Angiotensin-(1-9) Attenuates Cardiac Fibrosis in the Stroke-Prone Spontaneously Hypertensive Rat via the Angiotensin Type 2 Receptor

Monica Flores-Munoz, Lorraine M. Work, Kirsten Douglas, Laura Denby, Anna F. Dominiczak, Delyth Graham, Stuart A. Nicklin

Abstract—The renin-angiotensin system regulates cardiovascular physiology via angiotensin II engaging the angiotensin type 1 or type 2 receptors. Classic actions are type 1 receptor mediated, whereas the type 2 receptor may counteract type 1 receptor activity. Angiotensin-converting enzyme 2 metabolizes angiotensin II to angiotensin-(1-7) and angiotensin I to angiotensin-(1-9). Angiotensin-(1-7) antagonizes angiotensin II actions via the receptor Mas. Angiotensin-(1-9) was shown recently to block cardiomyocyte hypertrophy via the angiotensin type 2 receptor. Here, we investigated in vivo effects of angiotensin-(1-9) via the angiotensin type 2 receptor. Angiotensin-(1-9) (100 ng/kg per minute) with or without the angiotensin type 2 receptor antagonist PD123 319 (100 ng/kg per minute) or PD123 319 alone was infused via osmotic minipump for 4 weeks into stroke-prone spontaneously hypertensive rats. We measured blood pressure by radiotelemetry and cardiac structure and function by echocardiography. Angiotensin-(1-9) did not affect blood pressure or left ventricular mass index but reduced cardiac fibrosis by 50% (P<0.01) through modulating collagen I expression, reversed by PD123 319 coinfusion. In addition, angiotensin-(1-9) inhibited fibroblast proliferation in vitro in a PD123 319-sensitive manner. Aortic myography revealed that angiotensin-(1-9) significantly increased contraction to phenylephrine compared with controls after N-nitro-L-arginine methyl ester treatment, an effect abolished by PD123 319 coinfusion (area under the curve: angiotensin-(1-9) N-nitro-L-arginine methyl ester=98.9±11.8%; control+N-nitro-L-arginine methyl ester=74.0±10.4%; P<0.01), suggesting that angiotensin-(1-9) improved basal NO bioavailability in an angiotensin type 2 receptor–sensitive manner. In summary, angiotensin-(1-9) reduced cardiac fibrosis and altered aortic contraction via the angiotensin type 2 receptor supporting a direct role for angiotensin-(1-9) in the renin-angiotensin system. (Hypertension. 2012;59:300-307.) • Online Data Supplement

Key Words: renin-angiotensin system • angiotensin-(1-9) • cardiac fibrosis • angiotensin type 2 receptor • stroke-prone spontaneously hypertensive rat

Angiotensin II (Ang II) is the main effector of the renin-angiotensin system (RAS), classically acting via the angiotensin type 1 receptor (AT₁R) to stimulate effects such as sodium reabsorption, vasoconstriction, proliferation, and inflammation. Ang II, acting via the AT₁R, contributes to the pathophysiology of cardiovascular disease. The angiotensin type 2 receptor (AT₂R) is 34% homologous to the AT₁R, and its actions differ. AT₂R expression is limited to fetal/neonatal tissues; however, in adult rodents it is upregulated in heart failure and postmyocardial infarction. In human adult myocardium, 41% of angiotensin binding sites are AT₂R. Ang II signaling via the AT₁R counteracts AT₂R signaling; for example, blocking AT₂R activation promotes cardiomyocyte hypertrophy, whereas AT₂R overexpression in stroke-prone spontaneously hypertensive rat (SHRSP) hearts prevents increased left ventricular mass index (LVMI). Transgenic overexpression of the AT₂R in mice reduces cardiac fibrosis. Furthermore, the bradykinin B₂ receptor associates with the AT₂R, inhibiting fibrosis and promoting vasodilatation through endothelial NO synthase. Stimulating the AT₂R with the oral agonist C21 postmyocardial infarction diminishes interleukin 6, interleukin 1β, and interleukin 2 expression and reduces scar size.

The angiotensin-converting enzyme (ACE) homologue ACE2 is an additional important RAS member with different enzymatic activity, substrate specification, and inhibitor specificity. ACE2 metabolizes angiotensin I and Ang II to form angiotensin 1-9 (Ang-[1-9]) and angiotensin 1-7 (Ang-[1-7]), respectively. Ang-(1-9) is also metabolized by ACE to Ang-(1-7). In the heart, vasculature, and kidneys, Ang-(1-7)
blocks detrimental effects of Ang II via the receptor Mas.\textsuperscript{12} For example, Ang-(1-7) blocks Ang II–induced fibrosis, cardiac hypertrophy, and hypertension\textsuperscript{13} and in the heart reduces re-entrant arrhythmias.\textsuperscript{14} Ang-(1-7) reverses Ang II–induced endothelial dysfunction via Mas-mediated stimulation of endothelial NO synthesis.\textsuperscript{15}

Ang-(1-9) is thought to reduce Ang II levels because it competes with angiotensin I at the ACE active site and increases Ang-(1-7) and stimulates bradykinin release in endothelial cells.\textsuperscript{16} Recently Ang-(1-9) was demonstrated to inhibit cardiac hypertrophy after myocardial infarction in rats.\textsuperscript{17} This was not dependent on Ang-(1-9) to Ang-(1-7) conversion, because coadministration of the Mas antagonist A779 did not influence the effects of Ang-(1-9). However, Ang-(1-9) administration did lead to decreased Ang II levels. Moreover, we have shown recently that Ang-(1-9) is able to compete with Ang II binding at the AT\textsubscript{2}R via radioligand binding assay and is able to block cardiomyocyte hypertrophy in vitro. Furthermore, inhibition of hypertrophy by Ang-(1-9) is blocked in the presence of the selective AT\textsubscript{2}R inhibitor PD123 319.\textsuperscript{18} Here, we have infused Ang-(1-9) into a model of essential hypertension, the SHRSP, to investigate its effects on blood pressure, cardiac remodeling and function, and vascular function and whether they are mediated via the AT\textsubscript{2}R.

\section*{Methods}
For the methods section for this article please see the online Data Supplement at http://hyper.ahajournals.org.

\section*{Results}
\subsection*{Effects of Ang-(1-9) on Blood Pressure}
Averaged weekly telemetric blood pressure readings were analyzed. All 4 of the groups (n=6) started with similar values of mean arterial blood pressure (control: 155.8±1.6 mm Hg; Ang-(1-9): 153.9±3.6 mm Hg; Ang-(1-9)+PD123 319: 156.2±2.2 mm Hg; PD123 319: 150.6±2.4 mm Hg; Figure 1). Two weeks after minipump implantation, mean arterial blood pressure increased in Ang-(1-9), Ang-(1-9)+PD123 319, and PD123 319 animals compared with control, although it did not reach statistical significance when comparing groups or when comparing the 4-week end point within a group to measurements at the point of minipump implantation (13 weeks). For example, daytime mean arterial blood pressures at 13 weeks were as follows: control, 146.9±0.9 mm Hg; Ang-(1-9), 148.3±3.5 mm Hg; Ang-(1-9)+PD123 319, 148.8±3.3 mm Hg; and PD123 319, 144.4±0.9 mm Hg (Figure 1). Daytime mean arterial blood pressure values at 17 weeks were as follows: control, 152.7±0.9 mm Hg; Ang-(1-9), 164.3±9.9 mm Hg; Ang-(1-9)+PD123 319, 171.9±8.6 mm Hg; and PD123 319, 164.8±8.8 mm Hg.

\subsection*{Effects of Ang-(1-9) on Cardiac Function}
Baseline measurements between groups at the start of the study did not differ (data not shown). Analysis of echocardiograms at both 2 and 4 weeks after minipump implantation (15 and 17 weeks of age) revealed that LVMI normalized through tibia length was not different between groups (control: 1.8±0.1 mm; Ang-(1-9): 1.9±0.1 mm; Ang-(1-9)+PD123 319: 1.8±0.1 mm; PD123 319: 1.7±0.5 mm; Table S1, available in the online Data Supplement). Extrapolation of data from the echocardiograms showed no significant difference in cardiac output, stroke volume, fractional shortening, or ejection fraction between groups (Table S1). Calculation of early/atrial ratio as an indirect indicator of diastolic function also revealed no significant differences between groups (Figure S1).

\subsection*{Effects of Ang-(1-9) on Endothelial Function}
Aortic wire myography was used to evaluate the effect of Ang-(1-9) on endothelial function. Carbachol induced relaxation in all of the groups confirming functional integrity of the endothelium of the aortic rings because there were no significant differences between any of the groups (Figure S2). In control SHRSPs, inhibition of endothelial NO synthase with N\textsuperscript{G}-nitro-L-arginine methyl ester (l-NNAME) did not modify basal arterial tension induced by phenylephrine (PE) because there was no significant difference between the contraction curves (control PE: 64.1±10.3% of maximal contraction; control PE+l-NNAME: 74.0±10.4%, area under the curve; Figure 2). This indicated reduced basal NO availability in the control animals, consistent with other SHRSP studies.\textsuperscript{19} In Ang-(1-9)-infused animals l-NNAME significantly increased contractile response, inducing a 30%
difference between the curves (Ang-(1-9) PE: 68.3±10.8%; Ang-(1-9) PE+L-NAME: 98.9±11.8; P<0.01; Figure 2), indicating that Ang-(1-9) improved NO bioavailability. In contrast, animals coinfused with Ang-(1-9) and PD123 319 showed no difference between basal and PE/L-NAME contraction (Ang-(1-9)+PD123 319 PE: 62.2±10.8%; Ang-(1-9)+PD123 319 PE+L-NAME: 72.5±17.7%), indicating that PD123 319 abrogated effects of Ang-(1-9) on endothelial function (Figure 2). PD123 319 alone had no effect on endothelial function. Next, we analyzed expression of the reactive oxygen species–generating enzyme NADPH oxidase 4 in aortas of SHRSPs infused with water (control), Ang-(1-9), Ang-(1-9)+PD123 319, or PD123 319 alone for 4 weeks was assessed by immunohistochemistry and quantified using Image-ProPlus. #P<0.05 vs control. Scale bar=50 μm, top panel magnification: ×20; bottom panel magnification: ×40.

**Figure 2.** Effects of angiotensin (Ang)-(1-9) infusion on endothelial function. Large-vessel myography was performed in aortic rings from stroke-prone spontaneously hypertensive rats (SHRSPs) infused with water (control; A), Ang-(1-9) (B), Ang-(1-9)+PD123 319 (C), or PD123 319 alone (D) for 4 weeks. Basal contractile response was induced by phenylephrine (PE) and endogenous production of NO inhibited with N\(^{-}\)-nitro-L-arginine methyl ester (L-NAME) before inducing contraction with PE. Endothelial function was evaluated by NO bioavailability, which was assessed as the difference between contractile response curves. *P<0.0001; n=6 animals per group. E, NADPH oxidase 4 (NOX4) expression in aortas of SHRSPs infused with water (control), Ang-(1-9), Ang-(1-9)+PD123 319 (PD+Ang1-9), or PD123 319 alone for 4 weeks was assessed by immunohistochemistry and quantified using Image-ProPlus. #P<0.05 vs control. Scale bar=50 μm, top panel magnification: ×20; bottom panel magnification: ×40.

**Histological Assessment of Ang-(1-9) Effects in the Heart**

There was no obvious evidence of irregularity in cardiac structure between groups by hematoxylin and eosin staining.
Masson trichrome in control SHRSPs revealed significant collagen staining in the perivascular region (Figure 3B), whereas perivascular fibrosis was attenuated in SHRSPs infused with Ang-(1-9), suggesting that it blocked fibrosis development. Interestingly, after coinfusion of PD123 319 and Ang-(1-9), quantification of cardiac fibrosis was similar to that observed in control animals; however, the distribution of staining in PD123 319 and Ang-(1-9)–infused animals was altered as interstitial fibrosis was observed, as opposed to the predominantly perivascular staining in control and PD123 319 alone animals (Figure 3B). Picrosirius red staining of control hearts revealed increased perivascular and interstitial deposits of collagen fibers, which was blocked by Ang-(1-9) infusion in a PD123 319-sensitive manner (Figure 3C). Similar to what was observed with Picrosirius red staining, immunohistochemical staining of collagen Ia fibers showed a significant reduction in SHRSPs infused with Ang-(1-9), an effect that was reversed when Ang-(1-9) was coinfused with PD123 319 (Figure 4A and 4B). However, no significant difference between groups was observed for collagen IIIa fibers (Figure 4B through 4D). Myocardial mRNA expression of tissue inhibitor of metalloproteinase 1, tissue inhibitor of metalloproteinase 2, and matrix metalloproteinase (MMP) 2 showed no significant difference between groups (Figure S3). However, a significant increase in MMP-14 gene expression was observed in SHRSP hearts infused with PD123 319 alone. Gene expression levels of MMP-9 were undetectable in all of the groups.
In Vitro Proliferation Assays in Cardiac Fibroblasts

To confirm direct effects of Ang-(1-9) on fibroblast phenotype, isolated primary cultures of neonatal fibroblasts were assessed. Coaddition of Ang-(1-9) to 5% serum-stimulated fibroblasts inhibited proliferation (Figure 5). However, the addition of PD123 319 to Ang-(1-9)–stimulated fibroblasts reversed the antiproliferative effect of Ang-(1-9) (Figure 5A). Quantification of collagen Ia and IIIa expression by quantitative RT-PCR revealed that Ang-(1-9) induced significant downregulation of collagen Ia, but not IIIa, an effect prevented by coaddition of PD123 319 (Figure 5B). These results demonstrated that Ang-(1-9) was able to mediate antiproliferative effects on fibroblasts via the AT2R.

Discussion

Previously we have shown that regression of increased LVMI in the SHRSP can be achieved via AT1R antagonism or mitochondrial-targeted antioxidants.20–22 Here, SHRSPs were used to study the effects of Ang-(1-9) on blood pressure, cardiac structure and function, and endothelial function via the AT2R. SHRSPs were infused with water (control), angiotensin (Ang)-1-9, Ang-(1-9)/H11001 PD123 319 (PD/H11001 Ang-(1-9)), or PD123 319 alone was assessed by immunohistochemistry and quantified using ImageProPlus. Scale bar=10 μm. Magnification: ×20. *P<0.05 vs control animals.

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changes in blood pressure. Furthermore, there was a tendency for animals infused with Ang-(1-9) to have higher blood pressure, although only longer-term studies would reveal whether this reached significance. Previously, lentiviral overexpression of the AT2R in spontaneously hypertensive rat hearts did not affect blood pressure,6 and transgenic cardiac-selective AT2R overexpression did not affect blood pressure or cardiac hypertrophy compared with wild-type mice; however, it did reduce cardiac fibrosis.29 Here, coinfusion of Ang-(1-9) and the AT2R antagonist PD123 319 also appeared to invoke redistribution of fibrosis from perivascular sites to the interstitium. In both Ang-(1-9) and PD123 319 and PD123 319 alone groups, increased collagen I staining compared with control animals was observed. Furthermore, PD123 319 infusion alone increased MMP-14 gene expression. This is consistent with previous observations regarding endogenous AT2R function. Infusion of PD123 319 in a rat model of glomerulosclerosis induced by 5/6 nephrectomy significantly increased glomerular cell proliferation and kidney fibrosis compared with animals that were nephrectomized alone.30 Increased collagen I levels have also been shown previously to upregulate MMP-14 expression at the mRNA level,31 which supports our findings. Moreover, fibrotic tissue remodeling after injury is associated with vascular damage and leakage, which has been shown to be associated with type I collagen remodeling and increased MMP-14 activity in mouse models of age-related dermal fibrosis.32

We demonstrated recently that Ang-(1-9) was able to block cardiomyocyte hypertrophy in vitro in both adult and neonatal cardiomyocytes, an effect that was mediated via the AT2R and not via Mas and was distinguishable from the effects of Ang-(1-7) that did act via Mas.18 Furthermore, Ocaranza et al17 demonstrated that Ang-(1-9) also prevented cardiomyocyte hypertrophy in a rat myocardial infarction model. In that model, preventing cardiomyocyte hypertrophy was also not dependent on Ang-(1-9) increasing Ang-(1-7) activity via Mas, because the Mas antagonist A779 had no effect but was thought to be through Ang-(1-9) binding ACE and preventing Ang II generation. Both of these articles suggest that Ang-(1-9) actions are independent of conversion to Ang-(1-7) by ACE. Moreover, our lack of effect on cardiac hypertrophy is
consistent with previous effects of AT₂R signaling.²⁹ In SHRSPs aged 12 weeks, hypertension and increased LVMI are well established,²¹ although SHRSPs only develop increased LVMI as a compensatory response to increased afterload and do not develop heart failure until ~2 years of age and, therefore, have normal cardiac function.³³,³⁴ Here, this is supported by calculation of early/atrial ratio, which indicated normal diastolic function. Although early/atrial ratio is an indirect analysis of diastolic function and use of pressure-volume loop catheters in future studies would accurately assess diastolic function. However, in the SHRSPs, Ang-(1–9) was able to block development of cardiac fibrosis, an effect that was blocked by coinfusion with PD123 319. These data further support a role for the AT₂R, because other studies support an antifibrotic role for the AT₂R without affecting cardiac hypertrophy or systemic blood pressure.³⁵,³⁶ Although circulating Ang-(1–9) levels are low, in heart failure patients the myocardium forms 1 nmol/L min⁻¹ mg⁻¹ Ang-(1–9), and the heart contains 170 nmol/L of Ang I, with 85% converted to equivalent levels of Ang-(1–9) and Ang II, supporting a potential role for Ang-(1–9) in human cardiovascular disease by potentially acting at the AT₂R to counteract detrimental effects of Ang II at the AT₁R.³⁷ With evidence that Ang-(1–9) is a functional agonist at the AT₂R, this has important translational implications for understanding and developing new therapeutic avenues in cardiovascular disease.

Perspectives

We show a direct in vivo role for Ang-(1–9) acting via the AT₂R to reduce cardiac fibrosis and improve endothelial function in the SHRSP. These findings suggest that Ang-(1–9), a relatively unexplored peptide of the RAS, may be an additional member of the increasingly important counterregulatory RAS and, like Ang-(1–7), an important target in our understanding of cardiovascular disease and in identifying novel therapeutic targets.

Acknowledgments

We thank Prof Andrew H. Baker (University of Glasgow) for helpful discussions and advice and Nicola Britton and Gregor Aitchison for technical assistance.

Sources of Funding

We thank the University of Glasgow Postgraduate Scholarship Scheme, CONACYT (Consejo Nacional de Ciencia y Tecnologia), BHF (British Heart Foundation) Chair (CH98001; to A.F.D.), and BHF programme grant (RG1071005), as well as a Capacity Building Award in Integrative Mammalian Biology funded by the BBSRC (Biotechnology and Biological Sciences Research Council), BPS (British Pharmacological Society), KTIN (Knowledge Transfer Network), MRC (Medical Research Council), and SFC (Scottish Funding Council).

Disclosures

None.

References


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Hypertension. 2012;59:300-307; originally published online December 19, 2011; doi: 10.1161/HYPERTENSIONAHA.111.177485

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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ANGIOTENSIN-(1-9) ATTENUATES CARDIAC FIBROSIS IN THE STROKE PRONE SPONTANEOUSLY HYPERTENSIVE RAT VIA THE ANGIOTENSIN TYPE 2 RECEPTOR

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Short title: Ang-(1-9) attenuates cardiac fibrosis via the AT2R

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Methods

Animals

The stroke prone spontaneously hypertensive rat (SHRSP) are maintained “in-house” by brother-sister mating and microsatellite and single nucleotide polymorphism genotyping is routinely performed to confirm homozygosity of all screened loci. All animals were housed under 12h light/dark cycles at ambient temperature, fed standard rat chow (Rat and Mouse No 1, Special Diet Servcies) and water provided ad libitum. Male rats were used. All studies in experimental animals were in accordance with the Animals Scientific Procedures Act 1986 and all studies were approved by the University of Glasgow’s Ethics Review Committee. 6 animals were used for each group, 24 in total.

Surgical Procedures

For radiotelemetry probe and osmotic minipump implantation rats were anesthetized with 2.5% isofluorane in 1.5 L/min O2 for the duration of the surgical implantation procedure. Carprofen (5mg/kg), a Non-Steroidal Anti-inflammatory analgesic is administered immediately prior to surgery. For transthoracic echocardiography SHRSP were mildly anaesthetized with 1.25% to 1.5% isoflurane in 1.5 L/min O2 before image acquisition and were positioned in left lateral decubitus on a heated pad.

Radiotelemetry

Hemodynamics of SHRSP were monitored using the Dataquest IV (Art Gold) Radiotelemetry System (Data Science International, Minnesota, USA). Eleven week old SHRSP were anesthetised with isoflurane and the transmitter inserted into the abdominal aorta as previously described 1. Rats were housed in individual cages following the procedure. Ten days later osmotic minipumps (Alzet, CA, USA) were subcutaneously implanted to secrete Ang-(1-9) (Phoenix Pharmaceuticals, Burlingame, CA, USA) (100ng·kg⁻¹·min⁻¹) +/- the AT2R blocker PD123,319 (Sigma Aldrich, Poole, Dorset, UK) (100ng·kg⁻¹·min⁻¹) for 4 weeks. Control SHRSP were implanted with a minipump secreting dH₂O. At the end of the protocol rats were sacrificed and tissues harvested for myography, histology and Q-RT-PCR.

Echocardiography

Three echocardiograms were performed at 11, 15 and 17 weeks of age. An Acuson Sequoia c512 ultrasound system was used to acquire non-invasive 2-D guided M-mode images at a depth of 20 mm and at the tip of the papillary muscles which were measured in a short axis view using the leading edge-to-leading edge convention during both systole and diastole over at least three consecutive cardiac cycles. Echocardiographic indices assessed included left ventricular anterior and posterior wall thickness (LVAW and LVPW, respectively), LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), LV end diastolic diameter (LVEDD), LV end-systolic diameter (LVESD) endocardial fractional shortening (FS), ejection fraction (EF) to assess left ventricular systolic function. Internal left ventricular diameter (ILVD) and wall thickness was measured in diastole and systole to allow accurate estimation of LV mass index (LVMI) and left ventricular systolic function. Early (E) and late (atrial; A) ventricular filling velocity were calculated from measuring blood velocities across the mitral valve during each cardiac cycle. E/A ratio was calculated as an indirect measure of diastolic function. Ejection fraction (EF)= \( \frac{[LVEDV - LVESV]}{LVEDD} \times 100\% \); LVEDV= left ventricular end diastolic volume; LVESV = left ventricular end systolic volume. Fractional shortening (FS)= \( \frac{[(LVEDD - LVESD)]}{LVEDD \times 100\%} \). Cardiac output (CO)= \((ESV - EDV) \times HR\); ESV= end systolic volume; EDV= end diastolic volume; HR= heart rate. Stroke volume (SV)= ESV – EDV; ESV= end systolic volume; EDV= end diastolic volume. Left ventricular mass index (LVMI) was calculated as: cube function formula (ASE-cube formula) with Devereux correction 2 and normalized to tibia length: 0.8 (1.04 \( (LVAWd + LVIDd + LVPWd)^{3} - (LVIDd)^{3} \) + 0.6); LVAWd= left ventricular anterior wall in diastole; LVIDd= left ventricular internal diameter in diastole; LVPWd= left ventricular posterior wall in diastole.

Large vessel wire myography
Thoracic aortic endothelial function was assessed by wire myography. Three mm aortic rings were isolated from 17 week old SHRSP at the end of the study, mounted, pre-treated with potassium-buffered Kreb’s followed by stimulation with phenylephrine (PE) and carbachol (1 µM). Drugs were washed out and aortas rested before cumulative concentration-response curves to PE (for contraction) and carbachol (for relaxation) (1 nM to 10 µM) were constructed. Aortic rings were then washed and rested before exposure to L-NAME (100 mM) and a concentration-response curve to PE (1 nM to 10 µM) constructed.

**Histology**

Fibrosis was assessed in 5 µm myocardial sections using Masson’s Trichrome kit (Sigma, Dorset, UK) as per manufacturer’s instructions. Picrosirius red staining was performed on sections incubated under dark conditions in 0.1% Picrosirius Red solution. Sections were washed, stained with eosin and nuclei were counterstained in haematoxylin. To quantify fibrosis Image ProPlus (Media Cybernetics, Basingstoke, UK) was used. The area for quantification was selected using the Area of Interest Macro and pixel values were transformed to optical density units as previously described 3. Immunohistochemistry analysis was performed using primary antibodies against collagen 1a (1:200), collagen 3a (1:200) and Nox-4 (1:250) (clone numbers Col-1, FH-7a and NOX4 respectively, Abcam, Cambridge, UK). Briefly, sections were incubated for 30 minutes at room temperature in 1% hydrogen peroxide in methanol followed by citrate buffer antigen retrieval. Sections were incubated with the primary antibody overnight at 4°C, followed by 1 hr incubation with biotinylated secondary antibody (DAKO, Cambridgeshire, UK). Antibody binding was visualized using the avidin-biotin complex method according to the manufacturer’s instructions (Vectastain ABC; Vector, Peterborough, UK). Sections were subsequently counterstained with haematoxylin. Collagen content was measured using ImageProPlus as previously described from five fields of view (x10) from each section.

**Real-time quantitative polymerase chain reaction assays**

The expression of matrix metalloproteinases (MMP) 2, 9 and 14, and tissue inhibitors of MMPs (TIMP) 1 and 2 were determined by quantitative real time polymerase chain reaction (qRT-PCR). Total RNA from paraffin embedded heart was isolated using RecoverAll FFPE RNA isolation kit (Ambion, Warrington, UK) following manufacturer’s instructions. DNaseI treated RNA samples were reverse transcribed to cDNA using the Applied Biosystems Reverse Transcription Kit following manufacturer’s instructions. Resulting cDNA was used qRT-PCR for matrix metalloproteinase (MMP)-2, MMP-9, MMP-14, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2 and beta-2 microglobulin (B2m) using inventoried TaqMan gene expression assays (Applied Biosystems). Gene expression was normalized to the expression of B2m using an Applied Biosystems 7900HT Sequence Detection System following manufacturer’s instructions. All samples were analysed in triplicate using TaqMan data analysis software. For expression of Collagen Ia and Collagen IIIa in rat neonatal cardiac fibroblast, cells plated in 12 well plates at 1x10^5 cells per well and incubated for 24 hours. Cells were then quiesced in serum free media for 24 h, followed by addition of 1 µM Ang-(1-9) and 5% FCS for 48 h at 37°C. PD123,319 (500 nM) was added 15 minutes before Ang-(1-9). RNA was isolated at 48 hours using the RNeasy mini kit (Qiagen, Sussex, UK) following the manufacturer’s protocols. Following DNase treatment (Applied Biosystems, Warrington, UK), RNA was reverse transcribed to cDNA using Reverse Transcription Kit (Applied Biosystems, Warrington, UK) following the manufacturer’s instructions. qRT-PCR was performed using inventoried TaqMan gene expression assays (Applied Biosystems, Warrington, UK) for Collagen I and Collagen III and normalized to the housekeeper gene peptidylpropyl isomerase B (PPIB) expression (Applied Biosystems, Warrington, UK) using an Applied Biosystems 7900HT Sequence Detection System (Warrington, UK) following the manufacturer’s instructions.

**Cardiac Fibroblast culture**
Cardiac fibroblasts were isolated from 1 to 3 day old neonatal rat hearts by enzymatic digestion (310 U/mg collagenase type 2 [Worthington Biochemical Corporation, USA] and 310 U/mg Pancreatin [Sigma, Dorset, UK]). Fibroblasts were allowed to attach and non-adherent cells removed. Cells were cultured in DMEM supplemented with 20% fetal calf serum (FCS) until confluence. Three different fibroblast preparations were used between passages 3-5.

**Cell Proliferation assay**

Fibroblasts were seeded at 5x10^3 cells/well 24 h prior to experiments and quiesced in serum free media for 24 h, followed by addition of 1 μM Ang-(1-9) and 5% FCS for 48 h at 37°C. PD123,319 (500nM) was added 15 minutes before Ang-(1-9). Fibroblast proliferation was quantified by MTS Assay (Promega, Southampton, UK) according to manufacturer's instructions and absorbance recorded at 490nm using a Wallac Victor2 (Wallac, Turku, Finland).

**Statistical analysis**

*In vivo* experiments were performed with n=6 per group. Myography comparisons were performed by repeated measures ANOVA as described previously 4. Radio-telemetry was analysed by two way repeated measures ANOVA with Bonferroni's correction. *In vitro* experiments were performed in triplicate on 3 independent occasions. Data are shown as mean ± standard error of the mean. One way ANOVA with Dunnett’s correction for multiple comparisons were applied and statistical difference was considered with p values <0.05.

**References**

<table>
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<th>Groups</th>
<th>Age</th>
<th>LDMI</th>
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<th>SV</th>
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Table S1. Echocardiography. Left ventricular mass index (LDMI), heart rate (HR), stroke volume (SV), cardiac output (CO), ejection fraction (EF) and fractional shortening (FS) were calculated from echocardiography images. Age of rats in weeks. * n=2 rats/group.
Figure S1. Echocardiography analysis of diastolic function. E/A ratio, as an indicator of diastolic function, was calculated from echocardiograph images measuring blood velocity across the mitral valve in both early and late (atrial) phase of each cardiac cycle in control animals and animals infused with Ang-(1-9) at 11, 15 and 17 weeks of age, (n=4 per group).
Figure S2. Functional integrity of the endothelium. At the end of the PE contractile response curves, cumulative concentrations of carbachol (1 nM to 10 µM) was added and the relaxant response evaluated in aortic rings from SHRSP infused with (A) water (control), (B) Ang-(1-9), (C) Ang-(1-9) + PD123,319 or (D) PD123,319 alone for 4 weeks.
Figure S3. Quantitative RT-PCR. RNA was isolated from SHRSP infused for 4 weeks with water (control), Ang1-9, Ang1-9 + PD123,319 (PD+Ang1-9), or PD123,319 alone, reverse transcribed and (A) MMP-2 and MMP-14 and (B) TIMP-1 and TIMP-2 expression quantified via real-time qRT-PCR and normalised to B2m RNA expression.