) in neonatal rat cardiac fibroblasts [29], we were unable to detect this effect in the adult cells. In adult rat cardiac fibroblasts, we found that Ang-(1–7) preincubation inhibited subsequent Ang II-stimulated upregulation of ET-1 and LIF mRNA. We also found that Ang II-induced secretion by adult rat cardiac fibroblasts of unknown paracrine factors that induced hypertrophy in cultured cardiomyocytes could be significantly inhibited by preincubation of the fibroblasts with Ang-(1–7) (Fig. 30.1 and [28]).

30.2.2.2 Autocrine/Paracrine Second Messengers

Fibroblasts can originate from other sources in addition to the cardiac interstitium, especially when the heart is actively remodeling. Sources of nonresident fibroblasts

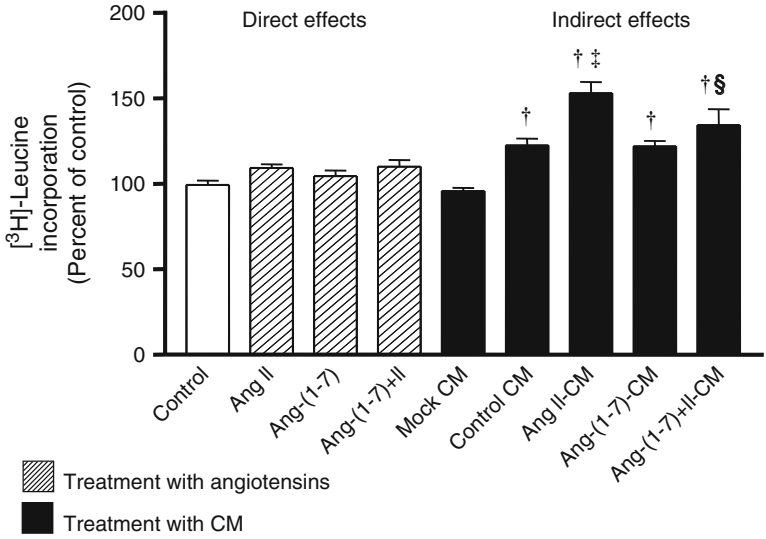

Fig. 30.1 Effects of angiotensins and conditioned media (CM) from adult rat cardiac fibroblasts (ARCFs) treated with angiotensins on [^{3}H]leucine incorporation in cardiomyocytes. Cardiomyocytes were stimulated for 24 h with Ang II, Ang-(1–7), both peptides after 1 h pretreatment with Ang-(1–7) or CM from ARCFs to assess cardiomyocyte hypertrophy measured by [^{3}H] leucine incorporation. Ang II-CM, CM from Ang II-treated ARCFs; Ang-(1–7)-CM, CM from Ang-(1–7)-treated ARCFs; Ang-(1–7) + II-CM, CM from ARCFs treated with both peptides after 1 h pretreatment with Ang-(1–7). † $P < 0.001$ vs. mock CM; ‡ $P < 0.001$ vs. control CM; § $P < 0.05$ vs. Ang II-CM (Reproduced with permission [28])

in the remodeling heart include circulating fibrocytes and mesenchymal cells generated from epithelial-to-mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndMT). It is still not clear whether these additional cells serve some unique purpose or simply elevate the pool of fibroblasts during increased need. However, the possibilities exist that (1) these cells may be affected by Ang-(1–7) differently than the resident fibroblasts and (2) the recruitment and/or differentiation of these cells could be altered by Ang-(1–7). Indeed, Ang-(1–7) has been shown to reduce the number, proliferative capacity, and collagen secretion of human circulating fibrocytes, possibly by increasing nitric oxide production [37]. Also, cGMP-dependent protein kinase (PKG) has been shown previously to disrupt transforming growth factor-β1 (TGF-β1)-induced nuclear translocation of pSmad3 [38]. Considering (1) the involvement of TGF-β1 in EMT (or EndMT) and myofibroblast differentiation and (2) the activation of guanylate cyclase and subsequent cGMP generation after nitric oxide exposure, there is a strong possibility that Ang-(1–7)-induced nitric oxide synthesis could inhibit fibroblast functions in a remodeling tissue. Ang-(1–7) has been shown to directly induce the release of nitric oxide in platelets, cardiomyocytes, and aortic endothelial cells [26, 30, 39], a process which involves activation of Akt and eNos in cardiomyocytes. As noted above, Ang-(1–7) has also been shown to augment the release of nitric oxide from endothelial cells

that have been stimulated with bradykinin [32]. Many of these indirect effects can theoretically influence cardiac fibroblasts during cardiac remodeling, but their effects in animal models or human patients have not been studied.

30.3 Effects of the ACE2/Ang-(1–7)/Mas Pathway on Cardiac Remodeling

Germline ablation of ACE2 results in a phenotype of cardiac dilatation and dysfunction in some [40], but not all [41], mouse models, and it predisposes to cardiac failure when the heart is stressed [42]. We have shown that pharmacologic inhibition of ACE2 in the post-MI rat heart using the selective inhibitor C16 increases infarct size and adversely affects contractile function [43]. Treatment with C16 had no significant effect on the increased level of apoptosis in the infarct and border zones nor did it significantly affect capillary density surrounding the MI. It did, however, significantly reduce the number of c-kit(+) cells in the border region, a finding consistent with the possibility that Ang-(1–7) or some other product of ACE2 helped reduce infarct size by enhancing the viability of cardiomyocytes in the border zone either directly or via paracrine effects of mediators released from the c-kit(+) cells. The observation that C16 inhibited increases in wall thickness and fibrosis in non-infarcted LV, however, raises the possibility that ACE2 activity has diverse (and perhaps even some adverse) effects on post-MI remodeling. Others have reported that loss of ACE2 accelerates maladaptive post-MI LV remodeling [44], while increasing ACE2 levels by gene transfer therapy inhibits post-MI remodeling [45].

In addition to degrading Ang ll, many favorable effects of ACE2 appear to be due to increased Ang-(1–7) levels. Elevated Ang-(1–7) levels are predicted to be inhibitory to both cardiomyocyte hypertrophy and fibrosis. In rats, post-MI remodeling can be inhibited by infusing Ang-(1–7) [46] or ingestion of either AVE 0991 (a nonpeptide Ang-(1–7) analogue) [47], an Ang-(1–7) hydroxypropyl β-cyclodextrin preparation [48], or a stabilized Ang-(1–7) analogue [49]. Angiotensin II-stimulated cardiac hypertrophy in mice is inhibited by cardiac-specific Ang-(1–7) overexpression [50, 51]. Whether Ang-(1–7) cardioprotection is due to effects of the peptide within the heart or effects of the peptide on extracardiac cells/tissues, however, is uncertain as some evidence suggests that circulating rather than cardiac Ang-(1–7) produces the beneficial effects of the peptide on post-MI remodeling [52]. We have shown that ACE2 inhibition increases post-MI infarct expansion [43] so that there is also uncertainty whether Ang-(1–7) inhibits remodeling due to an early reduction in infarct size or due to later effects on the remodeling process. Among strategies for using Ang-(1–7) to treat post-MI remodeling, gene transfer therapy is one of the most appealing [53–55]. Evidence from experiments in which a lentiviral vector encoding an engineered Ang-(1–7) fusion protein was injected directly into the LV wall several weeks prior to coronary ligation supports this possibility [56].

Germline deletion of Mas in mice has been reported to cause decreased cardiac performance, which is believed to be the result of increased levels of

extracellular matrix proteins in the right ventricles and AV valves [57]. Pharmacologic stimulation of the Mas receptor has shown promise in animal models of cardiac dysfunction. The nonpeptide Mas agonist, AVE 0991, improves cardiac function in rats with diabetes [58, 59], with isoproterenol treatment [60] and following experimentally induced MI [61]. AVE 0991 has also been shown to ameliorate progression of atherosclerosis in apoE-null mice [62]. Another Mas agonist peptide, CGEN-856S, which has been reported to be more stable than Ang-(1–7), demonstrated anti-arrhythmogenic effects in isolated rat hearts [63], but this peptide has not been extensively studied. To our knowledge, no human data exist regarding these Mas agonists, but their clinical potential as cardioprotective agents is recognized. Based on these considerations, further exploration of the effects of stable Ang-(1–7) analogues or Mas receptor agonists in cardiovascular disease is clearly warranted.

30.4 Future Considerations

There are many questions that still need to be answered about the relevance of this relatively new axis of the RAS and its potential role in regulating cardiac remodeling. Ang-(1–7)-induced signaling pathways (whether direct or indirect) remain unclear and require further study. Although Mas is widely regarded as the Ang-(1–7) receptor, there are still indications that other receptors are utilized in certain contexts [15–17]. Regarding cardiac fibroblasts, very little study has been devoted to understanding how ACE2/Ang-(1–7)/Mas can influence the recruitment and/or production of other cell types that contribute to the interstitial cell pool in the remodeling heart. Positive outcomes have been obtained when perturbing the ACE2/Ang-(1–7)/Mas system in animal models of disease. However, to our knowledge, no clinical use of such strategies has yet been reported. Since ACE2/Ang-(1–7)/Mas is considered cardioprotective, stimulation of this pathway, rather than inhibition, is expected to be beneficial. Ang-(1–7) infusion would be clinically difficult due to its short half-life. However, synthetic genes have been engineered to enhance Ang-(1–7) secretion from transfected or transduced cells [64]. Theoretically, increased expression of Ang-(1–7) would act cooperatively with existing ACE inhibitor or ARB pharmacologic treatment regimes, but this has yet to be tested. Enhanced ACE2 activity alone could demonstrate considerable improvements over existing therapies since it would simultaneously degrade Ang II and generate the cardioprotective Ang-(1–7). Stimulation of ACE2 enzymatic activity may prove challenging using pharmacology, but overexpression of ACE2 is always possible by transfection or viral transduction. We must remain cautious, however, since ACE2 overexpression by such methods has proven to be detrimental in at least one published work [65]. Also, discrepancies as to whether cardioprotective effects are due to activation of this pathway in the myocardium or in extracardiac tissues demand further studies [52, 56].

30.5 Conclusions

Although the past several decades have witnessed substantial improvements in heart failure outcomes, morbidity and mortality still remain unacceptably high. This, along with the relentless increase in new cases of heart failure throughout the world, makes it clear that novel treatment strategies are still needed. The RAS has been a pharmacologic target for heart failure treatment for many years. A more recently discovered branch of the RAS, namely, the ACE2/Ang-(1–7)/Mas system, has demonstrated cardioprotection when activated during remodeling in animal models of heart failure. The ACE2/Ang-(1–7)/Mas system can affect many cell types, but the cardiac fibroblast has been shown to be an important target of its action. Effects of Ang-(1–7) on cardiac fibroblasts can involve direct activation of signaling pathways or indirect effects on their function that involve either the paracrine release of second messengers, alterations in the differentiation/recruitment of fibroblast "precursors," or crosstalk with other signaling pathways. Ultimately, activation of the ACE2/Ang-(1–7)/Mas system is predicted to produce an "antifibrotic" phenotype and should improve cardiac remodeling. Despite favorable results in animal models, however, adaptation of the promising strategies for enhancing the effects of this alternate pathway and proof of efficacy in well-designed human trials are still needed.

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