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Regulation of cardiac angiotensin-converting enzyme and angiotensin AT₁ receptor gene expression in *Npr1* gene-disrupted mice

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SUMMARY

1. Understanding of the regulatory mechanisms of gene expression in the control of blood pressure and fluid volume is a key issue in cardiovascular medicine. Guanylyl cyclase/natriuretic peptide receptor-A (GC-A/NPRA) signalling antagonizes the physiological and pathophysiological effects mediated by the renin–angiotensin–aldosterone system (RAAS) in the regulation of cardiovascular homeostasis.
2. The targeted-disruption of the *Npr1* gene (coding for GC-A/PRA) leads to activation of the cardiac RAAS involved in the hypertrophic remodelling process, which influences cardiac size, expression of pro-inflammatory cytokine genes and the behaviour of various hypertrophy marker genes. The *Npr1* gene-knockout (*Npr1*^{−/−}) mice exhibit 35–40 mmHg higher systolic blood pressure and a significantly greater heart weight to bodyweight ratio than wild-type (*Npr1*^{+/+}) mice.
3. The expression of both angiotensin-converting enzyme (ACE) and angiotensin II AT_{1a} receptors are significantly increased in hearts from *Npr1*^{−/−} mice compared with hearts from *Npr1*^{+/+} mice. In parallel, the expression of interleukin-6 and tumour necrosis factor-α is also markedly increased in hearts from *Npr1*^{−/−} mice.
4. These findings indicate that disruption of NPRA/cGMP signalling leads to augmented expression of the cardiac RAAS in conjunction with pro-inflammatory cytokines in *Npr1*-null mutant mice, which promotes the development of cardiac hypertrophy and remodelling.

Keywords

angiotensin-converting enzyme; AT₁ receptor; atrial natriuretic peptide; cardiac hypertrophy; fibrosis; gene expression; gene knockout; guanylyl cyclase/natriuretic peptide receptor-A; pro-inflammatory cytokines

INTRODUCTION

The cardiac hormones atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are expressed in high concentrations in the heart, are released into the circulation and can elicit natriuretic, diuretic, vasorelaxant and antiproliferative responses, all directed to the reduction of blood pressure and blood volume.^{1–6} Conversely, C-type natriuretic peptide (CNP) is found primarily in vascular endothelial cells and the central nervous system.⁵ Three natriuretic peptide receptor systems have been described.⁷ Both ANP and BNP bind to guanylyl cyclase-A/natriuretic peptide receptor-A (GC-A/NPRA), which is considered the principal natriuretic peptide hormone receptor that synthesises the intracellular second messenger cGMP.^{7–11} Previous studies have suggested that ANP/NPRA signalling locally antagonizes cardiac growth responses to hypertrophic stimuli.¹² In particular, mice carrying targeted disruption of the *Npr1* gene (encoding for NPRA) exhibit hypertension and congestive heart failure, with sudden death occurring in mice after 6 months of age.^{13–16} Overexpression of NPRA reduces blood pressure and attenuates hypertrophic agonist-induced cardiomyocyte growth.^{5,17,18} Interestingly, ANP gene delivery has been shown to attenuate cardiac hypertrophy in spontaneously hypertensive rat models.^{19–21} Nevertheless, the cellular mechanisms by which the ANP/NPRA system blocks cell proliferation and hypertrophic growth is not well understood.

The renin–angiotensin–aldosterone system (RAAS) plays an important role in the endocrine/paracrine regulation of arterial pressure and blood volume. Angiotensin (Ang) II, the main active component of the RAAS, is a potent vasoconstrictor; it increases sympathetic tone and elicits trophic effects in the cardiovascular remodelling process associated with hypertension.^{22–27} Thus, in addition to its systemic effects, AngII is implicated in the development of cardiac hypertrophy and cardiac fibrosis in humans and in experimental animal models.^{25,27–30} Most of the effects of AngII in the cardiovascular system are mediated through AT₁ receptors.^{30–32} Treatment with angiotensin-converting enzyme (ACE) inhibitors or AT₁ receptor antagonists effectively lowers blood pressure and prevents or ameliorates myocardial hypertrophy.^{24,25,33} In addition, AngII is considered to be a pro-inflammatory mediator that has a pivotal function in the inflammatory process underlying the development of vascular complications.^{32,34} Furthermore, AngII has been shown to mediate hypertrophic growth in neonatal and adult myocytes by activating transforming growth factor (TGF)- β and endothelin signalling pathways.^{35,36}

At the cellular and molecular levels, ANP/NPRA signalling has been suggested to inhibit the AngII-mediated induction of protein kinase C and mitogen-activated protein kinases in vascular smooth muscle cells and mesangial cells.^{37–39} Conversely, AngII has been shown to modulate and repress *Npr1* gene transcription and expression.^{40–42} In addition, the ANP/NPRA system has been reported to have an anti-inflammatory role, inhibiting tumour necrosis factor (TNF)- α production in interferon- γ -activated macrophages and inhibiting TNF- α -induced adhesion molecule expression in endothelial cells.^{43,44} The present review focuses on the effect of disruption of the GC-A/NPRA signalling pathway in the activation of cardiac RAAS components and inflammatory mediators in ventricular tissues of hypertrophied hearts of mice lacking NPRA.

SYSTOLIC BLOOD PRESSURE AND DEVELOPMENT OF CARDIAC HYPERTROPHY AND FIBROSIS IN *Npr1* GENE-DISRUPTED MICE

The *Npr1*^{-/-} mutant mice exhibit significantly increased systolic blood pressure (SBP) and an increased heart weight (HW) to bodyweight (BW) ratio compared with *Npr1*^{+/+} control mice.^{16,45} Table 1 lists the SBP, HW/BW ratio and indices of fibrosis in hearts from both *Npr1*^{+/+} and *Npr1*^{-/-} mice. On average, SBP, HW/BW ratio, total collagen and fibrosis are significantly higher in hearts from *Npr1*^{-/-} mutant mice compared with age-matched wild-type control mice.¹⁶ The coronary vessel wall thickness and myocyte cross-sectional area were also significantly increased in mutant mice compared with wild-type mice.^{13,14,45} Interestingly, the antihypertensive drugs captopril and hydralazine were effective in reducing the elevated SBP in mutant mice.¹⁶ However, captopril alone attenuated the HW/BW ratio while significantly reducing fibrosis and collagen content, suggesting the involvement of the RAAS in the hypertrophic growth of mutant mice hearts. These studies also demonstrated a correlation between decreased ventricular cGMP and increased ventricular AngII. Previously it was shown that *Npr1*^{-/-} mice have cardiac hypertrophy disproportionate to their increased blood pressure, suggesting that the ANP/NPRA system exerts a local antihypertrophic effect on cardiac cells.⁴⁶ Furthermore, mouse pups lacking NPRA showed a significant increase in heart size compared with age-matched wild-type control pups as early as 16–30 days after birth, supporting the notion that blood pressure alone is not likely to be the primary cause for the development of cardiac hypertrophy in *Npr1*^{-/-} mutant mice.^{13,47,48} In a previous study, the ACE inhibitor captopril was shown to reduce blood pressure and decrease the HW/BW ratio in *Npr1*^{-/-} mice.¹⁶ However, another study⁴⁷ reported that enalapril was able to reduce blood pressure only and not the HW/BW ratio in *Npr1*^{-/-} mice. We believe that the discrepancy between these two studies may be due to the different methodologies used for drug delivery in *Npr1* mice.^{16,47}

To examine the local effect of the ANP/NPRA system in counteracting the effect of AngII *in vivo*, conditional cardiomyocyte (CM)-restricted disruption of *Npr1* (CM-*Npr1*^{-/-}) mice have also been used.⁴⁹ In that study, the authors reported that the hypertensive responses of CM-*Npr1*^{-/-} mice to exogenous AngII were not altered; however, AngII-induced cardiac hypertrophy and fibrosis were greatly enhanced. Their findings also indicated that ANP/NPRA signalling can moderate cardiac events independent of its blood pressure-lowering effects, even though the higher basal blood pressure and higher left ventricular weight themselves contribute towards the increased susceptibility to heart failure in *Npr1*^{-/-} mice. In ANP gene-knockout (-/-) mice with volume overload due to an aortocaval fistula, higher ventricular weight and wall thickness were observed compared with control ANP wild-type (+/+) mice;⁵⁰ despite this, blood pressure did not differ significantly, suggesting that ANP can exert an antihypertrophic effect independent of blood pressure.⁵⁰ Similarly, a *Npr1* gene-knockout aortocaval fistula mouse model produced a greater degree of congestive heart failure and impaired haemodynamics with increased left and right ventricular weights compared with sham-operated control mice.⁵¹ Those authors suggested that, in the absence of NPRA signalling, volume overload induced a greater degree of heart failure.

GENE EXPRESSION AND ROLES OF ACE AND AT₁ RECEPTORS IN BLOOD PRESSURE REGULATION AND CARDIAC REMODELING IN *Npr1* GENE-DISRUPTED MICE

The ventricular expression of ACE and AT_{1a} were found to be up-regulated by almost three- to fourfold in hearts from *Npr1*-null mutant mice compared with hearts from wild-type mice.¹⁶ Surprisingly, both AngII content and AT_{1a} mRNA expression were found to be increased in hearts from mutant mice, further suggesting that RAAS components were significantly activated in the absence of *Npr1*^{-/-} signalling. Accordingly, the increased AngII levels in hearts from mutant mice were found to be negatively correlated with ventricular cGMP levels, further supporting the finding that the ANP/NPRA signalling system antagonizes RAAS signalling in a tissue-specific manner.^{45,52} Although systemic and renal AngII levels are increased in newborn mouse pups lacking NPRA, both circulating and intrarenal AngII and renin levels were reduced in adult *Npr1*-null mutant mice compared with age-matched *Npr1*^{+/+} mice.⁵² It has been suggested that the possible cause for the reduction in circulating and renal AngII and renin levels may be the increased arterial pressure, which would lead to baroreceptor activation and reflex neural inhibition of renin release in the absence of a functional *Npr1* gene in adult mice.^{4,45,52} It is implied that the cardiac RAAS is activated in hearts from adult mutant mice, even though the circulating levels of AngII are decreased, further supporting previous studies indicating that the cardiac RAAS can be activated independently of and dissociated from the circulating RAAS.^{53,54} The evidence also suggests that tissue levels of RAAS components are greatly increased in contrast with circulating levels of RAAS components in AngII-infused hypertensive rat models with elevated arterial pressure.⁵⁵

It has been demonstrated that all the components of the RAAS can be synthesised in local tissues and that locally produced AngII can serve as a paracrine and/or autocrine growth stimulatory factor.^{52,55-57} It has also been shown that inhibition of plasma renin activity with salt overload does not affect ventricular remodelling after myocardial infarction in rats; however, the local cardiac RAAS is activated, which plays a predominant role in local adaptation of the heart after myocardial infarction.⁵⁸ Our studies have established that the cardiac RAAS is greatly activated and plays a critical role in the ventricular remodelling process in mice lacking NPRA.⁴⁵

In another mouse model lacking NPRA, blockade of the AT₁ receptor reduced the cardiac hypertrophy and fibrosis to a great extent, suggesting a role for AT₁ receptor function in cardiac remodelling in *Npr1*^{-/-} mice.⁵⁹ Interestingly, coactivation of ACE and AT_{1a} expression has been reported in ventricular tissue of *N*^G-nitro-L-arginine methyl ester (L-NAME)-induced hypertensive rat models.³³ Treatment with ACE or AT₁ receptor antagonists reduced the inflammatory phenotype in the arterial vessel wall of L-NAME-induced hypertensive rat models.^{33,60} Previous studies have demonstrated that treatment with captopril, but not hydralazine, significantly attenuates indices of cardiac hypertrophy and coronary vessel wall thickening in *Npr1*^{-/-} mutant mice (Table 1).

The increased expression of both ACE and AT_{1a} receptor mRNA in hearts of *Npr1*^{-/-} mice was significantly reduced by captopril, but not hydralazine, treatment compared with wild-type mice (Fig. 1a–d). Increased expression of AT_{1a} (Fig. 2a,e) and nuclear factor (NF)-κB (Fig. 2b,f) immunoreactivity was also evident in the vascular tissue of hearts from *Npr1*^{-/-} mutant mice compared with hearts from wild-type mice. Masson's trichrome staining showed increased perivascular fibrosis in the vascular tissue of hearts from mutant mice compared with hearts from wild-type mice (Fig. 2c,g). Toluidine blue staining of infiltrating mast/monocyte cells showed no significant increase in the number of these cells in the arteries of *Npr1*^{-/-} compared with *Npr1*^{+/+} mice (Fig. 2d,h). Figure 2i shows the average mast/monocyte positive cell infiltration per section in mutant and wild-type mice. It was concluded that *Npr1*^{-/-} mutant mice hearts showed no significant increase in the infiltration of mast/monocyte cells compared with wild-type mice hearts.¹⁶ However, blockade of AngII seems to contribute to improvement in the cardiac remodelling and inflammatory process in *Npr1*^{-/-} mice. Because both captopril and hydralazine equally attenuate SBP, but only captopril significantly decreases the HW/BW ratio, it is implied that AngII elicits cardiac remodelling in a blood pressure-independent manner.¹⁶ Similarly, the mineralocorticoid receptor blocker eplerenone has been shown to attenuate cardiac remodelling in *Npr1*^{-/-} mice in a blood pressure-independent manner.⁶¹ In another model of *Npr1*-null mutant mice in which both *Npr1* and AT₁ receptor genes are ablated, the HW/BW ratio and levels of fibrosis were similar to those of wild-type mice and it was suggested that cardiac remodelling in *Npr1*^{-/-} mice occurs in an AT₁ receptor-dependent manner.⁵⁹

CARDIAC REMODELLING AND VENTRICULAR CYTOKINE GENE EXPRESSION IN *Npr1* GENE-DISRUPTED MICE

Previous findings have shown that disruption of ANP/NPRA signalling results in augmented expression of both circulating and ventricular cytokines (interleukin (IL)-2, IL-6 and TNF-α), suggesting that activation of the inflammatory process in vascular and ventricular tissues of hearts from *Npr1*^{-/-} mutant mice is correlated with enhanced cardiac fibrosis and hypertrophic growth, independent of blood pressure.^{13,16} It has been proposed that pro-inflammatory cytokines such as IL-6 and TNF-α are important mediators in the development of cardiac hypertrophy and heart failure.^{62–66} Cytokines have been shown to be expressed within the heart in response to either mechanical overload or ischaemic injury.^{64,66–68} In hearts from *Npr1*^{-/-} mice, significant upregulation was observed in the ventricular expression of cytokine genes compared with hearts from age-matched wild-type mice.¹⁶ Parallel increases of almost fourfold in plasma levels of IL-6 and TNF-α occurred in *Npr1*^{-/-} mice. The mechanisms underlying the stimulation of ventricular and plasma concentrations of inflammatory markers are not well understood. Participation of early gene activation has been proposed, with progressive mechanical stress associated with hypertension and cardiovascular events.¹³ In previous studies, the reduced expression of inflammatory markers was more pronounced in captopril-treated *Npr1*^{-/-} mice than in hydralazine-treated mice,¹⁶ suggesting that haemodynamic overload may not to be the sole mechanism accounting for improvement in the inflammatory process in these hearts.

We have reported that a significant increase occurs in NF- κ B levels and inhibitory κ B kinase- β (IKK- β) activity in hearts from *Npr1*^{-/-} mice compared with *Npr1*^{+/+} mice, suggesting that the NF- κ B signalling pathway is activated in the mutant mouse heart.^{13,16} The increased NF- κ B binding activity was positively correlated with increased expression of cytokine genes in hearts from *Npr1*-null mice. Recent studies by several groups have implicated the activation of NF- κ B as a causal event in cardiac hypertrophy.⁶⁹⁻⁷¹ We have also shown that ventricular levels of thiobarbituric acid-reactive substances (TBARS) are increased in hearts from *Npr1*-null mutant mice compared with hearts from wild-type mice, indicating an increase in overall oxidative stress.¹⁶ The increased levels of TBARS, an end-product of lipid peroxidation, have been considered as a potential marker for oxidative stress in the pathophysiology of many forms of hypertension.^{72,73} Furthermore, treatment with free radical-scavenging agents such as tempol (4-hydroxy tetramethylpiperidine-1-oxyl) significantly ameliorates oxidative stress, blood pressure and hypertrophic growth in different hypertensive models.⁷⁴ It has been reported that AngII functions a strong activator of reactive oxygen species and NF- κ B, mediated by its binding to the AT_{1a} receptor *in vivo* and *in vitro*.^{30,75,76}

Conversely, it has been shown that the ANP/NPRA system attenuates the production of inflammatory mediators such as TNF- α and IL-6 by regulating the NF- κ B pathway.^{44,77} Furthermore, a recent study demonstrated that augmentation of the second messenger cGMP via chronic inhibition of cGMP-specific phosphodiesterase 5A attenuates load-induced hypertrophy, fibrosis and myocardial dysfunction independent of changes in load, suggesting that cGMP locally antagonizes hypertrophic mediators and signalling pathways.⁷⁸ Immunohistochemical analyses have demonstrated increased expression of AT₁ receptors and NF- κ B in the vascular wall of hearts from *Npr1*^{-/-} mutant mice, suggesting that vascular wall remodelling and medial thickening play a crucial role in the development of hypertrophy in *Npr1*^{-/-} mutant mice.¹⁶ Figure 3 shows the signalling pathway proposed to be involved in the cardiac remodelling process in the absence of NPRA/cGMP signalling. Previous findings have implicated disruption of ANP/NPRA/cGMP signalling leading to increased activation of the RAAS and pro-inflammatory cytokines, in turn leading to medial thickening and perivascular fibrosis. These changes promote cardiac remodelling, hypertrophy and heart failure.^{13,16} Together, those studies have clearly demonstrated that NF- κ B signalling is critically involved in the activation of inflammatory cytokine gene expression in the vascular and ventricular tissues of hearts from *Npr1*^{-/-} mutant mice.

CONCLUSION AND PERSPECTIVES

In conclusion, the evidence suggests that disruption of the ANP/NPRA signalling pathway results in increased blood pressure and cardiac hypertrophy associated with augmented expression of cytokines, such as IL-6 and TNF- α . Furthermore, both antihypertensive drugs captopril and hydralazine are effective in reducing the elevated SBP, but captopril alone attenuated fibrosis and collagen content, implicating direct involvement of RAAS components in hypertrophic growth of *Npr1*-null mice, independent of increased blood pressure. Interestingly, captopril treatment also reduced the augmented expression of cytokines in *Npr1*^{-/-} mice, suggesting that AngII is involved in the activation of cardiac inflammatory mediators that may be associated with pathways leading to cardiac

hypertrophy and congestive heart failure. Indeed, future studies are needed to explore molecular targets of natriuretic peptides and their receptor systems in the treatment, diagnosis and prevention of high blood pressure and cardiovascular events.

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List of abbreviations

ACE	Angiotensin-converting enzyme
AngII	Angiotensin II
ANP	Atrial natriuretic peptide
BNP	B-Type natriuretic peptide
CNP	C-Type natriuretic peptide
GC-A/NPRA	Guanylyl cyclase-A/natriuretic peptide receptor-A
IKK-β	Inhibitory κ B kinase- β
IL	Interleukin
L-NAME	N^G -Nitro-L-arginine methyl ester
NF-κB	Nuclear factor- κ B
RAAS	Renin-angiotensin-aldosterone system
SBP	Systolic blood pressure
TBARS	Thiobarbituric acid-reactive substances
TNF-α	Tumour necrosis factor- α

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Fig. 1.

Expression profiles of (a,b) angiotensin-converting enzyme (ACE) and (c,d) angiotensin AT_{1a} receptor genes in hearts from *Npr1*^{+/+} and *Npr1*^{-/-} mice. (a,c) Representative reverse transcription–polymerase chain reaction (RT-PCR) mRNA expression analysis of ACE and AT_{1a} receptor expression in *Npr1*^{+/+} and *Npr1*^{-/-} mice with and without antihypertensive drug treatment. UT, untreated; HYZ, hydralazine; CAP, captopril. (b,d) Densitometry analysis of mRNA transcripts normalized against the expression of GAPDH. The RT-PCR was performed using gene-specific primers. Values are expressed as the mean $\pm$ SEM ($n = 8$ /group). *** $P < 0.001$. Reproduced with permission from Vellaichamy *et al.*¹⁶

Fig. 2. Immunohistochemical analysis of angiotensin AT_{1a} receptor and nuclear factor (NF)-κB protein expression in coronary vessels of hearts from *Npr1*^{+/+} and *Npr1*^{-/-} mice. (a,e) Increased AT_{1a} receptor protein expression, (b,f) NF-κB protein expression, (c,g) perivascular fibrosis and (d,h) the number of inflammatory mast/monocyte infiltrating cells in coronary vessels of hearts from *Npr1*^{-/-} (a–d) and *Npr1*^{+/+} (e–h) mice. (i) Mast/monocyte positive cells infiltration/section. Values are expressed as the mean ± SEM (*n* = 8). UT, untreated; HYZ, hydralazine; CAP, captopril. Reproduced with permission from Vellaichamy *et al.*¹⁶

Fig. 3.

Diagram of the proposed signalling pathway involved in the cardiac remodelling process in the absence of natriuretic peptide receptor-A (NPRA)/cGMP signalling. Disruption of atrial natriuretic peptide (ANP)/NPRA signalling leads to increased activation of the renin–angiotensin system (RAS). Activation of the RAS has been shown to activate increased generation of reactive oxygen species (ROS). This leads to activation and dissociation of nuclear factor (NF)-κB and activator protein (AP)-1 transcription factor subunits from the complex in the cytoplasm and translocation into the nucleus, which activates pro-inflammatory cytokine genes, leading to medial thickening and perivascular fibrosis, in turn promoting cardiac remodelling, hypertrophy and heart failure. KHD, kinase homology domain; GC, guanylate cyclase; AngII, angiotensin II. Reproduced with permission from Vellaichamy *et al.*¹⁶

Table 1

Systolic blood pressure, heart rate, heart weight to bodyweight ratio, cardiac collagen content, cGMP, angiotensin II, Thiobarbituric acid-reactive substances and fibrosis in *NprI*^{+/+} and *NprI*^{-/-} mice treated with and without antihypertensive drugs

	Untreated		Hydralazine		Captopril	
	<i>NprI</i> ^{+/+}	<i>NprI</i> ^{-/-}	<i>NprI</i> ^{+/+}	<i>NprI</i> ^{-/-}	<i>NprI</i> ^{+/+}	<i>NprI</i> ^{-/-}
SBP (mmHg)	100 ± 4	130 ± 8***	97 ± 3	110 ± 6***††	98 ± 4	106 ± 5***††
HW/BW ratio	4.6 ± 0.3	7.5 ± 0.5*	4.7 ± 0.4	7.1 ± 0.7*	4.4 ± 0.2	5.3 ± 0.7*†
cGMP (pmol/mg protein)	26 ± 5	5 ± 1***	25 ± 3	6 ± 1**	28 ± 6	10 ± 2†
AngII (pmol/mg protein)	15 ± 2	32 ± 2***	14 ± 1	28 ± 3**	12 ± 1	17 ± 2††
TBARS (nmol MDA/mg protein)	25 ± 3	38 ± 3***	23 ± 2	32 ± 2*	22 ± 2	23 ± 2††
W/L ratio	0.10 ± 0.02	0.25 ± 0.07***	0.10 ± 0.03	0.2 ± 0.5**	0.1 ± 0.3	0.13 ± 0.03††
MCA (μm ²)	415 ± 22	915 ± 73***	413 ± 18	825 ± 34**	409 ± 16	562 ± 82*††
Collagen (mg/g tissue)	1.4 ± 0.1	3.5 ± 0.7***	1.3 ± 0.3	2.4 ± 0.2**	1.2 ± 0.3	1.6 ± 0.4***††
Fibrosis (%)	0.8 ± 0.3	25 ± 3***	0.9 ± 0.3	24 ± 2**	1.0 ± 0.2	7.6 ± 0.9***††