Chapter 18
Nitrite-Nitric Oxide Signaling and Cardioprotection

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An acute myocardial infarction remains the leading single cause of death worldwide. Although reperfusion strategies have significantly improved overall mortality rates, reduction of the I/R-associated cardiomyocyte damage in patients remains an unmet goal [1]. Mitochondria as central energy suppliers in cardiac cells are key players in I/R-driven lethal injury. Protection of mitochondrial integrity and function is therefore a pivotal target in cardioprotection. Therapies that modify NO levels protect the heart from lethal myocardial I/R injury but both the resolution of the downstream signaling as well as the translation into clinical practice have not been achieved so far [2, 3]. This pertains both to the exact nature of how the response to the NO donors are transferred to the heart and which exact signaling cascades are activated within the cardiomyocyte and in cardiac mitochondria. Transfer of NO to the cardiomyocyte can be achieved by direct application of so-called NO-donors – compounds that continuously release the free radical NO. Recent experimental evidence also points to a potentially mitochondria-selective NO donor (mito-*S*NO) [4]. NO metabolizes to nitrite and nitrate. These chemically more stable forms distribute throughout the circulation and tissues but may be recycled to NO under hypoxic and ischemic conditions – an activation mechanism, which is canalized by metalloproteins, e.g. myoglobin in the heart [5–12]. Exogenous application of nitrate or nitrite can significantly enhance the bodily provision of these compounds and has been shown to contribute to cardioprotection in experimental studies. Furthermore, endogenous NO production can be enhanced by mechanical maneuvers, particularly rIPC – short alternating phases of extremity ischemia by cuff occlusion followed by reperfusion [3, 5]. Here, we briefly recapitulate the events that characterize myocardial I/R injury. This is followed by an outline of the NO-related signaling in

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I/R with particular emphasis on mitochondrial functions. Finally, we discuss how NO signaling can be effectively enhanced and how this could translate into cardioprotection.

Myocardial I/R Injury: Underlying Mechanisms

The events leading to myocardial I/R injury are complex and still under intense debate. The three major modifiers of I/R injury are ROS, a deranged Ca^{2+} signaling and the destruction of mitochondrial integrity and function.

Reperfusion therapy by percutaneous coronary intervention or pharmacological thrombolysis rapidly increases intracellular oxygen levels. A subsequent insufficient electron transport over mitochondrial membranes can result in the formation of ROS. This may overcome the availability of endogenous antioxidant mechanisms. The relative imbalance between the generation and the decomposition of ROS is one of the key initiators of I/R injury with consecutive organelle membrane and protein damage and the induction of apoptosis and necrosis [13]. Paralleled by increased ROS concentrations, the levels of bioactive NO appear to decrease during the early reperfusion phase. NO fulfills numerous functions in the heart including scavenging of radicals, modulating immune response, improvement of blood flow and left ventricular function [14–16]. As a consequence, these functions are impaired.

Evidence from experimental studies also implicates that a rapid burst-like increase in Ca^{2+} concentration contributes to the lethal injury of the reperfused myocardium [17–20]. Elevated Ca^{2+} levels can trigger arrhythmias, hypercontracture [21] and the activation of deleterious signaling cascades (see Chap. 10). In addition, hypercontracture and necrosis is not limited to one cell but also disseminated by directed contracture from cell to cell and by cell-to-cell progression through intercellular gap junctions [22].

A third central mediator/target of I/R injury are the mitochondria and specifically the mitochondrial membranes [23–25]. Mitochondria are not only the main source of energy production but can also signal cell death and cardiomyocyte disintegration [26]. However, modification of mitochondrial function is regarded a promising therapeutic option given to modulate functional recovery, reduce infarct size, improve cardiac function and prevent heart failure progression after ischemic injuries in experimental approaches [27]. Results from our studies implicate that administration of exogenous nitrite during myocardial I/R injury yields bioactive NO leading in particular to an *S*-nitrosation modification of mitochondrial complex I. This in turn regulates mitochondrial respiration and limits formation of mitochondria- derived ROS. *S*-nitrosation of complex I is furthermore associated with an adaption of myocardial functions to a reduced oxygen supply — a mechanism which is known as hibernation. Finally, nitrite reduction to NO contributes to a decrease in myocardial necrosis and apoptosis [8, 12, 28, 29].

In addition to the regulation of mitochondrial function by hypoxic nitrite signaling, mitochondrial integrity is maintained by the intraorganellar proteolytic system and the dynamic nature of the mitochondrial population in the cell. Membrane fusion and fission allow mitochondrial content adaption for cell integrity, and damaged mitochondria are selectively removed by a process, termed mitophagy, which protects against cell death [27, 30]. When mitochondria are damaged thus losing membrane potential, the kinase PTEN-induced putative kinase protein 1 (PINK1) accumulates and recruits the E3 ubiquitin ligase parkin, which then ubiquitylates mitochondrial proteins and causes mitochondria to become enclosed by membranes. BNIP3 as well as NIX, both related to the BH3-only family, also contribute to mitophagy in response to hypoxia. The current knowledge of the cellular mechanism behind mitochondrial quality control promotes interest in associated interventions in physiology and diseases such as aging, cancer, degenerative disorders and coronary heart disease. Notably, initial experimental evidence suggests that mitophagy is involved in the protection from ischaemic preconditioning (IPC) applied locally to a myocardial region. This mechanism leads to cardioprotection of the reperfused areas [27, 31]. It is speculated that the inhibition of the formation of mitochondrial membrane pores, e.g. the so-called mitochondrial permeability transition pore (mPTP), are involved in protection from ischemic preconditioning applied to the heart before index ischemia as well as in the regulation of mitophagy to reduce the numbers of dysfunctional organelles [27, 30, 31] (see Chap. 9). Less is known about the relative contribution to cardioprotection when the protective stimulus is applied to a heart-distant organ – rIPC.

Cardiomyocytes die primarily by apoptosis or necrosis with mitochondria at the center of regulation [32]. Apoptosis causes cell shrinkage, cellular and compartment fragmentation, and phagocytosis. The general characteristics of necrosis include cellular swelling and rupture, marked depletion of energy carriers, and an inflammatory response. Newer studies argue that both apoptosis and necrosis are regulated by a complex signaling machinery with overlapping processes. The critical step for apoptosis is the permeabilization of the MOM pore (MOMP). This should occur by oligomerization of BAX and other pro-apoptotic BH3 proteins. Subsequently, a release of apoptogenic factors, e.g. of apoptosis-inducing factor (AIF) which is also cleaved and activated by calpains, and endonuclease G, cause DNA damage [33, 34]. By contrast, the key characteristic of necrotic cell death is the permeabilization of the mitochondrial inner membrane. This may be further substantiated by a destruction of mitochondrial complex I. However, also BAX substantially contributes to necrotic cell death and *Bax* deficient mice show a remarkable reduction in necrosis. Interestingly, BAX-signaling is not only involved as critical step in apoptosis and necrosis, but also furthermore involved in mitochondrial structural dynamism [35].

Mitochondrial morphology and structural dynamism is regulated by a fine equilibrium between fission and fusion, repeated cycles of which re-distribute mitochondrial constituents by separation and fusion of mitochondria [36]. Fission is mediated by dynamin-related protein 1 (DRP1), a GTPase that transits from cytosol to mitochondria. Fusion is controlled by three dynamin-related GTPases: mitofusin

(Mfn1) and Mfn2 in the MOM and optic atrophy (OPA1) in the mitochondrial inner membrane. Interestingly, *Bax* knockout mice (formerly examined to have reduced apoptosis and necrosis) also show smaller, more fragmented mitochondria, which are less susceptible to cell death induction in I/R. Comparable characteristics were found in *Mfn2* knockout mice and cell lines. BAX reconstitution has been shown to cause an increase in mitochondrial fusion with mitochondria much more sensible to I/R related cellular stress. Although BAX is principally involved in both mitochondria-driven cell death and structural dynamism, the relationship between mitochondrial dynamics and cell death is poorly understood [37–42].

NO in Acute I/R Injury

As demonstrated in numerous experimental studies, myocardial I/R injury can be modulated. However, although numerous animal model-based studies showed much-reduced I/R injury, the respective translational clinical trials failed to demonstrate the same benefit in humans. Several of these experimental studies point to a protective role of the NO pool during myocardial I/R (Fig. 18.1) [28–31]. Increase of the circulating and the tissue (cardiac) NO pool exerts protective tissue effects in a mouse model of I/R injury in vivo [28, 30, 32]. The protective effects can derive from a hemeprotein-dependent reduction of nitrite to NO, as demonstrated in mice without myoglobin [30].

NO derived from nitrite reduction via cardiac myoglobin reversibly modulates mitochondrial electron transport, thus decreasing reperfusion-derived oxidative stress and inhibiting cellular apoptosis leading to a smaller final infarct size. In details, under hypoxic conditions the non-enzymatically formed NO binds to the aa_3 side of cytochrome oxidase (complex IV in the mitochondria) and thus inhibits respiration. As a result of this, a reduction in the energy status occurs, which results in an attenuation of the myocardial pump function and consequently, in reduced oxygen consumption. If the myocardium is again adequately supplied with oxygen, the non-enzymatic NO formation via myoglobin ceases and concurrently the inhibition of energy production as well as the restriction (hibernation) of the myocardial pump function also ends. Myoglobin thus appears to assume the role of an oxygen sensor in the myocardium via NO adjusts the myocardial energetics to the diminished oxygen supply [28].

Posttranslational modifications of relevant cardiomyocyte proteins are the second major aspect of hypoxic NO signaling. *S*-nitrosation is the addition of an NO moiety to a protein sulfhydryls. This can effectively change proteins conformation and function [8]. We and others have demonstrated that within the cardiomyocyte, NO reacts with numerous yet incompletely identified proteins [43]. Macrophage-migration inhibitory factor (MIF) plays a very important role in myocardial I/R injury. *S*-nitrosation of MIF was able to enhance the ROS decomposition properties of this molecule resulting in less necrosis and apoptosis in the reperfused myocardium [8]. *S*-nitrosation may further affect mitochondrial elements. It has recently

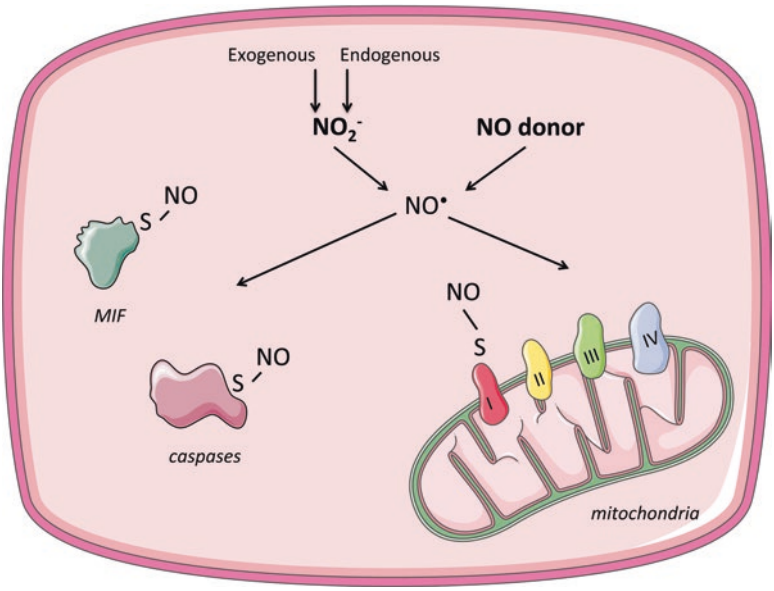

Fig. 18.1 Hypoxic NO signaling in myocardial I/R. Hypoxic NO signaling can be enhanced by e.g. increasing the levels of cardiomyocyte nitrite either by direct intravenous injection of by intake of dietary nitrate (exogenous) or be increase of endogenous NO/nitrite generation through mechanical endothelium stimulation (e.g. by rIPC). Alternatively, NO donors directly release NO which then causes an S-nitrosation modification of cytoplasmatic proteins involved in apoptosis (e.g. caspases) or related to ROS decomposition (e.g. MIF). NO can furthermore modifiy mitochondrial respiratory chain elements by S-nitrosation of complex I

been forwarded that elements of the fusion and fission machinery were significantly altered by NO-dependent modification [44]. While this may relate to a preservation of mitochondrial structure, *S*-nitrosation of mitochondrial complex I, has been significantly associated with a decrease in mitochondria-derived ROS through a decrease in misguided respiration [12, 45]. Taken together, multiple effects of NO have been characterized during I/R. The relative contribution of each of these signaling aspects remains obscure. However, the majority was in support of the notion that NO is cardioprotective in the scope of myocardial I/R.

Delivery of NO

Novel therapeutic strategies are required to alleviate the burden of I/R injury. As demonstrated in numerous infarction studies in animals and a small number of clinical proof-of-concept investigations, I/R injury can be effectively reduced by mechanical and pharmacological interventions [1].

Several pathways have been characterized that may affect the levels of NO in the reperfused myocardium. This ranges from chemical compounds that release NO on a continuous basis (NO donors) to inorganic nitrate and nitrite and mechanical maneuvers in particularly rIPC. While the next section provides and overview of these three entities, the reader is kindly referred to previous reviews discussing the use and benefits of organic nitrates which are characterized by a completely different mechanism of NO release [2].

NO Donors

A wide range of NO-releasing molecules has been forwarded with some interesting pharmacological agents among them. The reader is kindly referred to recent relevant reviews of this topic [2]. However, some very interesting aspects must be mentioned. Recent studies have evaluated compounds that combine standard medications in ischemic heart disease with NO-releasing agents. Among these were NO releasing pravastatin and nitro-aspirin. For both substances preclinical data implicate a therapeutical benefit for the reduction of myocardial I/R injury. Clinical data have to be awaited for.

While these donors cause a more or less continuous release of NO regardless of the localization (circulation, cytoplasma, mitochondria), the mitochondria-selective S-nitrosating agent mito-SNO potentially avoids these caveats by selective activation when crossing mitochondrial borders only [4]. This caused a significant S-nitrosation of mitochondrial complex I, which was reversible and protected the heart in the first few minutes of reperfusion. It remains to be evaluated whether this pertains to the clinical setting.

The Nitrate-Nitrite-NO Pathway

Nitrate and nitrite are now regarded as source for NO under hypoxic, ischemic, or injury conditions [11, 46]. Nitrite is reduced significantly to bioactive NO with decreased oxygen levels and pH by a variety of mechanisms, including reaction with deoxygenated myoglobin, in the heart as well as other heme proteins in solid organs and blood cells [28, 47–49]. Nitrite levels result from the reduction of dietary inorganic nitrate intake, which is reduced to nitrite by oral cavitiy bacteria [50, 51]. A dose of 1.2 nmoles of nitrite was sufficient in reducting myocardial and liver injury in I/R also these cause a moderate increase of 10% of the circulating nitrite levels [52, 53]. Furthermore, mice with decreased endgenous circulating nitrite concentrations are more susceptible to IR injury, an effect that is attenuated by exogenous nitrate [54]. These data suggest that a diet rich in nitrate and nitrite may have profound cytoprotective effects. In addition, eldery participants given dietary nitrate supplementation showed a marked incresase in endothelial function, which is a hallmark of arteriosclerosis, which, in turn, precedes myocardial infaction [55].

rIPC

Mechanical strategies that reduce I/R injury have been named IPC and postcondioning (PostC) [56, 57] They are applied as brief non-deleterious cycles of I/R before (IPC) or after (PostC) the main ischemic event. It is widely accepted that these mechanical interventions condition the heart and activate endogenous, protective modalities [58, 59]. Interestingly, the conditioning stimulus can also be applied to an organ distant (remote) to the heart such as the limb, kidney or intestine (rIPC) [60] and rPostC [61] respectively. The protective assets of IPC and rIPC rely on a distinct cellular signaling cascade, whose individual members can be subdivided into triggers, effectors, mediators and end-effectors [21, 62–66]. Endogenous triggering molecules are e.g. opioids, bradykinin, prostaglandins and adenosine and effects are mostly exerted by G-protein coupled receptors (GPCR) [67–70]. The rIPC downstream signaling cascade is structurally complex [65, 71, 72]. Activation of GPCR leads to an activation of phosphoinositide 3-kinase (PI3K) and further downstream targets [73–78]. Mitochondria are generally regarded to be the end-effector of the IPC signaling cascade [79–81]. Disruption may lead to cell swelling and disintegration of mitochondrial membranes.

rIPC is one of the most effective techniques in rendering the myocardium capable of protecting itself against I/R injury. rIPC is induced via short non-deleterious phases of I/R prior to an index ischemic event. While the cardioprotective effects of rIPC are generally acknowledged, the underlying mechanism and specifically a role for NO remain under intense debate. The signaling initiated by the rIPC stimulus involves a trigger, and a distinct cardiac signal transduction mechanism finally protecting the cardiomyocyte from I/R injury. As triggering pathways both humoral/blood borne factors [82–84] and nerval transmission [85] have been proposed. Given that the cardioprotective effects can be transferred when transfusing blood from preconditioned donor animals to unconditioned recipients in both in vivo and ex vivo preparations [82, 84, 86], a contribution of a blood borne factor appears rather presumable. Using a mouse model of warm liver I/R, it was recently demonstrated that ablation of endothelial NO Snythase (eNOS) abrogates the protective effects seen with rIPC on microscopic liver damage [87], eNOS generates NO which can modulate cardiovascular functions either locally or at a distance when transported as nitrite or nitroso species [88]. Changes in shear stress, e.g. due to an increase in blood flow, are the strongest physiological stimulus of eNOS activity, which is mirrored in higher circulating NO metabolites. In a recent investigation in humans, we determined that a 5 min phase of forearm occlusion massively increased postischemic blood flow in the proximal conduit arteries. This was paralleled by an increase in nitrite in the plasma of these individuals [89]. This rIPC maneuver causes an increase of circulating endogenous nitrite both in an experimental and clinical setting, enhances nitrite levels in the myocardium and thus protects from myocardial I/R injury [5]. The relative importance of this pathway remains to be elucidated.

Conclusion

Mitochondria remain a central target of cardioprotection strategies. This relates to the preservation of mitochondrial structure and function. Experimental evidence points to a benficial role NO in mediating protection during I/R. Therefore an increase of NO in the reperfused myocardium is a desired approach to protect the heart. The strategies to achiev this range from NO donors, inorganic nitrate and nitrite to an endogenouse enhancement of NO/nitrite production, e.g. by rIPC: the relative importance and a potential clinical application must be evaluated in future clinical trials.

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