Npr1-regulated gene pathways contributing to cardiac hypertrophy and fibrosis

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Abstract

The natriuretic peptides, atrial (ANP) and brain natriuretic peptide (BNP) are known to suppress cardiac hypertrophy and fibrosis. Both ANP and BNP exert their bioactivities through the Npr1 receptor, and Npr1 knockout mice (Npr1 $-/-$) exhibit marked cardiac hypertrophy and fibrosis. In this study, we investigated which genes within the hypertrophic and fibrotic pathways are influenced by the lack of Npr1 signalling. cDNA microarray and quantitative real-time PCR (RT-PCR) analyses were performed on cardiac ventricles from Npr1 $-/-$ mice. Gene expression at early and late stages during development of hypertrophy was investigated in male and female Npr1 $-/-$ mice at 8 weeks and 6 months of age. Heart weight to body weight ratios (HW:BW) were maximally increased in 8-week males ($P < 0.01$), whilst HW:BW in females continued to increase progressively up to 6 months ($P < 0.01$). This was despite blood pressure being similarly elevated at both the ages in male and female knockout when compared with wild-type (WT) mice ($P < 0.001$). Microarray analysis identified altered gene expression at the earliest steps in the hypertrophy-signalling cascade in Npr1 $-/-$ mice, particularly calcium-calmodulin signalling and ion channels, with subsequent changes in the expression of intracellular messengers including protein kinases and transcription factors. Real-time PCR analysis confirmed significant differences in gene expression of ANP, BNP, calmodulin 1, histone deacetylase 7a (HDAC7a), protein kinase C (PKC) ϵ , (GATA) 4, collagen 1, phospholamban and transforming growth factor- β 1 in Npr1 $-/-$ mice when compared with WT ($P < 0.05$). The present study implicates the calmodulin-CaMK-Hdac-Mef2 and PKC-MAPK-GATA4 pathways in Npr1 mediation of cardiac hypertrophy.

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Introduction

The term cardiac remodelling is used to describe the changes in size, shape and function of the heart observed during the development of cardiac disease, and is characterised not only by cardiomyocyte hypertrophy and cell death, but also by cardiac fibroblast proliferation and fibrosis (Swynghedauw 1999). Cardiac hypertrophy occurs when terminally differentiated cardiac myocytes respond to an increase in workload or injury (such as hypertension or myocardial infarction) by increasing cell size to maintain a normal cardiac output.

The cardioprotective hormones, the natriuretic peptides, regulate cardiac remodelling by inhibiting both myocyte hypertrophy and cardiac fibrosis (Cao & Gardner 1995, Horio *et al.* 2000, Ogawa *et al.* 2001). Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are secreted predominantly from the cardiac atria and the ventricles respectively in response to increased cardiac stretch, and regulate blood pressure through their combined actions on vasculature, kidneys and adrenal glands. The actions of both

these peptides are mediated by the natriuretic peptide receptor-A (NPR-A or Npr1), a guanylyl cyclase-linked receptor whose activation generates the second messenger cyclic guanosine monophosphate (cGMP). This intracellular signal elicits the well-characterised hypotensive, diuretic and natriuretic effects of these peptides (Esperiner *et al.* 1995).

Evidence for a role of the natriuretic peptides in suppressing cardiac remodelling is supported by both *in vitro* and *in vivo* data. Hypertrophy of cardiac myocytes in culture is inhibited by ANP (Horio *et al.* 2000). Gene delivery of ANP is reported to attenuate hypertension and cardiac hypertrophy in a salt-sensitive rat model (Lin *et al.* 1998). In addition, all the three members of the natriuretic peptide family, ANP, BNP and C-type natriuretic peptide (CNP), inhibit DNA synthesis in cultured fibroblasts (Cao & Gardner 1995). The lack of ANP and BNP bioactivities in Npr1 gene knockout (Npr1 $-/-$) mice leads to both cardiac hypertrophy and fibrosis (Oliver *et al.* 1997). The Npr1-signalling pathway is reported to directly oppose the hypertrophic response, an effect that is independent of

the elevated blood pressure observed in these mice (Oliver *et al.* 1997, Knowles *et al.* 2001). When blood pressures in *Npr1*^{−/−} mice were maintained within the normal range by antihypertensive drugs, their cardiac hypertrophy was not ameliorated (Knowles *et al.* 2001). Furthermore, transgenic mice with the *Npr1* gene deletion targeted specifically to cardiac tissue exhibited cardiac hypertrophy in the absence of systemic hypertension (Holtwick *et al.* 2003), demonstrating conclusively that *Npr1*-signalling functions as an intrinsic inhibitor of myocyte growth.

Cardiac hypertrophy and fibrosis in *Npr1*^{−/−} mice have been partially ascribed to activation of the angiotensin 1 receptor (AT1R; Li *et al.* 2002, 2004). The calcineurin–nuclear factor of activated T cells (NFAT) pathway mediates AT1R signalling and has also been implicated since blockade of calcineurin activation significantly ameliorated the cardiac hypertrophy and the activation of cardiac gene expression in *Npr1* knockout mice (Tokudome *et al.* 2005). This study aimed to identify the global changes in gene expression that contribute to the hypertrophic and fibrotic pathways influenced by the lack of *Npr1* signalling, using the methods of cDNA microarray and quantitative real-time PCR (RT-PCR) analysis of cardiac ventricles from *Npr1*^{−/−} mice. The influence on gene expression at early and late stages during the development of hypertrophy and fibrosis was investigated in hearts collected from male and female *Npr1*^{−/−} mice at 8 weeks and 6 months of age. Isolated perfused heart studies were also performed to investigate the contractile response to elevated ventricular stretch in wild type (WT) and *Npr1*^{−/−} knockout mice associated with development of hypertrophy.

Materials and methods

Npr1^{−/−} mice

The original heterozygote breeding stock was kindly donated by Professor Oliver Smithies, Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC, USA. Mouse experiments were performed on *Npr1*^{−/−} and WT control mice backcrossed at least 15 generations to C57BL/6 mice derived from the original mutants, as described previously (Oliver *et al.* 1997). Experiments were performed according to the protocols approved by the Animal Ethics Committee of the University of Otago.

Adult male and female *Npr1*^{−/−} and WT (*n* = 6 per group) at 8 weeks and 6 months of age were used in this study. Blood pressures were measured on conscious mice by a non-invasive computerised tail cuff system (ADInstruments, Dunedin, New Zealand). The mice were familiarised with being placed in the restrainer

and the tail cuff system over 7 days of training, after which daily blood pressure measurements were made for each animal (mean of at least five recordings), and were repeated in subsequent sessions on each of 5 days.

For cardiac gene expression studies, mice were euthanised with an anaesthetic overdose (Halothane; Merial Australia Pty Ltd, Sydney, Australia) before cervical dislocation, hearts were rapidly excised, the atria dissected from the ventricles, weighed and immediately snap-frozen in liquid nitrogen for RNA isolation. The mice were weighed prior to killing to allow heart weight to body weight ratios to be calculated.

RNA isolation

For each sample, total ventricular RNA was isolated by automated grinding in a Retsch MM301 tissue mill at 30 Hz for 10 min in 800 µl pre-chilled TRIzol (Invitrogen). Chloroform (160 µl) was added and samples were centrifuged at 12 000 *g* for 15 min. The RNA-containing supernatant was purified by RNeasy Midi Columns (Qiagen).

cDNA microarray analysis

Mouse 22K Compugen oligonucleotide microarray slides used in this study (purchased from The Clive and Vera Ramaciotti Centre for Gene Function Analysis, The University of New South Wales, Australia, <http://www.ramaciotti.unsw.edu.au>), consisted of 22 464 60mer oligonucleotides spotted onto GeneMachines OmniGrid Epoxy coated glass slides.

A reference design (Simon & Dobbin 2003) was employed in the microarray study in which each heart RNA sample was compared with a common reference RNA pool. This allowed the hybridisation intensity for a sample (WT or *Npr* gene knockout (KO)) to be measured relative to an identical reference sample in all arrays. The reference RNA used in this study was pooled from *Npr1* heterozygote (*Npr1*^{+/−}) atria and ventricle tissue of mice ranging in age from 8-week- to 1 year-old.

cDNA probes were generated for each *Npr1* WT and KO RNA sample of each gender at each age (*n* = 6 per group) and also for the reference RNA sample, using the Superscript III Indirect cDNA labelling System (Invitrogen) according to the manufacturer's instructions. For each sample, 10 µg total ventricle RNA were transcribed. Each cDNA probe (sample or reference) was resuspended in 2 µl 10 mM EDTA before being combined and denatured at 95 °C for 10 min. After incubating on ice for 1 min, the probe mix was combined with 70 µl pre-heated (65 °C) Slidehybe-1 hybridisation solution (Ambion, Austin, TX, USA) and pipetted onto the microarray slide. The oligonucleotide array slides were pre-treated prior to hybridisation by

washing in 0.1% SDS at 95 °C, 5% ethanol and double distilled water at room temperature. All washes were for 1 min under constant agitation. Slides were dried by centrifugation at 1200 r.p.m. for 6 min before proceeding to hybridisation under LifterSlips (Eerie Scientific, Portsmouth, NH, USA) at 42 °C overnight in a humid hybridisation chamber.

Post-hybridisation, the slides were washed for 10 min at room temperature in the following solutions: 2×SSC + 0.2% SDS, 2×SSC and 0.2×SSC before being spun dry and scanned. They were scanned at 635 (Cy5 Dye) and 532 nm (Cy3 Dye) in a GenePix 4000B Scanner (Axon Instruments, Foster City, CA, USA). Median fluorescent data were collected for each wavelength for each gene. Fluorescent data were analysed by the statistical software BRB Array Tools (Biometric Research Branch, National Cancer Institute, MD, USA, <http://linus.nci.nih.gov/BRB-ArrayTools.html>). Data were normalised by applying a Lowess smoother and genes were excluded if less than 20% of expression data had at least a 1.5-fold change in either direction from a gene's median value, or if more than 50% of data were missing or filtered out. Log ratios were calculated comparing the fluorescence data for the reference (at 532 nm) with the sample (WT or KO, at 635 nm). Class comparison analysis was performed by two sample *t*-tests comparing the WT log ratio with the KO log ratio. An increase or decrease that was statistically significant is expressed as up- (↑) or down- (↓) regulation of a gene in text and tables. Statistical significance was accepted when $P < 0.05$.

Genes which were significantly changed were analysed by Biorag software (Bio Resource for Array Genes,

<http://www.biorag.org>) and classified based on gene ontology terms.

Quantitative real-time PCR analysis

Quantitative RT-PCR analysis was performed on selected genes identified as significantly altered in Npr1^{-/-} versus WT by microarray analysis, in order to quantitate the expression of genes of interest. Genes were selected if several members of a signalling pathway were significantly altered in more than one of the Npr1^{-/-} versus WT age or gender groups. We used the total RNA samples from the same individuals for both microarray and RT-PCR analyses. The cDNA was generated from 2.5 µg ventricular total RNA (after treatment with RNase-free DNase I; Roche, Mannheim, Germany) for each Npr1 WT and Npr1^{-/-} RNA sample using Superscript III Reverse Transcriptase (200 U/µl, Invitrogen). cDNA products were then treated with 1 µl RNase H (2 U/µl, Invitrogen) for 20 min at 37 °C.

The PCR conditions were optimised for each gene of interest, and the sequences of PCR products were confirmed by sequencing on an ABI 3100-Avant Genetic Analyser (Foster City, CA, USA) before real-time PCR analysis. Levels of mRNA expression were evaluated by quantitative RT-PCR in a Rotor-Gene RG-3000 real-time PCR machine (Corbett Research, Sydney, Australia). Oligonucleotide primer sequences and PCR annealing temperatures for each gene studied are given in Table 1. Reactions incorporated the fluorescent dye SYBR Green 1 (Roche), and absolute

Table 1 Primer sequences and annealing temperatures for RT-PCR assays

	Primers	Annealing temperature (°C)
mRNA		
ANP	f GAACCTGCTAGACCACCT r CCTAGTCCACTCTGGCT	56
BNP	f AAGCTGCTGGAGCTGATAAGA r GTTACAGCCCAAACGACTGAC	56
Mef2C	f ATTTGGGAAGTGAAGCTGTGC r CGCTCATCCATTATCCTCGT	56
Hdac 7a	f ATCTCTTCCTGGCAGGCTTA r TTCTGCTTGACCACTGCT	54
PKC ϵ	f CTACCGCGGGGATATTATGA r TCAGCTCGTACAGCCTGAAA	54
GATA4	f AATGCCTGTGGCCTCTATCA r CTGGTTTGAATCCCCTCCTT	58
Collagen 1	f AGGCTTCAGTGGTTTGGATG r CACCAACAGCACCATCGTTA	54
Calmodulin 1	f AGGGGTTTGGAGGTGACTTT r TTCCTCGGAGGTTAGGGTTT	54
Phospholamban	f ATTTGCCTCCTTACCTCCA r AGCTTCAGCGTCACGTTTCT	54
TGF- β 1	f TGAGTGGCTGTCTTTTGACG r GGTTTCATGTCATGGATGGTG	54

Sequences are listed 5'–3'. Forward primers are designated by f and reverse primers by r.

gene expression levels were calculated by generating individual standard curves for each gene as described by Karsai *et al.* (2002). Standard curves comprising at least six points were run with each assay, with concentrations ranging from 0 to 2000 pg, with each concentration run in triplicate. Hotmaster Taq DNA Polymerase (Eppendorf, Hamburg, Germany) was used in all reactions. For each assay, a hotstart at 96 °C for 2 min was performed before the following PCR cycling parameters: denaturation at 94 °C for 30 s, annealing for 35 s at the gene-specific annealing temperature (Table 1) and extension at 72 °C for 30 s. Each sample underwent 30 cycles, after which a melt curve was performed. Each sample was assayed in duplicate and gene levels expressed as picograms of message per microgram of total RNA (pg/ μ g total RNA). Fluorescent data were acquired at each extension step.

Cardiac function

Cardiac function experiments were performed on 8-week- and 6-month-old male WT, heterozygous and *Npr1* $-/-$ mice. The isolated perfused mouse heart preparation was similar to that described previously (Eklund *et al.* 2001). Briefly, mice were anaesthetised with sodium pentobarbitone (50 mg/kg, i.p.), decapitated and the hearts rapidly cooled with ice-cold buffer, prior to retrograde perfusion by the Langendorff technique. The flow rate was adjusted according to the weight and genotype of the animal to achieve a similar perfusion per gram of tissue. To measure the isometric force of contraction, the left atrium was cut off and an empty plastic balloon was inserted into the left ventricle. The balloon was filled with 50% ethanol to give an end-diastolic pressure of approximately 5 mmHg, and the developed pressure inside the balloon was recorded with a pressure transducer (Capto, model SP844). A similar pressure transducer was connected to a sidearm cannula to monitor the aortic perfusion pressure. The hearts were paced with a stimulator (Digitimer Ltd, Hertfordshire, UK; 400 beats per minute at baseline), and all recordings were made by an ADInstruments Powerlab system. The comparison of contractile function was done at corresponding levels of end-diastolic pressure because of the difference in the cardiac size between the experimental groups. The data were obtained during the stepwise filling of the left ventricular balloon as described previously (Stromer *et al.* 1997, Piuhola *et al.* 2003).

ANP secretion

The cardiac secretion of ANP was analysed from coronary effluents from isolated hearts from 8-week- and 6-month-old male mice. At baseline, the 8-week

hearts were perfused with a coronary flow rate of 21.7 ± 1.4 and 20.5 ± 1.1 ml/g cardiac weight, for WT and *Npr1* $-/-$ mice respectively, resulting in a coronary perfusion pressure of 83 ± 5 and 91 ± 10 mmHg. For the RIA of ANP, timed collection of coronary effluent was obtained, the samples were cooled on ice and stored at -20 °C until SepPak extraction and RIA was performed as described by Yandle *et al.* (1991).

Statistical analysis

All results (except microarray data) are expressed as means $\pm$ S.E.M. The effects of WT versus *Npr1* $-/-$, age and gender on cardiac gene expression data were tested by three-way factorial ANOVA. Subsequent comparisons between WT and *Npr1* $-/-$ of specific age and gender groups were conducted using independent *t*-tests. The effects of genotype on cardiac ANP secretion and developed pressure were tested by one-way ANOVA. Associations between variables were tested using Pearson's correlation coefficient. Values of $P < 0.05$ were considered statistically significant.

Results

Hypertension and cardiac hypertrophy

Mean arterial pressure (MAP) was significantly increased over all *Npr1* $-/-$ groups when compared with WT (Fig. 1A, $P < 0.001$), equally at 8 weeks and 6 months. On an average, there was a 32 mmHg difference in MAP between *Npr1* $-/-$ and WT groups, and there were no significant differences between blood pressures dependent on age or gender. In contrast, heart weight to body weight ratios (HW:BW) of *Npr1* $-/-$ mice increased significantly in all groups when compared with WT by ANOVA ($P < 0.01$, Fig. 1A). The most pronounced increase was observed in male *Npr1* $-/-$ mice at 8 weeks of age. In 8-week-old male mice, HW:BW was 169% when compared with WT (6.6 ± 0.5 vs 3.9 ± 0.3). However, the relative hypertrophy observed in young adult male *Npr1* $-/-$ mice was not sustained, and at 6 months the HW:BW of *Npr1* $-/-$ male mice was only 118% relative to WT (6.9 ± 0.5 vs 5.8 ± 0.1). In contrast, in female *Npr1* $-/-$ mice, the proportionate increase in HW:BW was similar at both 8 weeks and 6 months of age. The HW:BW of female *Npr1* $-/-$ mice at 8 weeks of age was 135% when compared with WT (5.3 ± 0.2 vs 3.9 ± 0.3), and at 6 months of age was 134% when compared with WT (6.76 ± 0.4 vs 5.04 ± 0.3). These results suggest that maximal hypertrophy occurred at an early age in males, whereas in females the hypertrophic response was sustained as the female mice aged.

Figure 1 MAP and HW:BW ratios (A), ventricular levels of ANP and BNP mRNAs (B) in male and female Npr1^{-/-} mice at 8 weeks and 6 months of age when compared with WT control mice (quantitated gene levels are expressed as picograms of message per microgram of total RNA), and ANP secretion rates from isolated perfused hearts of KO (-/-), heterozygote (-/+) and WT (+/+) Npr1 male mice at 8 weeks and 6 months of age (C). Black bars represent Npr1 KO and grey bars represent WT (A and B). Significant differences between WT and KO at individual age/gender points are indicated by * $P < 0.05$, [†] $P < 0.01$ and [‡] $P < 0.001$.

Microarray analysis of cardiac gene expression

To investigate the pathways regulated by Npr1 in the development of cardiac hypertrophy, cDNA microarray analysis was used as a screening tool to indicate genes

with altered expression in the ventricles of male and female Npr1^{-/-} mice at 8 weeks and 6 months. Out of a total of 22 656 genes represented on the Compugen microarray slides, 187 were significantly changed ($P < 0.05$) between 8-week male WT and Npr1^{-/-},

when compared with 248 genes in 8-week females. In contrast, at 6 months, 284 genes were significantly changed between WT and *Npr1*^{−/−} males, when compared with 567 genes in 6-month female mice ($n=6$ per group). The microarray data of hypertrophy-related genes that were significantly altered are shown in Table 2. The most significantly altered genes include ANP, calmodulin and calmodulin kinase, protein kinases and MAP kinases, and several transcription factors, notably members of the Hdac, Mef2, NFAT and GATA families. In addition, altered cell-signalling pathways involved in fibrosis were observed, including fibroblast growth factor, collagen and matrix metalloproteinases. This led us to focus the real-time PCR studies on key genes involved in these pathways. Other genes whose expression was significantly altered in the microarray data but have not been studied further in the present study included genes involved in cardiac development (including forkhead box proteins and catenin), receptor activity (guanine nucleotide binding protein) and the regulation of transcription (nuclear factor interleukin-3, insulinoma-associated 2, zinc-finger proteins, general transcription factors, ankyrins). In addition, genes involved in heart-rate regulation (nicotinic cholinergic receptor) and ion channel signalling (ryanodine receptor, solute carrier family, voltage-gated sodium channels and K⁺ channels) were also significantly altered but not examined further in this study. Genes involved in the regulation of transcription were particularly increased at 8 weeks of age in male *Npr1*^{−/−}; however, in female animals, up-regulation of transcription factor genes became more apparent at 6 months of age. These gender differences in the age at which transcription factors were elevated, paralleled the times when their maximal HW:BW ratio occurred (also at 8 weeks in male and 6 months in female knockouts). Intriguingly, gene ontology classification indicated that the largest proportion of genes with altered expression is those whose function is presently unknown.

Quantitative real-time PCR analysis

Quantitative real-time PCR analysis was used to characterise expression levels in genes of interest selected after collating the microarray data from all groups. As expected, ventricular ANP gene expression was significantly increased in KO mice over all age and gender groups ($P=0.002$), particularly in female *Npr1*^{−/−} mice at both 8 weeks and 6 months (Fig. 1B). Ventricular BNP expression was also greater over all *Npr1*^{−/−} groups compared with WT mice when compared by three-way factorial ANOVA ($P=0.005$). Levels of BNP mRNA significantly increased with age ($P<0.001$) and females had higher levels than males at both the ages ($P<0.001$). BNP mRNA was particularly increased in 6-month female *Npr1*^{−/−} mice, as indicated by *t*-test analysis ($P<0.05$).

In 8-week-old males, the average ANP secretion rate per minute was 4.7 ± 0.9 and 2.7 ± 0.4 fmol/min, for *Npr1*^{−/−} and WT mice respectively ($P<0.05$, $n=5$ for both the genotypes). Similar results were seen in the 6-month-old mice, with ANP secretion levels of 5.3 ± 1 and 2.8 ± 0.3 fmol/min ($P<0.05$), for *Npr1*^{−/−} and WT respectively (Fig. 1C).

A gene that is central to hypertrophic signalling is calmodulin. Gene expression of calmodulin 1 was significantly increased in *Npr1*^{−/−} when compared with WT overall ($P=0.013$), and especially in 8-week females (Fig. 2A). It should be noted, however, that the most significant change in calmodulin 1 was the remarkable increase in expression with age in both the genotypes ($P<0.001$). Ventricular *PKC α* gene expression showed an interaction with genotype, age and gender ($P=0.008$), whereby expression was significantly increased in female *Npr1*^{−/−} when compared with WT at 6 months, but tended to be decreased in 6-month *Npr1*^{−/−} males (Fig. 2B). Gene expression of Hdac 7a was increased in *Npr1*^{−/−} males versus WT at both 8 weeks and at 6 months ($P<0.05$). No change in Hdac 7a expression was seen in females at either age (Fig. 2E). Analysis of ventricular expression of the transcription factor, Mef2C, by RT-PCR did not show significant differences in *Npr1*^{−/−} when compared with WT mice at either age (Fig. 2C), in contrast to the microarray data. However, Mef2C expression was markedly increased with age in both the genotypes ($P<0.001$). Ventricular expression of GATA4 was significantly increased with genotype in the *Npr1*^{−/−} mice ($P=0.03$), particularly in 6-month female *Npr1*^{−/−} animals (Fig. 2D). In addition, there was a significant increase in GATA4 with age ($P<0.001$).

Among genes involved in fibrosis, collagen 1 expression was increased in *Npr1*^{−/−} mice of both the genders ($P=0.011$), although this was particularly evident in 6-month female *Npr1*^{−/−} mice (Fig. 3A). Ventricular expression of phospholamban and transforming growth factor- $\beta 1$ (*TGF- $\beta 1$*) were the only genes which significantly decreased with age ($P<0.001$). *TGF- $\beta 1$* gene expression was greater in *Npr1*^{−/−} versus WT males at 6 months (Fig. 3B). Phospholamban was significantly decreased in 6-month female *Npr1*^{−/−} versus WT (Fig. 3C).

Gene correlations

The majority of hypertrophy-related genes were strongly correlated with HW:BW (except collagen 1 and *TGF- $\beta 1$*), and with each other, including ANP, BNP, Mef2c, Hdac 7a, protein kinase C (PKC) α , GATA4 and calmodulin 1 ($r=0.322$ – 0.948). Collagen 1 expression was significantly correlated with HW:BW and MAP, but was not correlated with any of the hypertrophy-related genes. While MAP was

Table 2 Microarray analysis data showing hypertrophy related genes significantly altered in Npr1^{-/-} versus WT groups ($P < 0.05$)

Ca/Calmodulin	
Calmodulin kinase (NM_012040)	↑ 6-Month males
Calmodulin 1 (NM_009790)	↑ 6-Month females
Ion transport	
Ryanodine receptor 1 (X83932)	↓ 8-Week females, ↓ 6-month males
Solute carrier family 22, member 4 (NM_019687)	↓ 8-Week females
Chloride ion channel inducer protein (U53455)	↓ 8-Week females
Solute carrier family 4 (anion exchanger) (NM_011403)	↓ 6-Month males
K ⁺ channel subfamily U1 (NM_008432)	↓ 6-Month males
Na ⁺ /H ⁺ exchanger (AF139195)	↑ 6-Month males
K ⁺ channel protein (AJ291608)	↓ 6-Month males
Maxi K ⁺ channel-β subunit (AF020711)	↓ 6-Month males
Voltage gated sodium channel (L36179)	↓ 6-Month females
Voltage gated sodium channel type 1-β	↑ 6-Month females
Solute carrier family 22, member 21 (NM_019723)	↓ 6-Month females
Cell signalling	
Mitogen-activated protein kinase 7 (MAPK7) (NM_011841)	↑ 8-Week males
Protein kinase C, iota (NM_008857)	↑ 8-Week males
Protein kinase inhibitor, γ (NM_011106)	↑ 8-Week males
Mitogen-activated protein kinase 4 (NM_008696)	↓ 8-Week females
Mitogen-activated protein kinase kinase kinase 14 (Map3k14; NM_016896)	↓ 8-Week females
Guanine nucleotide-binding protein γ-11 (NM_025331)	↑ 6-Month males
Mek kinase 3 (U43187)	↓ 6-Month females
Mitogen-activated protein kinase kinase kinase 3 (BC006037)	↓ 6-Month females
Protein kinase C, ζ (NM_008860)	↑ 6-Month females
Mitogen-activated protein kinase 3 (Erk-1; Z14249)	↓ 6-Month females
Transcription factors	
Myocyte-specific enhancer factor 2 (Mef2C; L13171)	↑ 8-Week males
Insulinoma-associated 2 (NM_020287)	↑ 8-Week males
Nuclear factor, interleukin-3 regulated (NM_017373)	↑ 8-Week males
Transcription factor 3 (NM_009332)	↑ 8-Week males
Forkhead box N1 (NM_008238)	↑ 8-Week males, ↓ 6-month males
Kruppel-like factor 12 (NM_010636)	↓ 8-Week males
Histone deacetylase 7A (NM_019572)	↓ 8-Week females
Histone deacetylase 8 (AK011332)	↓ 8-Week females
General transcription factor II A,1 (NM_031391)	↓ 8-Week females
CCAAT/enhancer-binding protein (NM_009883)	↓ 8-Week females
Zinc-finger protein 57 (NM_009559)	↓ 8-Week females
Forkhead box G1 (NM_008241)	↑ 6-Month males
Forkhead box N4 (AF323488)	↓ 6-Month males
Catenin β (NM_007614)	↑ 6-Month males
Zinc-finger homeobox 1a (NM_011546)	↑ 6-Month males
General transcription factor III C1 (BC004708)	↓ 6-Month males
Transcription factor AP-2 α (NM_011547)	↓ 6-Month males
Ankyrin repeat domain 1 (NM_013468)	↑ 6-Month females
Ankyrin repeat domain 2S (BC006647)	↑ 6-Month females
Homeobox B13 (NM_008267)	↓ 6-Month females
Transcription factor 19 (BC004617)	↓ 6-Month females
TAF7 RNA polymerase II, TATA box-binding factor (NM_011901)	↑ 6-Month females
Fibrosis	
Fibroblast growth factor 3 (NM_008010)	↑ 8-Week males
Matrix metalloproteinase 15 (NM_008609)	↓ 8-Week males
Matrix metalloproteinase 7 (NM_010810)	↓ 8-Week males
Matrix metalloproteinase 3 (NM_010809)	↑ 8-Week males
Fibroblast growth factor inducible 15 (NM_008016)	↓ 8-Week females
Fibroblast growth factor homologous factor 3 isoform 1B (AF199604)	↓ 8-Week females
Matrix metalloproteinase 8 (NM_008611)	↑ 6-Month females
Fibroblast growth factor 3 (NM_008007)	↓ 6-Month females
Procollagen type 8 α-1 (NM_007739)	↑ 6-Month females
Other	
Atrial natriuretic peptide (K02781)	↑ 8-Week males, ↑ 8-week females
Purinergic receptor P2X, ligand gated ion channel 5 (NM_033321)	↓ 8-Week males
Cholinergic receptor, nicotinic, β 2 (NM_009602)	↓ 8-Week females
Cholinergic receptor, nicotinic, α-polypeptide1 (NM_007389)	↓ 6-Month females
Glyceraldehyde-3-phosphate dehydrogenase (NM_008084)	↑ 8-Week males

GenBank accession numbers are shown in brackets.

Figure 2 Ventricular levels of calmodulin 1 (A), PKC ϵ (B), Mef2C (C), GATA4 (D) and Hdac 7a (E) mRNAs in male and female *Npr1*^{-/-} mice at 8 weeks and 6 months of age when compared with WT control mice. Quantitated gene levels are expressed as picograms of message per microgram of total RNA (pg/μg total RNA). Black bars represent *Npr1* KO and grey bars represent WT. Significant differences between WT and KO mice are indicated by * $P < 0.05$ and † $P < 0.01$.

significantly correlated with ANP, BNP and PKC, it was not correlated with other hypertrophic or fibrotic genes, in agreement with previous reports that these pathways are independent of the blood pressure elevation in the *Npr1*^{-/-} mice.

Cardiac function

Cardiac contractility and response to stress

The cardiac contractility of WT and *Npr1*^{-/-} male mouse hearts was compared at different levels of

Figure 3 Ventricular levels of collagen 1 (A), TGFβ1 (B) and phospholamban (C) mRNAs in male and female Npr1 $-/-$ mice at 8 weeks and 6 months of age when compared with WT control mice. Quantitated gene levels are expressed as picograms of message per microgram of total RNA (pg/μg total RNA). Black bars represent Npr1 KO and grey bars represent WT. Significant differences between WT and KO mice are indicated by * $P < 0.05$ and † $P < 0.01$.

end-diastolic pressure in isolated Lagendorff-perfused hearts. Throughout the pressure range used, the hearts of 8-week-old Npr1 $-/-$ mice showed enhanced contractility when compared with those from WT animals (Fig. 4). In 8-week-old mice, the developed pressure (DP) was 18.7 ± 1.8 mmHg in WT and 27.2 ± 2.2 mmHg in Npr1 $-/-$ mice at the left ventricular end-diastolic pressure (LVEDP) of 10 mmHg ($P < 0.005$). In 6-month-old mice, this contractility enhancement was lost, and there was no difference in the contractility between the different genotypes (DP 33.5 ± 3.9 mmHg in WT and 34.0 ± 4.8 mmHg in Npr1 $-/-$; at LVEDP, 10 mmHg; P , not significant).

Discussion

The present study implicates the calmodulin–CaMK–Hdac–Mef2 and PKC–MAPK–GATA4 pathways in Npr1

mediation of cardiac hypertrophy, extending our previous understanding of Npr1 involvement in the AT1R–calcineurin–NFAT pathway. Increased expression of genes of the Ca/calmodulin pathways was observed early in the development of cardiac remodelling in Npr1 $-/-$ mice, whilst other genes involved in cardiac hypertrophic-signalling pathways including PKC ϵ , Hdac 7a and GATA4 tended to be increased in Npr1 $-/-$ mice at a more advanced stage. Structural molecules involved in cardiac fibrosis such as collagen 1 also tended to be increased later in the remodelling process.

One of the startling observations of this study was the age-related change in gene expression, independent of genotype. In particular, the expression of cardiac transcription factors Mef2C and GATA4, as well as calmodulin 1, were markedly increased at 6 months when compared with 8 weeks in both knockout and wild-type mice. Over the same period, there were significant decreases in expression levels of phospholamban and

Figure 4 The contractile function at different levels of left ventricular end-diastolic pressure (LVEDP) in WT (+/+), heterozygous (-/+) and KO (-/-) *Npr1* male mice at 8 weeks and 6 months of age. At 8 weeks, the KO *Npr1* mice had significantly enhanced contractility throughout the range of developed pressures (* $P < 0.05$, $^{\dagger}P < 0.01$, *Npr1* KO versus WT). At 6 months, this enhancement of contractility was lost and there were no differences in cardiac function among genotypes.

TGF- β 1. To our knowledge, there have been no other reports in the literature of marked changes in gene expression associated with normal cardiac aging in the absence of pathophysiological changes. However, the phenotypic plasticity of adult myocardium is well documented, associated with the reactivation of the foetal gene programme and the expression of at least four transcription factors, GATA4, MEF2, Csx/Nkx2-5 and c/dHAND (Swynghedaauw 2006). This activation is normally attributed to cardiac mechanical overload and wall stress, acting via stretch-activated Ca, K and Na channels and calcineurin (Swynghedaauw 2006). It may be that arterial stiffness in the aging cardiovascular system leads to increased arterial pre-load, wall stress in the heart and the gradual activation of cardiac transcription. These findings highlight the necessity that gene expression studies are performed on precisely age-matched animals. In the present study, however, significantly altered gene expression was demonstrated in *Npr1* -/- mice when compared with age-matched wild-type animals, indicative of cardiac remodelling over and above the age-related changes.

To determine whether the cardiac remodelling observed in aging *Npr1* -/- mice influences cardiac function, the contractile response to elevated ventricular stretch was studied in perfused isolated hearts. Throughout the pressure range used, 8-week-old male *Npr1* -/- mice showed enhanced contractility when compared with the wild-type animals. In 6-month-old animals, this enhancement of contractility was lost. These results suggest that in 8-week-old *Npr1* -/- mice, the left ventricular hypertrophy enhances contractile force, whereas older *Npr1* -/- mice show impaired contractile function, possibly as a result of the increasing collagen deposition and developing heart

failure. Enhancement of cardiac contractility has been reported in other models of left ventricular hypertrophy in rats (Stromer *et al.* 1997, Piuhola *et al.* 2003). Consistent with the present study, in another strain of *Npr1* -/- mice, Kuhn *et al.* (2002) also reported impaired cardiac function in older mice (12 months old), but no differences in cardiac function between mouse genotypes at 4 months of age.

Taken together, the microarray analysis revealed marked differences between genders in gene expression patterns, with male *Npr1* -/- mouse hearts demonstrating a greater activation of hypertrophy signals in early adulthood when compared with female *Npr1* -/- animals. Female *Npr1* -/- mice exhibited their greatest increase in HW:BW and gene expression of ANP, BNP, PKC-MAPK and GATA4 at 6 months, suggesting a more delayed but sustained hypertrophic response in female mice. Many previous studies have established that male mice are more sensitive than female to various genetic interventions leading to cardiac hypertrophy, as has been comprehensively summarised by Du (2004). These findings are in keeping with the observations from other species, including humans, that males in general have an earlier transition into heart failure than females. However, the mechanism underlying sex differences in cardiovascular risk is poorly understood. Although deleterious effects of androgens and beneficial effects of ovarian hormones have been proposed, recent studies suggest that male hormones have a neutral or beneficial effect on the heart (Muller *et al.* 2003). A previous study of *Npr1* -/- mice proposed a role for testosterone in aggravating the hypertrophy in young, male mice (Li *et al.* 2004), in which the castration of *Npr1* KO male mice reduced cardiac hypertrophy and fibrosis, whereas

testosterone infusion in ovariectomised female Npr1^{-/-} mice increased cardiac mass and fibrosis. The castration experiments in the study demonstrated that androgens accounted for most of the gender differences in hypertrophy and 50% of the gender-related cardiac fibrosis. Furthermore, Li *et al.* (2004) showed that gender differences in Npr1^{-/-} mice were almost abolished by deleting the angiotensin II type 1A receptor (AT1A), suggesting that androgens contribute to gender-related differences in cardiac remodelling via an interaction of Npr1 and AT1A receptors. These authors suggested that this may involve TGF- β 1 and TGF- β 3, which are activated by AT1A and are responsible for interstitial fibrosis. In the present study, a significant increase in TGF- β 1 expression was observed in 6-month-old male knock-outs, consistent with the influence of testosterone on this pathway. Other molecular pathways proposed to mediate the gender difference in cardiac remodelling include tissue hormones and transcription factors, including Akt and Mef2 and ion channels, particularly calcium channels (Du 2004).

Calmodulin is a key signalling messenger mediating the actions of calcium (Frey *et al.* 2000), and is a central mediator of several hypertrophic-signalling pathways (Fig. 5). The calcium/calmodulin-dependent enzymes, calcium/calmodulin-dependent protein kinase

(CaMK) and phosphatase calcineurin, have been implicated in the control of cardiac hypertrophy and failure. In this study, calmodulin 1 was shown by RT-PCR to be increased over all Npr1^{-/-} groups. These findings suggest that calmodulin may be a key signal in the cardiac hypertrophy that occurs in response to the lack of Npr1 bioactivity.

One important signal transduction cascade regulated by calmodulin in hypertrophy involves PKC and MAPK. Whilst PKC ϵ is not a classically expressed isoform in cardiac tissue, microarray analysis indicated that this isoform was increased in Npr1^{-/-} mice, and this was confirmed by RT-PCR. Previous studies suggest that PKC ϵ is involved in myocardial ischaemia (Albert & Ford 1998) and plays a role in actin cytoskeleton organisation (Spitaler *et al.* 2000). PKC is hypothesised to modulate cardiac hypertrophy by phosphorylation of transcription factors, including Mef2 and NFAT that regulate transcription of hypertrophic genes.

Histone deacetylases are also regulated by calmodulin and calmodulin kinase, and are negative regulators of cardiac hypertrophy that repress activity of the transcription factor Mef2 (Zhang *et al.* 2002). These chromatin-modifying enzymes have important roles in the control of cardiac hypertrophy (Zhang *et al.* 2002, Gusterson *et al.* 2003). In the present study, Hdac 7A and 8 were indicated by both microarray and RT-PCR to

Figure 5 Proposed interaction of Npr1 signalling with pathways contributing to cardiac hypertrophy.

have sexually dimorphic effects in male and female *Npr1* $-/-$ mice, suggesting that Hdacs have a complex role in the development of cardiac hypertrophy in combination with gender, as reported for other genetic mouse models (Du 2004).

GATA4 is another key regulator of inducible cardiac gene expression and a potential mediator of cardiac hypertrophy. The MAP kinases p38 and ERK1/2 stimulate GATA4 activity (Tenhunen *et al.* 2004), and this transcriptional activity is regulated through physical interaction with NFAT, Mef2 and serum response factor (Adazawa & Komuro 2003). GATA4 is responsible for regulating the basal expression of cardiac hypertrophy genes including α -myosin heavy chain, myosin light chain 1/3, cardiac troponin C and I, ANP, BNP and the sodium–calcium exchanger in cardiac tissue (Adazawa & Komuro 2003). In our studies, increased GATA4 gene expression was observed in all *Npr1* $-/-$ mice especially in 6-month-old *Npr1* KO females. Consistent with this, increased levels of both ANP and BNP mRNA expression in the *Npr1* $-/-$ ventricle were most marked in 6-month-old females. The reactivation of ANP expression in adult ventricular myocardium has become one of the most sensitive markers of hypertrophy (Day *et al.* 1987). This increased expression was reflected in increased ANP hormone secretion.

Cardiac fibrosis is a classical feature of hypertrophy and is characterised by the expansion of the extracellular matrix due to the accumulation of collagen, particularly collagen types I and III (Manabe *et al.* 2002). Such stiffening impedes both the contraction and the relaxation of cardiomyocytes, impairs electrical coupling and can lead to myocyte hypoxia due to reduced capillary density and increased oxygen diffusion distance (Manabe *et al.* 2002). In the present study, collagen I gene expression was increased in both male and female 6-month *Npr1* $-/-$ animals, but was not apparent in younger mice. TGF- β promotes the proliferation of fibroblasts, stimulates extracellular matrix protein production while inhibiting its degradation by induction of antiproteases or reduction of metalloproteases (Manabe *et al.* 2002). Transgenic mice overexpressing TGF- β 1 develop cardiac hypertrophy and interstitial fibrosis (Rosenkranz *et al.* 2002). The late increase in the expression of collagen I and TGF- β in 6-month-old *Npr1* $-/-$ mice implies that in this mouse model of cardiac disease, hypertrophy occurs in early adulthood, whilst the fibrotic response occurs later as the animals age, concomitant with the decline in cardiac contractility we observed in *Npr1* $-/-$ hearts between 8 weeks and 6 months. Finally, a decrease in the expression of phospholamban, a molecule responsible for mediating cardiac relaxation, was observed in 6-month female *Npr1* $-/-$ animals. Phospholamban mutations have been shown to cause dilated cardiomyopathy and

resultant heart failure, but this pathway has not previously been associated with *Npr1* activity.

In summary, we have shown that *Npr1* signalling influences the very earliest steps in the cellular pathways leading to cardiac hypertrophy and fibrosis by first altering the levels of Ca/calmodulin signalling. This, in turn may lead to observed alterations in the expression of the multiple intracellular messengers, including Hdac's, PKC α and the transcription factor GATA4. Male mice demonstrated an earlier onset of cardiac remodelling, consistent with the onset of human heart disease at a younger age in men when compared with women. We also report marked age-related changes in the levels of expression of several transcription factor genes, independent of genotype, which has implications for the phenotypic plasticity of adult myocardium even in the absence of overt cardiovascular disease. The microarray studies have raised intriguing questions about gene pathways not previously identified with *Npr1* signalling. Approximately one-third of genes identified as significantly altered by microarray are of unknown function. Characterisation of these gene pathways will be the subject of ongoing studies.

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