Natriuretic Peptides: Critical Regulators of Cardiac Fibroblasts and the Extracellular Matrix in the Heart

Hailey J. Jansen and Robert A. Rose

Abstract The mammalian heart contains numerous cell types with cardiac fibroblasts accounting for the majority of cells. These fibroblasts play essential roles in the heart including the synthesis and remodeling of the extracellular matrix (ECM), which is the component of the heart that includes interstitial collagens. In the setting of heart disease, including heart failure (HF), abnormal fibroblast proliferation and deposition of collagens leads to adverse structural remodeling, which is a major contributing factor to the progression of heart disease. Structural remodeling of the ECM in HF can increase stiffness of the myocardium leading to impaired cardiac performance and also increase the occurrence of cardiac arrhythmias due to impaired electrical conduction. Natriuretic peptides (NPs) are a family of cardioprotective hormones with numerous effects in the cardiovascular system. Included among these is the ability to prevent fibroblast proliferation and abnormal collagen deposition in the ECM. NPs elicit their effects by binding to three NP receptors denoted NPR-A, NPR-B and NPR-C. NPR-A and NPR-B are guanylyl cyclase-linked NPRs that elicit their effects by increasing cGMP levels. NPR-C is linked to the activation of inhibitory G-proteins (G_i). All three NPRs are expressed in cardiac fibroblasts and each has been shown to play a role in the ability of NPs to protect against adverse structural remodeling in the heart. The purpose of this chapter is to provide an overview of NPs and how they affect remodeling of the ECM in HF.

Keywords Natriuretic peptides · Natriuretic peptide receptors · Guanylyl cyclase · Adenylyl cyclase · Cyclic GMP · Cyclic AMP · Cardiac fibroblasts · Fibrosis

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1 Introduction

The mammalian heart contains numerous cells types, including cardiac myocytes, cardiac fibroblasts, vascular smooth muscle cells, endothelial cells and others. Although cardiac myocytes account for the majority of the myocardial volume, cardiac fibroblasts are the most abundant cell type in the heart [1, 2]. These fibroblasts play essential roles in myocardial function in the normal heart and in the setting of heart disease. One critical function of the cardiac fibroblast is the synthesis and remodeling of the extracellular matrix (ECM), which is the component of the heart that includes interstitial collagens, proteoglycans, and glycoproteins. These components form a complex three dimensional network that is intricately involved in cardiac function. Some of the essential roles of the ECM include the formation of an organizational network that surrounds cellular structures, the creation of a scaffold for the myocyte and nonmyocyte cell populations in the heart, distribution of mechanical forces through the myocardium, mechanotransduction and fluid movement in the extracellular spaces.

The organization, composition and density of the ECM are highly dynamic and modulated under different physiological and pathophysiological conditions and this profoundly impacts cardiac function [1]. Collagen expression and accumulation are increased in the setting of heart failure (HF) in a process referred to as structural remodeling. As the density of the ECM affects compliance, the process of remodeling can be a pathological condition that leads to inappropriately enhanced fibrosis in the setting of HF. Specifically, this enhanced fibrosis in the diseased heart results in myocardial stiffness and diastolic dysfunction [3]. Enhanced fibrosis is also thought to increase the susceptibility to cardiac arrhythmias by slowing conduction and interfering with normal electrical propagation, which can lead to electrical reentry [4, 5]. Although remodeling of the ECM and enhanced fibrosis are hallmarks of HF there is still much that is unknown in terms of how the process is initiated and regulated.

Natriuretic peptides (NPs) are a family of cardioprotective hormones with numerous beneficial effects in the cardiovascular system [6, 7]. Although best known for their ability to regulate blood volume and blood pressure through effects in the kidneys and the vasculature, it is now known that NPs also have numerous additional effects. Included amongst these are potent effects on cardiac fibroblast function, ECM deposition and fibrosis. The purpose of this chapter is to provide an overview of natriuretic peptides and their role in the remodeling of the ECM that occurs in HF.

2 Natriuretic Peptides

In 1981, de Bold *et al*. infused atrial homogenates into rats and observed rapid and potent diuretic and natriuretic effects [8]. This landmark study ultimately led to the isolation and discovery of the first NP, atrial natriuretic peptide (ANP). B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) were subsequently

identified and isolated from porcine brain extracts [9, 10]. BNP and CNP each exhibit profound relaxant effects on smooth muscle and following their discovery their presence in the heart was confirmed [7, 11]. *Dendroaspis* natriuretic peptide (DNP), a fourth member of the NP family, was initially identified in the venom of the Green Mamba snake [12]. There is evidence that DNP may also be present in human plasma and this NP has been shown to elicit relaxant responses in contracted aortic strips [13].

All NPs are synthesized as pre-pro-hormones that undergo posttranslational processing to form smaller, cyclical, biologically functional peptides [7]. NPs are structurally related and homology is observed in conserved residues within a 17 amino acid sequence flanked by cysteine residues (Fig. 1). A disulphide bridge is formed between these cysteine residues, creating a peptide ring. Structural variation occurs both within the cyclical structure and the amino- and carboxy-terminal tails of the NPs [7].

Pro-ANP and low levels of pro-BNP are stored within granules located in atrial myocytes [14, 15]. The dominant stimulus for release of these NPs from granules is atrial stretch in association with increased intravascular volume [16]. During exocytosis, pro-ANP is cleaved into the biologically active ANP by the transmembrane cardiac serine protease corin [17, 18]. ANP expression in the heart undergoes changes throughout development and in cardiac disease. For example, in addition to being expressed in the atria, ANP is expressed in fetal and neonate ventricles as well as hypertrophied ventricles in adults [19, 20]. ANP expression in normal adult ventricular tissue is very low. Circulating ANP levels increase by 10–30 fold in patients with congestive HF [21–23].

Within the ventricular myocardium BNP is constitutively expressed and released into the circulatory system. Ventricular BNP secretion is transcriptionally regulated and expression significantly increases in response to load induced ventricular wall stretch [24–26]. Plasma levels of BNP are 200–300 fold higher in patients with ventricular hypertrophy or those with congestive HF and, in some cases, circulating BNP levels exceed ANP levels [23].

Multiple CNP molecules have been identified. Pro-CNP is cleaved by the intracellular endoprotease furin to form a CNP molecule that is 53 amino acids in length (CNP-53) [27]. CNP-53 is located in cardiac tissue whereas a smaller 22 amino acid form of CNP (CNP-22) is detected in plasma [28, 29]. The enzyme responsible for the conversion between CNP-53 and CNP-22 remains unknown. Circulating levels of CNP are extremely low, approximately 1 fmol/l, and it is thought that CNP acts primarily as a paracrine molecule [30, 31]. As with ANP and BNP, circulating CNP levels are elevated in patients with congestive HF [7, 32].

NPs are rapidly cleared from the circulation via two mechanisms. First, NPs can be degraded by a membrane neutral endopeptidase called neprilysin, which cleaves peptides on the amino side of hydrophobic residues [33]. Interestingly, human BNP is more resistant to neprilysin hydrolysis compared to ANP [34]. The second component of NP degradation is coupled with the termination of surface receptor-mediated signaling though the internalization of the peptide-receptor complex. This is followed by hydrolytic degradation by lysosomes and recycling of a small pool of receptors back to the cell membrane [7].

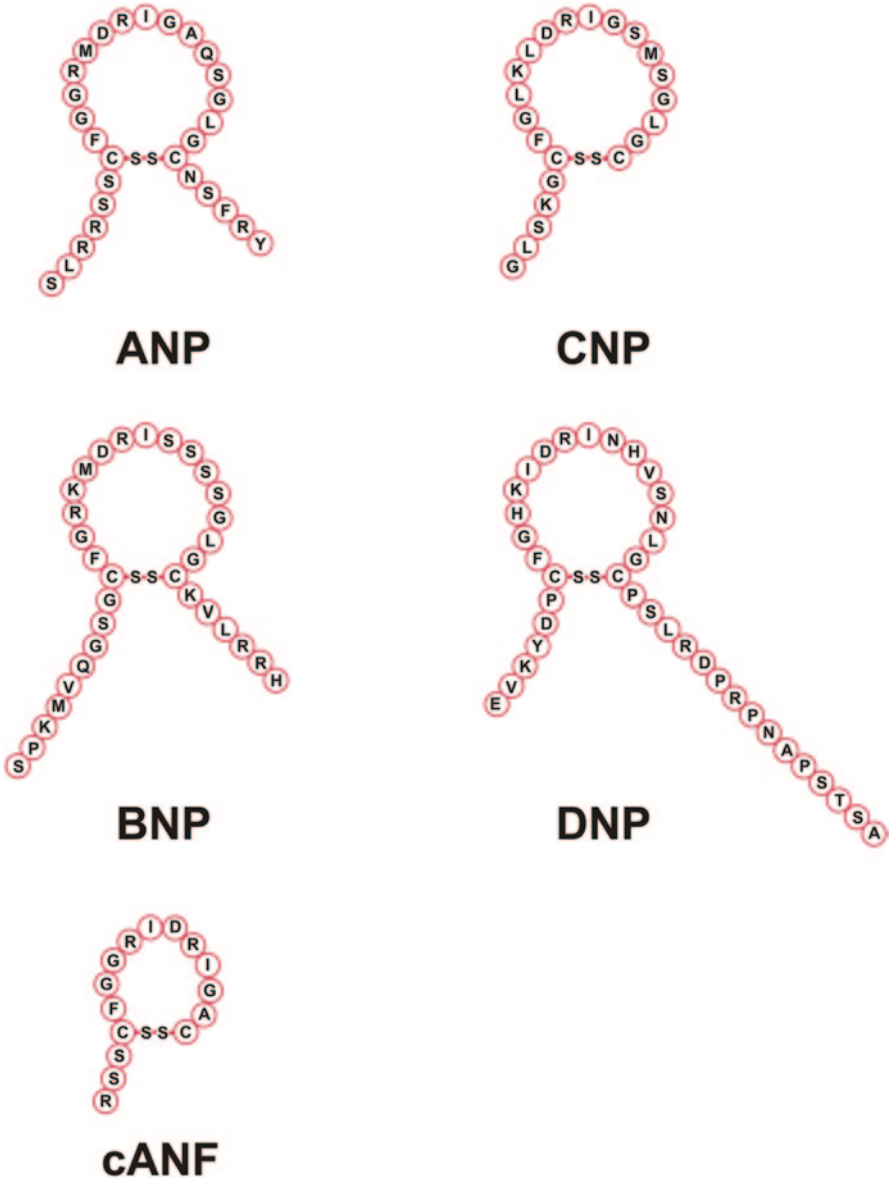

Fig. 1 Structure and amino acid sequence of natriuretic peptides. *ANP* atrial natriuretic peptide; *BNP* B-type natriuretic peptide; *CNP* C-type natriuretic peptide; *DNP Dendroaspis* natriuretic peptide; *cANF* synthetic natriuretic peptide receptor C (NPR-C) agonist

3 Natriuretic Peptide Receptors

NPs elicit their effects by binding to specific NP receptors (NPRs). There are currently three known NPRs denoted NPR-A, NPR-B and NPR-C (Fig. 2). NPR-A has binding affinity for ANP and BNP, while NPR-B preferentially binds CNP [7, 35].

NPR-A and NPR-B are coupled to intracellular particulate guanylyl cyclase (GC) enzymes. Following activation of these NPRs, GTP is converted into the second messenger cyclic guanosine monophosphate (cGMP); thus, NPR-A and NPR-B elicit their effects via changes in cGMP levels. Several downstream signaling molecules may be modulated by cGMP signaling including a cGMP-dependent protein kinase (PKG), cGMP regulated phosphodiesterases (PDEs), and cyclic nucleotide-gated ion channels [7].

NPR-C is the most abundantly expressed NPR and demonstrates similar binding affinity for all NPs [36, 37] (Fig. 2). In contrast to NPR-A and NPR-B, NPR-C is not directly coupled to changes in guanylyl cyclase signaling. Instead, NPR-C is coupled to the activation of inhibitory G-proteins (G_i) via specific 'G_i-activator domains' located within the 17 amino acid intracellular domain of the receptor [38, 39]. Following G_i activation, adenylyl cyclase (AC) activity is inhibited in a GTP-dependent fashion, which results in reductions in cAMP. Activation of NPR-C also results in the activation of the β isoform of phospholipase C (PLCβ), which converts phosphatidyl inositol bisphosphate (PIP_2) into inositol triphosphate (IP_3) and

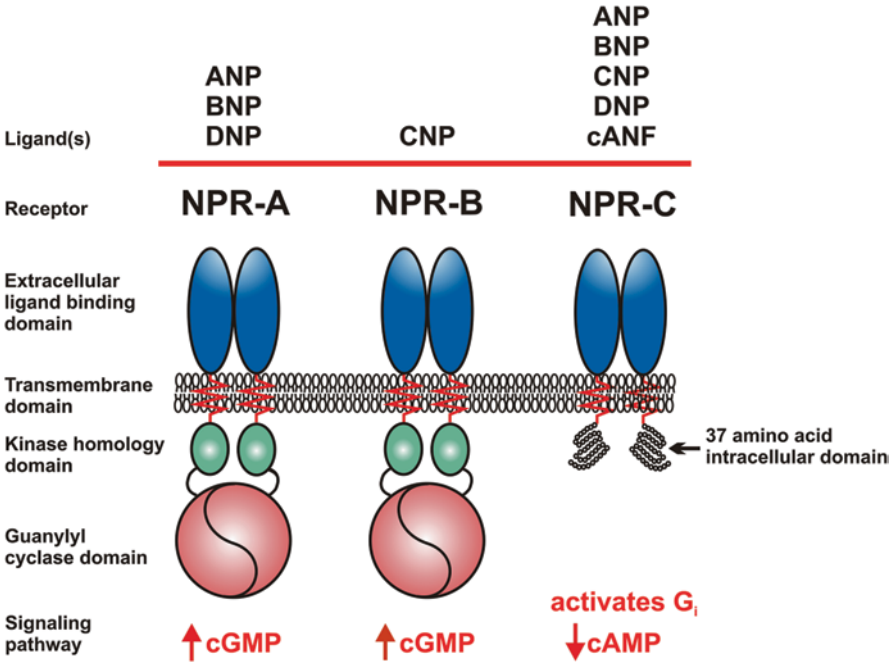

Fig. 2 Natriuretic peptide receptors and their ligand binding patterns. *NPR-A* natriuretic peptide receptor A; *NPR-B* natriuretic peptide receptor B; *NPR-C* natriuretic peptide receptor C. Note that *NPR-A* and *NPR-B* are guanylyl cyclase-linked receptors that mediate increases in cyclic guanosine monophosphate (*cGMP*). *NPR-C* has a short 37 amino acid intracellular domain that contains inhibitory G protein (G_i) activator sequences. As such NPR-C mediates a reduction in cyclic adenosine monophosphate (*cAMP*) levels

diacylglycerol (DAG). This leads to Ca^{2+}mobilization and protein kinase C (PKC) activation [36].

4 Natriuretic Peptides and Cardiac Fibrosis

NPs have been implicated in ECM remodeling and fibrosis in the heart. Some of this insight has been obtained from studies of genetically altered mice in which the NP system has been targeted. Specifically, enhanced cardiac fibrosis can be observed in NPR-A, BNP and ANP knockout animals depending on experimental conditions. Mice with global deletion of BNP ($Nppb^{-/-}$; $BNP^{-/-}$) display multifocal fibrotic lesions in the ventricles that are not observed in age matched wild type mice (Fig. 3a) [40]. When $BNP^{-/-}$ mice were subjected to aortic constriction, the level of ventricular fibrosis tripled compared to $BNP^{-/-}$ mice that received sham operations [40]. $BNP^{-/-}$ mice also exhibit increased transforming growth factor β3 (TGFβ3) and angiotensin converting enzyme (ACE) expression, suggesting that these pathways may be involved in the enhanced fibrosis characteristic of these mice.

ANP knockout mice ($Nppa^{-/-}$; $ANP^{-/-}$) are both hypertensive and hypertrophic at baseline [41, 42]. These mice display modest increases in collagen expression and collagen volume relative to wildtype controls; however, $ANP^{-/-}$ mice do not appear to exhibit the same degree of fibrosis as $BNP^{-/-}$ mice at baseline [43–45]. Nevertheless, $ANP^{-/-}$ mice subjected to pressure overload following transverse aortic constriction display profoundly worse fibrosis compared to sham operated $ANP^{-/-}$ mice, indicating that ANP is importantly involved in structural remodeling of the ECM in the heart, particularly in the setting of cardiac stress. This enhanced fibrotic response in $ANP^{-/-}$ mice occurred in association with increased expression of ECM proteins such as collagen I and III, matrix metalloproteinase 2 and tissue inhibitor of metalloproteinase 3 [44, 46].

Hearts from global NPR-A knockout ($NPR\text{-}A^{-/-}$) mice are both fibrotic (Fig. 3b) and hypertrophic in association with increased collagen deposition, increased procollagen I mRNA expression, and increased ANP and BNP mRNA expression in the ventricles [42, 47–49]. A cDNA microarray study in wild type and $NPR\text{-}A^{-/-}$ animals revealed significant alterations in gene expression patterns for genes from cell signaling pathways known to be involved in the development of cardiac fibrosis. These include, for example, fibroblast growth factor (FGF), collagens, matrix metalloproteinases, and multiple transcription factors including the histone deacetyltransferase 7a, myocyte-specific enhancer factor 2, calcineurin-nuclear factor of activated T cells, and GATA families [47, 50]. These observations in $NPR\text{-}A^{-/-}$ mice clearly suggest that the fibrotic phonotypes present in ANP and BNP knockout mice are at least partially due to a loss of NPR-A-dependent signaling. NPR-C has also been demonstrated to play an integral role in structural remodeling in the heart based on evidence that $NPR\text{-}C^{-/-}$ mice display enhanced fibrosis leading to atrial arrhythmias. Interestingly, fibrosis was restricted to the atrial myocardium in $NPR\text{-}C^{-/-}$ mice, while the ventricular myocardium was unaffected [51]. Collectively,

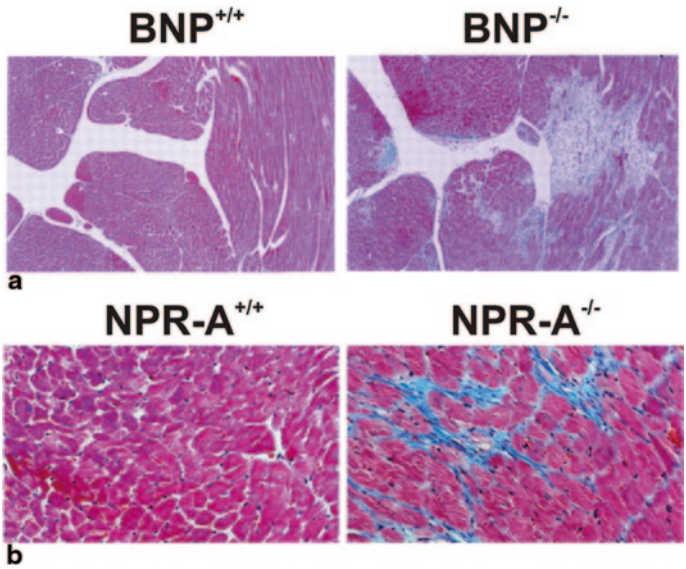

Fig. 3 Histological images of the ventricular myocardium in BNP and NPR-A knockout mice. Masson's trichrome stains from $BNP^{+/+}$ and $BNP^{-/-}$ mice (*panel A*) or $NPR\text{-}A^{+/+}$ and $NPR\text{-}A^{-/-}$ mice (*panel B*). Both $BNP^{-/-}$ and $NPR\text{-}A^{-/-}$ mice are characterized by ventricular fibrosis (*blue* color), which is not seen in wildtype mice. Data in *panel A* reproduced with permission from Tamura et al. (2000). Data in *panel B* reproduced with permission from Oliver et al. (1997)

these studies in genetically altered mice indicate that NPs play an essential protective role against adverse structural remodeling in the heart.

5 Effects of Natriuretic Peptides on Cardiac Fibroblasts

Although NPs are well known to be secreted from atrial granules located within atrial myocytes, it is now known that NPs are also made in, and secreted from cardiac fibroblasts [52, 53]. ANP and BNP mRNA can be detected in cultured fibroblasts from rats as young as 1 day old [54]. Furthermore, ANP and BNP proteins are readily detected by radioimmunoassay in the media from cultured fibroblasts. Similarly, CNP mRNA was detected in cultured ventricular fibroblasts isolated from 7-week-old rats and immunoreactive CNP was detected in the culture media [53]. Thus, NPs are synthesized in and secreted by cardiac fibroblasts.

In cultured neonatal rat ventricular fibroblasts, NPR-A, NPR-B, and NPR-C mRNAs are all expressed [55]. To determine the relative abundance of these NPRs, a Scatchard analysis was performed using cANF (Fig. 1), which is a selective agonist for NPR-C (Fig. 2) [56]. Using this approach, it has been estimated that 80% of the total NPR population is NPR-C in cultured rat and human cardiac fibroblasts [35, 55, 57]. Interestingly, NPR-B may be more highly expressed in ventricular

fibroblasts compared to cardiomyocytes [58]. All three NPRs have also been shown to be present in human cardiac fibroblasts [59].

NPs have potent antiproliferative and antimitogenic effects on cardiac fibroblasts. Using assays of radioactive thymidine incorporation into newly synthesized DNA, the potent effects of NPs on DNA synthesis have been quantified. In primary cultures of neonatal rat cardiac fibroblasts, ANP decreased the rate of DNA synthesis by approximately 40% under basal conditions [55, 60]. Cellular proliferation can also be induced by a number of hormones and growth factors including angiotensin II (Ang II), endothelin (ET), fibroblast growth factor (FGF), or insulin-like growth factor I (IGF-I), and the induction of proliferation by these compounds can be strongly antagonized by NPs. For example, in the presence of any of the above mentioned compounds, co-treatment with ANP inhibited agonist induced DNA synthesis [55]. In Ang II stimulated cultured adult rat cardiac fibroblasts, co-treatment with ANP (10^{-8} M) for 24 h resulted in 90% inhibition of cellular proliferation [61]. Similar antimitogenic effects were also observed in cells supplemented with BNP or CNP where FGF stimulated DNA synthesis rates were reduced by 25 and 21% respectively [55]. In primary human cardiac fibroblast cultures, co-treatment with BNP inhibited TGFβ induced cell proliferation by 65% [62]. In a separate study, application of BNP prevented 5-bromo-2′deoxyuridine (BrdU) incorporation into a human cardiac fibroblast cell line in which cellular proliferation was first stimulated with cardiotrophin-1 (CT-1) [59].

The vasoactive peptides Ang II and ET facilitate the enhanced cardiac fibroblast proliferation associated with cardiac fibrosis. Ang II, which is a peptide hormone, can elicit effects via the Ang II type 1 (AT_1) and type 2 (AT_2) receptors [63]. Cardiac fibroblasts express both AT_1 and AT_2 [64, 65] and it is thought that AT_1 mediates a number of the physiological and pathological effects of Ang II including fibroblast proliferation, collagen secretion, decreased collagenase activity, PLC activation, increased cytosolic calcium, and increased PKC activity [64–67].

ET-1 is a peptide growth factor initially described as a potent vasoconstrictor synthesized by cardiomyocytes and cardiac fibroblasts in the heart [68]. In cardiac fibroblasts, ET-1 levels are increased following activation of AT_1 [69, 70]. Interestingly, ET-1 levels are also increased in patients with HF. ET-1 promotes DNA synthesis following binding to the endothelin receptor ET_A, which stimulates cellular proliferation through the activation of PKC [71, 72].

Ang II and ET-1 stimulated DNA synthesis and cellular proliferation are inhibited in the presence of ANP, BNP, as well as 8-bromo-cGMP (a hydrolysis-resistant cGMP analogue) in culture media. The ET-1 promoter contains two regulatory elements responsible for basal transcriptional activity, a GATA element and activating protein-1 (AP-1) [73]. Mutation of specific sites in the proximal GATA element prevents the inhibitory effects of ANP on ET-1 induced DNA synthesis and cellular proliferation in cultured cardiac fibroblasts [60]. In this context, ANP is thought to function by inhibiting the ERK-dependent GATA4 phosphorylation required for binding to the ET-1 promoter. This in turn prevents ET-1 expression and subsequent

DNA synthesis and cellular proliferation. The fact that 8-bromo-cGMP elicits similar effects as ANP or BNP suggest that these NP effects are mediated by NPR-A.

6 Effects of Natriuretic Peptides on Collagen Synthesis

The interstitial collagens making up the ECM in the heart consist primarily of fibrillar collagen type I and collagen type III. The balance between the types of collagen present and the overall organization of these molecules within the heart play an important role in the mechanics of cardiac function. Increased levels of collagen type I is associated with myocardial stiffness whereas increased collagen type III is associated with compliance [74]. Collagen type I is several orders of magnitude stronger and stiffer than muscle [75].

NPs are very effective inhibitors of collagen synthesis in cardiac fibroblasts. In rat ventricular fibroblasts, the effects of ANP on collagen synthesis have been determined by quantifying hydroxyproline levels [57]. Treatment with TGFβ, Ang II, or serum results in a 1.3–3 fold increase in procollagen synthesis in cultured fibroblasts. The addition of ANP and zaprinast (a PDE5 inhibitor) to the culture media inhibited this increase [57]. In cultured canine ventricular fibroblasts, changes in *de novo* collagen synthesis were measured using [^{3}H]proline incorporation assays. In these experiments collagen synthesis was reduced by BNP in a concentration dependent manner. The maximum response was observed in the presence of 10^{-6} M BNP whereby [^{3}H]proline incorporation was inhibited by 29% [52]. Furthermore, RT-PCR experiments performed on TFGβ-stimulated primary human cardiac fibroblasts demonstrate increases in collagen I mRNA levels after 6, 24, and 48 h of exposure [62]; however, when cells were co-treated with BNP, this increase in collagen I expression was abolished. Western blots using collagen I antibodies further confirm these findings, whereby collagen levels increased by 3 fold in the presence of TFGβ and this effect was inhibited by 75% in the presence of BNP [62].

NPs also inhibit the effects of Ang II on collagen production by cardiac fibroblasts. For example, in Ang II stimulated rat cardiac fibroblasts, an 80% decrease in collagen synthesis was observed when cells were co-treated with ANP (10^{-8} M) for 24 h [61]. Similarly, in cultured neonatal rat cardiac fibroblasts, treatment with CNP (10^{-6} M) for 24 h caused a significant decrease in Ang II stimulated [^{3}H]proline incorporation [76]. This effect of CNP was blocked in the presence of Rp-8-pCPT-cGMP, a PKG inhibitor. Together, these experiments show that NPs have important inhibitory effects on collagen synthesis in cardiac fibroblasts.

Most of the effects of NPs on cardiac fibroblasts have been attributed to NPR-A and NPR-B activation. Consistent with this, intracellular levels of cGMP are dose dependently increased following exposure to NPs in cultured ventricular fibroblasts [52, 53, 58]. These increases in cGMP levels are correlated with decreases in collagen synthesis and DNA synthesis as determined by radioactive proline or thymidine incorporation assays, respectively. The addition of 8-bromo-cGMP to culture media mimics the effects of NPs on both DNA and collagen synthesis, thus sug-

gesting that inhibition of DNA and collagen synthesis occurs in a GC dependent fashion [52, 53, 55, 57, 61, 76]. Furthermore, NPR-A knockdown in cultured adult rat cardiac fibroblasts treated with Ang II results in a twofold increase in collagen I expression and a threefold increase in collagen III expression [61]. Addition of a synthetic cGMP analog to the media of NPR-A knockdown fibroblast cultures prevented these changes in collagen expression further confirming that the NPs can affect remodeling of the ECM via an NPR-A/cGMP pathway.

Although most studies have focused on NPR-A and NPR-B mediated effects on NPs, emerging evidence suggests that NPR-C also plays an important role in cardiac fibroblast function. As mentioned above, NPR-C is clearly expressed in cardiac fibroblasts [55, 59]. In cultured human cardiac fibroblasts, CT-1 increases BrdU incorporation which is decreased in the presence of BNP [59]. Thus, BNP (which binds NPR-A and NPR-C) inhibits CT-1 stimulated DNA synthesis. To determine the contribution of NPR-A to this effect of BNP the NPR-A antagonist HS-142-1 was added to CT-1 and BNP co-treated fibroblasts. HS-142-1 had no effect on the BNP mediated inhibition of fibroblast proliferation. In contrast, the NPR-C agonist cANF inhibited the effects of BNP on proliferation indicating a role for NPR-C in the modulation of cardiac fibroblast proliferation.

7 Transforming Growth Factor β

TGFβ is critically involved in the regulation of cellular differentiation, proliferation, as well as extracellular matrix deposition and composition [77]. The TGFβ pathway affects fibrotic remodeling within the heart as it potently modulates cardiac fibroblast proliferation and production of ECM proteins including collagens and fibronectin. TGFβ1 expression and activity is increased as a result of AT_1 activation by Ang II in cultured rat cardiac fibroblasts [67]. TGFβ1 functions by binding to two cell membrane receptor kinases, TGFβRI and TGFβRII. Once activated, these kinases facilitate the phosphorylation of two downstream proteins, Smad2 and Smad3 [78], which can then form a complex with Smad4. This Smad complex translocates to the nucleus where it activates profibrotic gene programs [77, 79, 80]. There is a positive correlation between TGFβ1 levels and collagen content in the heart. For example, TGFβ1 deficient mice have decreased levels of fibrosis whereas TGFβ1 levels are elevated in patients with HF exhibiting enhanced ECM remodeling and fibrosis [74, 81].

In cultured human cardiac fibroblasts changes in gene expression patterns in TGFβ stimulated cells were determined using microarray analysis. In this study, it was found that TGFβ stimulation induced 394 and 501 gene expression changes at 24 and 48 h of treatment, respectively. When co-treated with BNP, 88 and 85% of the TGFβ induced gene expression changes were abolished, including those involved in fibrosis and ECM production [62].

As discussed above, ANP acts as a negative modulator of cardiac remodeling and it appears that ANP functions, at least in part, by inhibiting the effects of TGFβ signaling in cardiac fibroblasts. This has been shown in cultured mouse cardiac fibroblasts pretreated with either ANP or cGMP prior to exposure to TGFβ1 for 24 h [82]. Pretreatment with ANP or cGMP resulted in a significant decrease in TGFβ induced collagen synthesis, fibroblast proliferation and pSmad3 translocation. Pretreatment with the PKG inhibitor KT5823 antagonized these inhibitory effects of ANP and cGMP. These findings indicate that ANP mediates its effects on TGFβ signaling and ECM remodeling via a cGMP-dependent mechanism. This study also shows that ANP inhibited the effect of TGFβ on collagen synthesis and fibroblast proliferation though the prevention of pSmad3 translocation into the nucleus [82].

8 Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases

The structure of the fibrillar collagen scaffold within the heart results from an interplay between collagen synthesis and degradation. Matrix metalloproteinases (MMPs) are a family of proteins that play an essential role in matrix degradation [74]. In the diseased heart, increased MMP activity results in degradation of normal collagen and the development of interstitial deposits of poorly cross-linked collagens characteristic of those present in the fibrotic heart [83]. In NPR-A deficient animals, MMP2 and MMP9 protein levels are increased by 3 and 4 fold respectively in 4 week old animals and further increased by 22 weeks of age [49]. Furthermore, as discussed above, collagen levels are doubled in adult NPR-A$^{-/-}$ mice compared to their wild type littermates. Stimulation of cultured rat cardiac fibroblasts with Ang II results in increases in MMP2 and MMP9 mRNA expression as well as activity [61]. These alterations are also observed in fibroblasts in which NPR-A is knocked down. Conversely, when fibroblasts isolated from wildtype mice are co-treated with Ang II and ANP, the increase in MMP2 and MMP9 activity and expression is abolished [61]. Together, these findings suggest that ANP and NPR-A oppose Ang II induced MMP2 and MMP9 synthesis.

To unravel the underlying mechanism for these observations, the effects of ANP on second messenger levels were evaluated in Ang II-stimulated fibroblasts. Ang II stimulation activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, resulting in the generation of reactive oxygen species (ROS) [84], which has been shown to induce cardiac fibroblast proliferation and increased fibrosis leading to the progression of end stage HF. Treatment of Ang II stimulated rat cardiac fibroblasts with ANP resulted in significantly decreased ROS levels as assessed by spectroflourometric analysis [61]. Conversely, in NPR-A knockdown experiments, ROS levels were further increased in the presence of Ang II relative to wildtype fibroblasts, but this could be abolished in the presence of 8-bromo-cGMP. Following ROS stimulation, nuclear factor-kappa-B (NF-κB) is translocated to the nucleus where binding of NF-κB to DNA results in the increased expression of ECM remodelers including collagens and MMP1, 3, and 9 [85]. In cultured rat cardiac

fibroblasts treated with Ang II, nuclear translocation of NF-κβ was examined using confocal microscopy and an NF-κB antibody. In these studies, addition of ANP to the cultures inhibited NF-κB nuclear translocation and DNA binding [61]. This in turn would result in decreased expression of collagen type I, collagen type III, MMP2, and MMP9 transcripts.

Tissue inhibitors of metalloproteinases (TIMPs) are potent endogenous inhibitors of MMP activity. There are four TIMP isoforms detected in the heart including TIMP1, TIMP2, TIMP3, and TIMP4. X-ray crystallography studies have shown that TIMPs bind to the active site of MMPs, thereby preventing ECM substrate binding and inhibition of MMP activity [86, 87]. BNP appears to exert its effects on ECM remodeling in part through its effects on TIMP expression levels. In left ventricular tissues isolated from NPR-A$^{-/-}$ mice, TIMP1 and TIMP2 protein levels were significantly lower compared to wildtype mice [49]. In a different model, TIMP2 protein expression displayed a 12% increase following 24 h of BNP treatment in cultured canine ventricular cardiac fibroblasts although TIMP1 levels remained unchanged [52]. Furthermore, in primary human cardiac fibroblast cultures, microarray analysis and RT-PCR experiments indicate that TIMP3 expression is increased in the presence of TGFβ [62]. The addition of BNP to these cells results in a downregulation of TIMP3 expression. Together, these studies suggest that NPs affect TIMP expression in cardiac fibroblasts; however, the mechanism by which NPs alter TIMP expression profiles or function remains largely unknown.

9 Chronic Natriuretic Peptide Treatment in the Diseased Heart

Myocardial infarction (MI) can be surgically induced in rodents by ligating the coronary artery, resulting in significant ventricular remodeling and a decline in cardiac function leading to HF. To study the effects of NPs in this disease model rats were subjected to MI and treated with a low dose (5 μg/kg/day) or a high dose (15 μg/kg/day) of BNP for 8 weeks beginning the day after the surgeries occurred [88]. Echocardiographic and hemodynamic measurements indicate that BNP treatment improved cardiac function compared to the untreated animals. In animals treated with BNP, histological analysis of excised hearts showed a significant decrease in the amount of collagen deposited within the ventricles. Both plasma and myocardium Ang II levels were significantly higher in vehicle-treated animals compared to those receiving BNP treatment. Furthermore, in animals treated with BNP there was a significant decrease in TGFβ1 and Smad2 mRNA and protein expression despite an increase in Ang II expression. This suggests that BNP both counteracts the harmful effects of increased Ang II levels and inhibits TGFβ1/Smad2 signaling resulting in less detrimental ECM remodeling following MI. These beneficial effects of BNP were more pronounced in animals treated with 15 μg/kg/day compared to the lower dose of 5 μg/kg/day.

A separate study infused CNP for 2 weeks in rats subjected to experimental MI. CNP (0.1 μg/kg/day) was delivered intravenously using osmotic mini-pumps starting 4 days following surgery and continuing for 2 weeks [76]. In this study, CNP infusion significantly prevented left ventricular enlargement and reduction in cardiac function caused by MI. Autoradiograms and qPCR experiments showed a significant decrease in the amount of collagen I and collagen III protein and mRNA expression in the ventricles of CNP treated animals. Interestingly, endogenous expression of CNP mRNA initially increased four-fold on day 3 in the infarcted left ventricle and gradually decreased to the end of the treatment period at day 18. Histological analysis revealed that CNP was concentrated at the infarct and border zone on day 7 following MI. Thus, CNP also acts as a cardioprotective agent following MI.

The cardioprotective effects of CNP have also been investigated in mice chronically treated with Ang II, which is a well-established model of cardiac hypertrophy and fibrosis [89, 90]. In a recent study using this model, mice were treated with Ang II (3.2 mg/kg/day) for 2 weeks and a subset of animals were co-treated with CNP (0.05 μg/kg/min) also for 2 weeks [91]. As expected, Ang II treated mice showed clear signs of cardiac dysfunction and had increased levels fibrosis, collagen expression, and ROS production. Co-treatment with CNP resulted in a significant decrease in the level of interstitial fibrosis and collagen type I and III mRNA expression compared to vehicle-treated animals (Fig. 4). CNP infusion completely prevented Ang

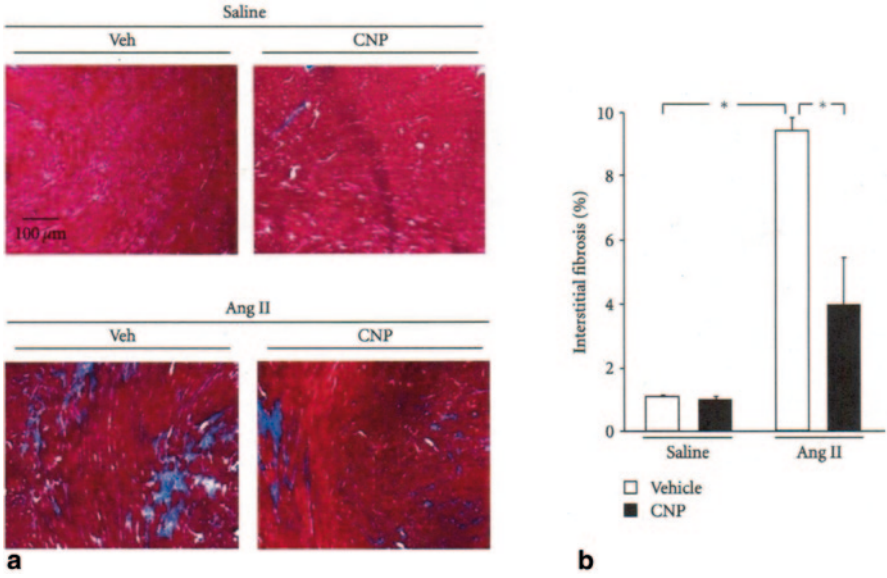

Fig. 4 Effects of CNP on Ang II induced ventricular fibrosis in mice. **a** Masson's trichrome stains of myocardium in saline and Ang II treated mice cotreated with CNP or vehicle. **b** quantification of interstitial fibrosis in Ang II and/or CNP treated mice. Ang II induces ventricular fibrosis, which is significantly attenuated by cotreatment with CNP. Data reproduced with permission from Izumiya et al. (2012)

II-induced cardiac superoxide production and significantly reduced the expression of the NADPH oxidase subunit NOX4. Interestingly, CNP infusion also prevented the upregulation of ANP and BNP mRNA expression seen in the vehicle treated control animals. Combined, these data further support the notion that CNP acts as a protective agent within the heart preventing Ang II-induced cardiac remodeling and superoxide production.

Most recently, attention has been given to the development of designer synthetic NPs that may be particularly effective in the treatment of HF and its associated complications. One example of this is the peptide CD-NP (also known as cenderitide), which is a chimeric peptide that combines CNP with the C-terminal tail of DNP [92, 93] (Fig. 5a). CD-NP is able to bind and activate all three NPRs [94]. The effects of CD-NP on ECM remodeling have been tested in an experimental model of cardiac fibrosis induced by unilateral nephrectomy in rats [95]. In this study, a 2 week subcutaneous infusion of CD-NP significantly suppressed left ventricular fibrosis (Fig. 5b) and preserved systolic and diastolic function compared to vehicle treated rats with unilateral nephrectomy. This same report also demonstrated that CD-NP could increase cGMP production in cells heterologously expressing NPR-A and NPR-B; however, this was not confirmed specifically in cardiac fibroblasts.

A separate investigation assessed the ability to slowly release CD-NP from biodegradable polymeric films [96], which could have important implications for the therapeutic use of CD-NP in conjunction with cardiac patches. Importantly, the bioactivity of CD-NP released from these patches was assessed by measuring the effects of released peptide on human cardiac fibroblasts. These studies demonstrate that the released CD-NP is able to inhibit fibroblast proliferation and suppress DNA synthesis in association with increased production of cGMP in these fibroblasts. This suggests that CD-NP may have beneficial therapeutic effects, which involve the prevention of ECM remodeling. Furthermore, these effects at least partially involve the NPR-A and NPR-B receptors.

10 Summary and Conclusions

When considered collectively, there is strong evidence that NPs have both potent antiproliferative and antifibrotic effects on cardiac fibroblasts. As such, NPs play an important protective role against adverse structural remodeling of the ECM in the normal heart and in the setting of cardiovascular disease. Despite these clear beneficial effects, there are several areas of ongoing investigation that will improve our understanding of how NPs protect against remodeling of the ECM. For example, most of the effects of NPs on cardiac fibroblasts have been attributed to the GC-linked NPR-A and NPR-B receptors. Nevertheless, there is some evidence that NPR-C, which is highly expressed in cardiac fibroblasts, may also be involved. As most naturally occurring and synthetic NPs are able to bind multiple NPRs, it seems critical that ongoing studies consider how simultaneous activation of the GC-linked NPRs and NPR-C results in the overall effects of NPs on the ECM.

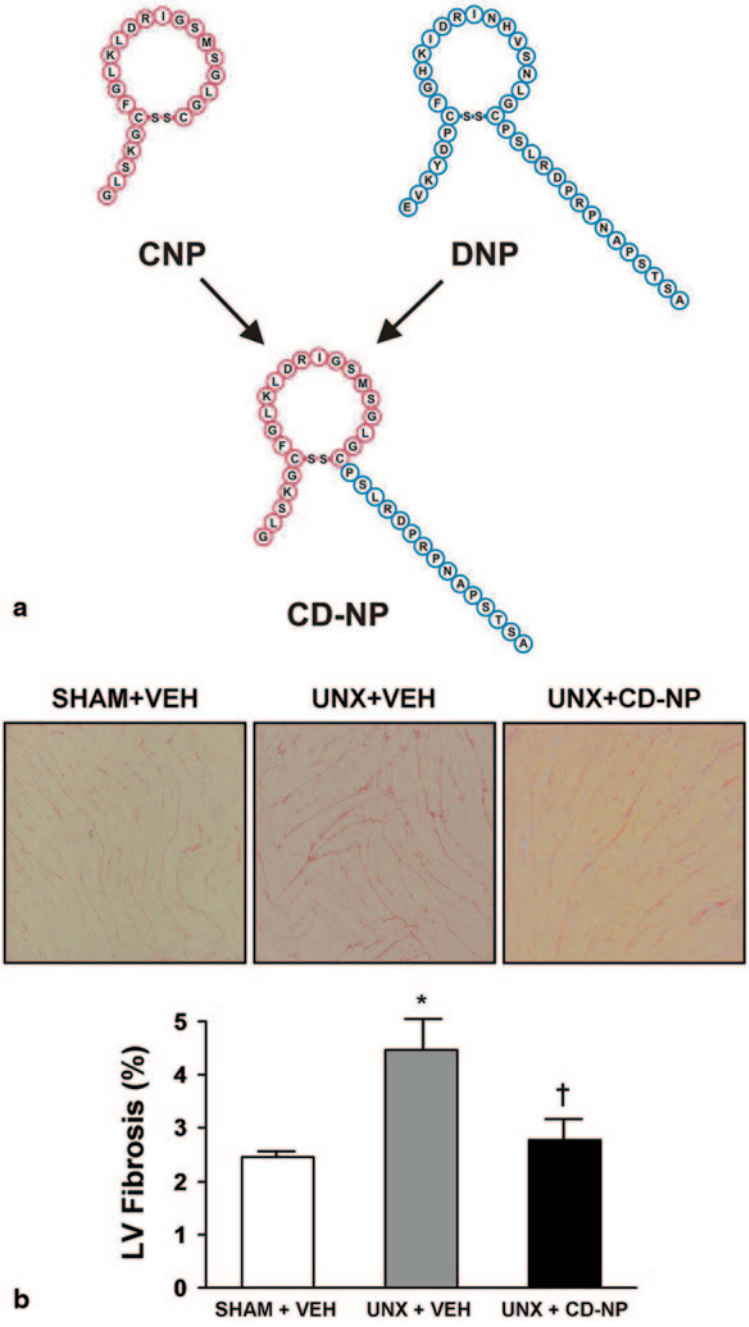

Fig. 5 Structure and antifibrotic effects of the chimeric natriuretic peptide CD-NP. **a** Structure and amino acid sequence of CD-NP, which is formed by combining CNP with the C-terminal tail of DNP. **b** Picrosirius red histology images and quantification of ventricular fibrosis from control rats (sham + vehicle), rats subjected to unilateral nephrectomy (*UNX*) to induce cardiac fibrosis, and rats subjected to UNX treated with CD-NP for 2 weeks. Data reproduced with permission from Martin et al. (2012)

Another emerging area of investigation is related to NP effects on ion channels in cardiac fibroblasts. These fibroblasts express a number of potassium and transient receptor potential (TRP) channels [97, 98]. NPs have been shown to activate non-selective cation currents that are likely carried by members of the TRP-C family of ion channels [98]. Furthermore, these same TRP-C channels have been shown to mediate an influx of Ca^{2+} into cardiac fibroblasts, which has implications for arrhythmogenesis in the heart [99, 100]. It is presently unknown whether the effects of NPs on fibroblast ion channels and Ca^{2+} homeostasis are directly linked to the protective effects of NPs against structural remodeling; thus, this will require ongoing investigation.

Finally, the recent development of chimeric NPs, such as CD-NP, and the possibility of delivering NPs via synthetic patches, highlights the exciting potential for the therapeutic use of NPs for the prevention of adverse structural remodeling and fibrosis in the heart. Continued investigation into the design of these synthetic NPs and the methods for their chronic delivery to patients is needed to bring this to fruition.

Progress in each of the above mentioned areas, in combination with the information already known regarding the effects of NPs on remodeling of the ECM, will greatly impact the strong potential for the use of NPs for the prevention of adverse structural remodeling and fibrosis in human HF patients.

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