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RESEARCH PAPER

Chronic treatment with angiotensin-(1-7) improves renal endothelial dysfunction in apolipoproteinE-deficient mice

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Keywords
endothelial dysfunction; angiotensin II; angiotensin-(1-7); NO bioavailability; reactive oxygen species; apolipoproteinE knockout mouse

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BACKGROUND AND PURPOSE

ApolipoproteinE-deficient [apoE (–/–)] mice, a model of human atherosclerosis, develop endothelial dysfunction caused by decreased levels of nitric oxide (NO). The endogenous peptide, angiotensin-(1-7) [Ang-(1-7)], acting through its specific GPCR, the Mas receptor, has endothelium-dependent vasodilator properties. Here we have investigated if chronic treatment with Ang-(1-7) improved endothelial dysfunction in apoE (–/–) mice.

EXPERIMENTAL APPROACH

ApoE (–/–) mice fed on a lipid-rich Western diet were divided into three groups and treated via osmotic minipumps with either saline, Ang-(1-7) (82 $\mu g \cdot kg^{-1} \cdot h^{-1}$) or the same dose of Ang-(1-7) together with D-Ala-Ang-(1-7) (125 $\mu g \cdot kg^{-1} \cdot h^{-1}$) for 6 weeks. Renal vascular function was assessed in isolated perfused kidneys.

KEY RESULTS

Ang-(1-7)-treated apoE (–/–) mice showed improved renal endothelium-dependent vasorelaxation induced by carbachol and increased renal basal cGMP production, compared with untreated apoE (–/–) mice. Tempol, a reactive oxygen species (ROS) scavenger, improved endothelium-dependent vasorelaxation in kidneys of saline-treated apoE (–/–) mice whereas no effect was observed in Ang-(1-7)-treated mice. Chronic treatment with D-Ala-Ang-(1-7), a specific Mas receptor antagonist, abolished the beneficial effects of Ang-(1-7) on endothelium-dependent vasorelaxation. Renal endothelium-independent vasorelaxation showed no differences between treated and untreated mice. ROS production and expression levels of the NAD(P)H oxidase subunits gp91phox and p47phox were reduced in isolated preglomerular arterioles of Ang-(1-7)-treated mice, compared with untreated mice, whereas eNOS expression was increased.

CONCLUSION AND IMPLICATIONS

Chronic infusion of Ang-(1-7) improved renal endothelial function via Mas receptors, in an experimental model of human cardiovascular disease, by increasing levels of endogenous NO.

Abbreviations

Ang, angiotensin; apoE, apolipoproteinE; AT_1 receptor, angiotensin II type 1 receptor; GSNO, S-nitrosoglutathione; IBMX, 3-isobutyl-1-methylxanthine; ROS, reactive oxygen species

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Introduction

Endothelial dysfunction is caused by decreased levels of nitric oxide (NO) derived from the endothelium. This deleterious condition is strongly associated with hypertension and therefore an important predictor of cardiovascular risk. The amount of biologically active NO, which plays a pivotal role in vascular homeostasis, is determined by the balance between the biosynthesis of NO and its degradation by endogenous reactive oxygen species (ROS) (Cai and Harrison, 2000).

Angiotensin (Ang) II, the major effector molecule of the renin-angiotensin-system (RAS) has an important effect on the genesis of endothelial dysfunction. Ang II, through its actions at the angiotensin II type 1 (AT_1) receptor (nomenclature follows Alexander *et al.*, 2009), increases ROS production which leads subsequently to NO degradation (Mehta and Griendling, 2007). Thus, inhibition of Ang II generation by angiotensin converting enzyme (ACE) inhibitors or blockade of the AT_1 receptor ameliorates endothelial dysfunction and thereby reduces cardiovascular morbidity and mortality. New components and functions of the RAS are still being uncovered. Besides the classical main effector Ang II, other peptides of the RAS, like Ang III, Ang IV and Ang-(1-7) have been shown to have biological actions (Ferrario *et al.*, 1998; Stegbauer *et al.*, 2003). Ang-(1-7) can be formed from Ang I or Ang II by several peptidases including the carboxypetidases ACE and ACE2 (Iusuf *et al.*, 2008) whereas the conversion of Ang II to Ang-(1-7) by ACE2 seems to be the preferred pathway of Ang-(1-7) generation. Ang-(1-7) activates its own GPCR, the Mas receptor and this receptor has been characterized as a physiological antagonist of the AT_1 receptor (Santos *et al.*, 2003; Kostenis *et al.*, 2005). Moreover, Ang-(1-7)-mediated activation of Mas receptors has been shown to inhibit endothelial cell growth, proliferation and cardiac remodelling by influencing MAP kinase signalling (Sampaio *et al.*, 2007a; Mercure *et al.*, 2008), and to improve cardiac function after myocardial infarction (Loot *et al.*, 2002; Grobe *et al.*, 2007). Moreover, activation of Mas receptors located in the endothelium induced vasodilation by generating NO and prostaglandins (Castro *et al.*, 2005; Sampaio *et al.*, 2007b). Also, Ang-(1-7) improved endothelial-dependent vasorelaxation in normotensive rats by increasing NO bioavailability (Faria-Silva *et al.*, 2005). Consequently, Mas receptor-deficient mice showed decreased endothelial NO synthase and increased NAD(P)H oxidase activity, leading to endothelial dysfunction and subsequently to hypertension (Xu *et al.*, 2008).

However, not much is known about the effects of Ang-(1-7) on endothelial dysfunction. To answer that question, we have used apolipoproteinE (apoE) (–/–) mice, as an experimental model for human atherosclerosis. These animals develop pronounced endothelial dysfunction when fed a lipid- and cholesterol-rich diet (Wassmann *et al.*, 2004). Thus, they provide an ideal model to dissect the consequences of Ang-(1-7)-mediated Mas receptor signalling for endothelial function.

Methods

Animals and osmotic minipump implantation

All animal care and experimental investigations were in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996) and were approved by the local Animal Care Committee (licence no. 50.8735.1 Nr. 109/6). Six-week-old male apoE (–/–) mice (C57Bl/6 background) and their littermates were obtained from Jackson Labs, Bar Harbor. Mice were fed with 'Western-type' diet (Sniff, Soest, Germany) (42% fat, 0.15% cholesterol) and allowed free access to tap water. After 6 weeks, mice were treated with either saline, Ang-(1-7) or with a combination of Ang-(1-7) and D-Ala-Ang-(1-7), delivered over 6 weeks by osmotic minipumps. The minipumps (Alzet Model 1004) were filled with either saline, Ang-(1-7) (to deliver 82 $\mu g \cdot kg^{-1} \cdot h^{-1}$) (Bachem) or D-Ala-Ang-(1-7) (to deliver 125 $\mu g \cdot kg^{-1} \cdot h^{-1}$) (Bachem). Osmotic minipumps were inserted subcutaneously during anaesthesia with ketamine (100 $mg \cdot kg^{-1}$, i.p.) and xylazine (5 $mg \cdot kg^{-1}$, i.p.) and replaced after 3 weeks. When both Ang-(1-7) and D-Ala-Ang-(1-7) were administered together, two osmotic minipumps were implanted.

Blood pressure measurements

Systolic blood pressure was measured in conscious mice by tail-cuff plethysmography (BP-98A; Softron Co.). For habituation, mice were trained daily for 5 days. After training period, 10 measurements per mouse were recorded daily for 5 days, 1 week before and 6 weeks after minipump implantation respectively.

Preparation of isolated perfused kidneys from mice

Eighteen-week-old apoE (–/–) mice and their wild-type littermates were anesthetized i.p. with ketamine (100 $mg \cdot kg^{-1}$) and xylazine (5 $mg \cdot kg^{-1}$). Kidneys were isolated under a microscope (Olympus CO11) and perfused with Krebs–Henseleit buffer according to an amended method described previously (Stegbauer *et al.*, 2005). Changes in perfusion pressure reflected changes in vascular resistance of renal resistance vessels.

Immediately after preparation, a bolus injection of 60 mM KCl was delivered to test the viability of the preparation followed by a stabilization period of 30 min. After the stabilization period, renal vasoconstriction was induced by noradrenaline (1 μM; Sigma-Aldrich) and concentration–response curves of the vasodilators carbachol (Sigma-Aldrich) in the presence or absence of Tempol (1 mM; Sigma-Aldrich) and S-nitrosoglutathione (GSNO, Alexis Corp.) were assessed. Vasodilation induced by GSNO was recorded in the presence of L-N^G-nitro-arginine methyl ester (L-NAME; 0.3 mM; Sigma-Aldrich) and diclofenac (3 μM). Renal relaxation was calculated as percentage of reduction in the precontracted kidneys which was set as 100%.

Measurement of H_2O_2

Isolation of preglomerular vessels does not yield enough tissue for reliable measurement of H_2O_2 production. Thus, H_2O_2 production of renal cortex including preglomerular vessels was measured with the Amplex Red H_2O_2/Peroxidase Assay Kit (Molecular Probes) in triplicates. Kidneys were perfused with cold Krebs–Henseleit buffer. After removing the renal capsule, renal cortex was cut into three 2 mm cubes and placed into warm buffer (37°C) and incubated in the reaction mixture for 1 h at 37°C in the dark. The supernatant was then

read in a fluorescence spectrophotometer (Cary eclipse, Varian Inc.). The fluorescent values were normalized to the protein content measured in the samples.

Determination of cGMP content in renal cortical slices

Renal cortical slices (250 µm) were cut with a vibratome (NVSLM1 from WPI) and equilibrated for 10 min in tempered (37°C), oxygenated (with 95% O_2, 5% CO_2) Krebs–Henseleit buffer. Slices were incubated with the non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 300 µM; Sigma Aldrich) for 10 min at 37°C. Subsequently, in the presence of IBMX the slices were incubated either with carbachol (100 µM; Sigma-Aldrich) or with diethylamine nitric oxide (DEA-NO) (100 µM; Alexis) for an additional 3 min. To extract cGMP, slices were frozen in liquid nitrogen, homogenized in 70% (v/v) ice-cold ethanol using a glass/glass homogenizer and then centrifuged (14 000x *g*, 15 min, 4°C). Supernatants were dried at 95°C and the cGMP content was measured by RIA (Brooker *et al.*, 1979). Preparation of tracer, acetylation of samples and standards and incubation with antibody were performed as described. In order to standardize the different samples, protein pellets were dissolved in 0.1 M NaOH/0.1% SDS and protein content was determined using the bicinchoninic acid method (Uptima).

Measurement of urinary nitrite/nitrate concentrations (Griess assay)

Twenty-four-hour urine samples were collected in metabolic cages at the end of the experimental period. Urinary concentrations of nitrite (NO_2) and nitrate (NO_3) were measured using a colorimetric assay kit (Cayman Chemical Company).

Measurement of urinary 8-isoprostane concentration

Twenty-four-hour urine samples were collected in metabolic cages at the end of the experimental period. Urinary concentrations of 8-isoprostane were measured using a colorimetric assay kit (Cayman Chemical Company) and normalized to urinary creatinine.

Isolation of preglomerular vessels

Preglomerular vessels, containing mainly interlobular arteries and afferent arterioles, were isolated by a modified iron oxide-sieving technique as described previously (Patzak *et al.*, 2008). As minor modifications, the kidneys were perfused via cannulation of the aorta, smaller needles (G20, G23) and pore sieves (100 µm) were used for tissue separation and separation of renal particles respectively.

Quantitative real-time PCR

Preglomerular vessels were used to study the expression of catalase and endothelial NO synthase (eNOS) and of the NAPH proteins, Nox1, gp91phox (Nox2), Nox4, p22pox and p47phox. After homogenization of isolated vessels with a Tissue Ruptor (Qiagen, Germany), total RNA was isolated using a RNA Micro Kit (Qiagen, Germany) according to the manufacturer's instructions. Quantitative real-time PCR was performed with an ABI PRISM 7300 (Applied Biosystem, Germany) and the SYBR Green master mix (Qiagen, Germany). The PCR reaction was performed in a total volume of 20 µL with 1 µL cDNA corresponding to 100 ng RNA as template and 1 pmol·µL^{-1} of each primer [Nox1 NM_172203, Nox2 NM_007807, Nox4 NM_015760, p22phox NM_007806, p47phox NM_010876, catalase NM_009804, eNOS NM_008713, Mas NM_008552; Qiagen, Germany and AT_{1A} receptor (forward 5′-GCTTGGTGGTGATCGTCACC-3′ and reverse 5′-GGGCGAGATTTAGAAGAACG-3′)]. The two-step PCR conditions were 2 min at 50°C, 15 min at 95°C, followed by 40 cycles (denaturation of 94°C for 15 s; annealing 55°C 30 s and extension at 72°C for 34 s). Experiments were performed in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH NM_008084) was chosen as the endogenous control (housekeeping gene). The levels of Nox1, Gp91phox, Nox4, p22pox, p47phox, catalase, eNOS, AT_{1A} and Mas cDNA were normalized to GAPDH by the ΔC_T method.

Immunoblotting for eNOS and phospho-eNOS

Renal cortex tissue was placed into ice-cold protein lysis buffer (600 µL EMPIGENE, 10 mL phosphate buffered saline, 44 nM phenylmethanesulphonylfluoride) containing a protease inhibitor cocktail (Sigma Aldrich) and immediately homogenized. Lysates were centrifuged at 4000 g for 10 min at 4°C. Protein concentrations from the supernatant were determined by a Bradford assay (Bioassay Systems). After treatment with dithiothreitol (100 mM) and denaturation (5 min at 95°C), 30 µg of total protein were loaded onto 8% SDS-PAGE gels and then transferred to nitrocellulose membranes according to manufacturer's instructions (X-Cell Blot Module, Invitrogen). Membranes were blocked in blocking buffer (5% BSA, and 0.1% tween 20 in PBS) for 1 h at room temperature and then incubated either with primary monoclonal mouse anti-eNOS antibody (1:3000) (BD Transduction Laboratories, material number 610297) or with primary monoclonal mouse Anti-phospho-eNOS (phospho-Ser^{1177}) antibody (1:1000) (BD Transduction Laboratories, material number 612392), and mouse anti-β-actin (1:5000) (Sigma Aldrich, St. Luis, MO, USA) over-night. Bound primary antibody was detected with anti-mouse HRP conjugated secondary antibody (1:10 000) (Dako, Germany) by 60 min incubation at room temperature. Antibody labelling was visualized by the addition of a chemiluminescence reagent. Chemiluminescence was visualized using a FluorChem FC2 Imager (Alpha Innotec, San Leandro, CA, USA). Immunoblots from each tissue were performed in triplicates.

Statistical analysis

Data are expressed as mean ± SEM (n = number of animals). Differences between dose–response curves were analysed by one-way ANOVA for repeated measurements, followed by unpaired Student's *t*-test. Statistical analyses of data not normally distributed were analysed by the Kruskal–Wallis test followed by Mann–Whitney *U*-test. Probability levels of $P < 0.05$ were considered statistically significant. The number of experiments indicates the number of mice.

Results

Six-week-old apoE (–/–) and wild-type mice were fed with a lipid-rich Western diet for the present study. After 6 weeks,

Table 1

Systemic blood pressure and circulating lipids in mice

	ApoE (+/+)	ApoE (–/–)	ApoE (–/–) + Ang-(1-7)
Blood pressure (mmHg)	117.8 ± 3.4	114 ± 4.1	113 ± 6.5
Cholesterol (mmol·L^{-1})	1.6 ± 0.3	13.7 ± 4.3**	15.7 ± 4.0**
Triglycerides (mmol·L^{-1})	0.6 ± 0.1	1.3 ± 0.4*	1.4 ± 0.5*
HDL (mmol·L^{-1})	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
LDL (direct) (mmol·L^{-1})	0.2 ± 0.1	6.3 ± 3.1***	6.4 ± 1.8***

Blood pressure was measured by tail-cuff and serum was collected for lipid analysis from six mice per group at the end of the study. Data are means ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001 different from values for apoE (+/+) mice: Student's t-test.
Ang, angiotensin; apoE, apolipoproteinE; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

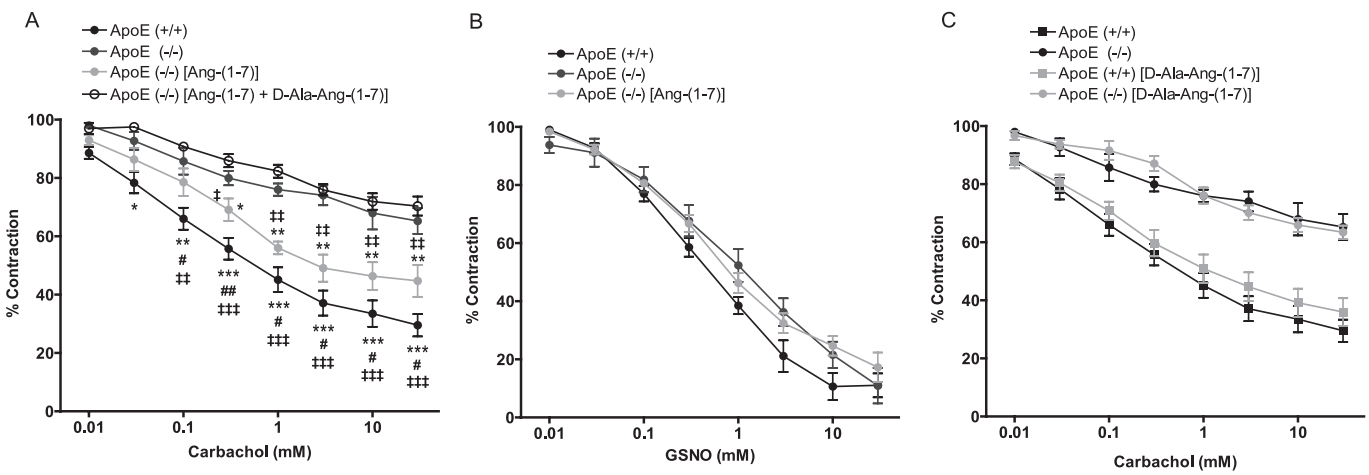

Figure 1

(A) The carbachol-induced endothelial-dependent vasorelaxation was impaired in isolated kidneys of apoE (–/–) mice (n = 7), compared with that in kidneys from apoE (+/+) mice (n = 6). Chronic Ang-(1-7) treatment (82 mg·kg^{-1}·h^{-1}) improved endothelium-dependent relaxation in kidneys of apoE (–/–) mice (n = 7). Combining Ang-(1-7) with D-Ala-Ang-(1-7) (125 mg·kg^{-1}·h^{-1}), a specific Mas receptor antagonist, attenuated the beneficial effects of Ang-(1-7) (n = 6). (B) Smooth muscle cell-dependent renal vasorelaxation, tested with the NO donor GSNO, did not differ between apoE (+/+) (n = 6), apoE (–/–) (n = 9) and Ang-(1-7)-treated apoE (–/–) (n = 9) mice. (C) D-Ala-Ang-(1-7) treatment alone showed no effects on carbachol-induced vasorelaxation in apoE (+/+) or apoE (–/–) mice. Data represent means ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001 versus apoE (–/–). #P < 0.05, ##P < 0.01 versus apoE (–/–) [Ang-(1-7)]. ‡P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.001 versus apoE (–/–) [Ang-(1-7) + D-Ala-Ang-(1-7)]. One-way ANOVA for repeated measurements followed by Student's t-test. Ang, angiotensin; apoE, apolipoproteinE; GSNO, S-nitrosoglutathione; NO, nitric oxide.

apoE (–/–) mice were divided into three groups and treated with either saline, Ang-(1-7) (82 μg·kg^{-1}·h^{-1}) or a combination of Ang-(1-7) (82 μg·kg^{-1}·h^{-1}) and the specific Mas receptor antagonist D-Ala-Ang-(1-7) (125 μg·kg^{-1}·h^{-1}) via osmotic minipump subcutaneously. Osmotic minipumps were replaced after 3 weeks. No differences were seen in blood pressure and lipid profiles between Ang-(1-7)-treated and untreated apoE (–/–) mice (Table 1).

Ang-(1-7) treatment improves endothelial-dependent renal vasorelaxation in apoE (–/–) mice through activation of Mas receptors

To evaluate the influence of Ang-(1-7) treatment on endothelial- and smooth muscle cell-dependent vasorelaxation in kidneys of apoE (–/–) mice, isolated kidneys were pre-constricted with noradrenaline (1 μM). Compared with apoE (+/+) mice, kidneys of apoE (–/–) showed a reduced endothelial-dependent vasorelaxation induced by carbachol (Figure 1A). Interestingly, chronic treatment with Ang-(1-7) improved endothelial-dependent renal vasorelaxation. This improved level of response was still less than that in the apoE (+/+) mice. To test whether the effect of chronic Ang-(1-7) treatment is restricted to the endothelium or in addition has an effect on muscle cell-dependent relaxation, the effects of GSNO, a NO donor, were assessed. GSNO-induced renal vasorelaxation did not differ between apoE (+/+), apoE (–/–) and Ang-(1-7)-treated apoE (–/–) mice (Figure 1B).

As Ang-(1-7) mediates its effects through Mas receptors, D-Ala-Ang-(1-7), a specific Mas receptor antagonist, was

tested. In kidneys of apoE (–/–) mice treated simultaneously with Ang-(1-7) and D-Ala-Ang-(1-7), endothelial-dependent vasorelaxation induced by carbachol was significantly reduced compared with apoE (–/–) mice treated only with Ang-(1-7). The dose–response curve was almost identical compared with untreated apoE (–/–) mice (Figure 1A). In contrast, treatment with the Mas receptor antagonist alone did not affect endothelial-dependent vasodilition in kidneys of apoE (+/+) or apoE (–/–) mice (Figure 1C).

In isolated preglomerular vessels, no differences of RNA expression levels of the Mas and AT_{1A} receptor were observed between apoE (+/+), apoE (–/–), Ang-(1-7)-treated apoE (+/+) and Ang-(1-7)-treated apoE (–/–) mice [Mas mRNA expression: apoE (+/+): 1.00 ± 0.10; apoE (–/–): 0.91 ± 0.08; apoE (–/–) + Ang-(1-7): 1.13 ± 0.06; apoE (+/+) + Ang-(1-7): 1.76 ± 0.76 arbitrary units; AT_{1A} mRNA expression apoE (+/+): 1.00 ± 0.15; apoE (–/–): 1.09 ± 0.13; apoE (–/–) + Ang-(1-7): 0.90 ± 0.07; apoE (+/+) + Ang-(1-7): 1.132 ± 0.12 arbitrary units).

Increased NO bioavailability in Ang-(1-7)-treated apoE (–/–) Mice

Endothelial dysfunction correlates closely with decreased NO bioavailability. Thus, we tested whether Ang-(1-7) treatment increased NO levels in apoE (–/–) mice. To prevent cGMP degradation, experiments were performed in the presence of IBMX, a non-specific phosphodiesterase inhibitor. Basal cGMP production was significantly increased in kidney slices from Ang-(1-7)-treated mice compared with those from saline-treated apoE (–/–) mice. In line with the results performed in isolated perfused kidneys, cGMP generation induced by carbachol was markedly increased in Ang-(1-7)-treated mice. Exogenous NO, provided by DEA-NO (100 μM), induced the same increases in cGMP generation in slices from Ang-(1-7)-treated as in slices from untreated mice, showing that the beneficial effects of Ang-(1-7) on renal vasorelaxation was restricted to the endothelium (Figure 2A). In addition, Ang-(1-7)-treated apoE (–/–) mice showed increased 24-hour urinary excretion rates of nitrate and nitrite compared with untreated apoE (–/–) mice (Figure 2B). Moreover, nitrate/nitrite urinary excretion rates were significantly greater in apoE (+/+) mice compared with the rates in Ang-(1-7)-treated and untreated apoE (–/–) mice. Finally, mRNA expression levels of eNOS, the main NO synthase in the renal vasculature, were measured and found to be significantly reduced in preglomerular arterioles of apoE (–/–) mice. Ang-(1-7) treatment slightly but significantly increased eNOS RNA expression (Figure 2C). These results were confirmed by measurement of total eNOS protein levels in the renal cortex of Ang-(1-7)-treated and saline-treated apoE (–/–) mice (Figure 2D). Interestingly, the ratio between expression levels of phospho-Ser^{1177}-eNOS, a marker for activated eNOS, and total eNOS were not significantly different between both groups (Figure 2D).

Chronic Ang-(1-7) treatment influences ROS production

It has been reported that production of ROS is increased in apoE (–/–) mice and responsible for endothelial dysfunction. To test if Ang-(1-7) mediated its effect by influencing ROS production, we used the ROS scavenger, tempol. In the presence of tempol (1 mM), endothelium-dependent vasorelaxation was significantly improved in kidneys of saline-treated apoE (–/–) mice (Figure 3A). This improvement was almost identical to that observed in Ang-(1-7)-treated mice. Interestingly, tempol had no additional effect on endothelial-dependent renal vasorelaxation in Ang-(1-7)-treated apoE (–/–) mice, providing the first evidence that Ang-(1-7) reduces ROS production.

To further support this observation, we measured H_2O_2 production in the renal cortex of apoE (+/+), apoE (–/–) and Ang-(1-7)-treated apoE (–/–) mice and urinary 8-isoprostane levels, a marker for oxidative stress. H_2O_2 production in the renal cortex as well as urinary 8-isoprostane levels were significantly increased in apoE (–/–) mice compared with apoE (+/+) mice and treatment with Ang-(1-7) attenuated H_2O_2 production and 8-isoprostane levels to baseline levels (Figure 3B).

Next, we analysed expression levels of enzymes involved in the metabolism of ROS. Expression levels of the NAD(P)H family differ between large conductance vessels and resistance arteries. Therefore, it was important to isolate preglomerular arterioles of mice kidneys. As the amount of isolated vessels is very limited, RNA instead of protein expression levels of Nox1, gp91phox, Nox 4, p22phox, p47phox and catalase, involved in the generation and catabolism of ROS, were measured by using quantitative real-time PCR. RNA levels of catalase which degrades H_2O_2 to H_2O were decreased in isolated preglomerular arterioles of apoE (–/–) compared with apoE (+/+) mice. However, treatment with Ang-(1-7) did not significantly increase catalase expression in isolated preglomerular arterioles of apoE (–/–) mice (Figure 4A). Next, we investigated whether expression levels of the NAD(P)H family involved in the generation of ROS were influenced by chronic Ang-(1-7) treatment. A significant increase in RNA expression levels was found for gp91phox and p47phox in preglomerular vessels of apoE (–/–) mice. Chronic treatment with Ang-(1-7) significantly reduced gp91phox and p47phox. However, gp91phox and p47phox expression levels were still increased compared with those in apoE (+/+) mice (Figure 4B). In contrast, RNA expression levels of Nox1, Nox4 and p22phox were unaltered in Ang-(1-7)-treated and untreated apoE (–/–) compared with apoE (+/+) mice (Figure 4C).

Discussion

Endothelial dysfunction is defined as decreased levels of NO, caused by either diminished NO generation or increased NO degradation and precedes vascular diseases at several sites of the vasculature, including kidney and heart. ApoE (–/–) mice fed a cholesterol- and lipid-rich diet develop endothelial dysfunction (Osada *et al.*, 2000; d'Uscio *et al.*, 2001; Laursen *et al.*, 2001). Despite the significantly impaired endothelium-dependent vasorelaxation observed in the renovascular bed of apoE (–/–) mice, we could not detect an increase in systemic blood pressure, relative to that of wild-type littermates. This, at first sight surprising finding, is in line with results obtained by others (Hartley *et al.*, 2000; Arruda *et al.*, 2005; Custodis *et al.*, 2008). The underlying compensatory mechanisms are still not fully understood but may be related to the genetic background (Rabelo *et al.*, 2008), the age of the

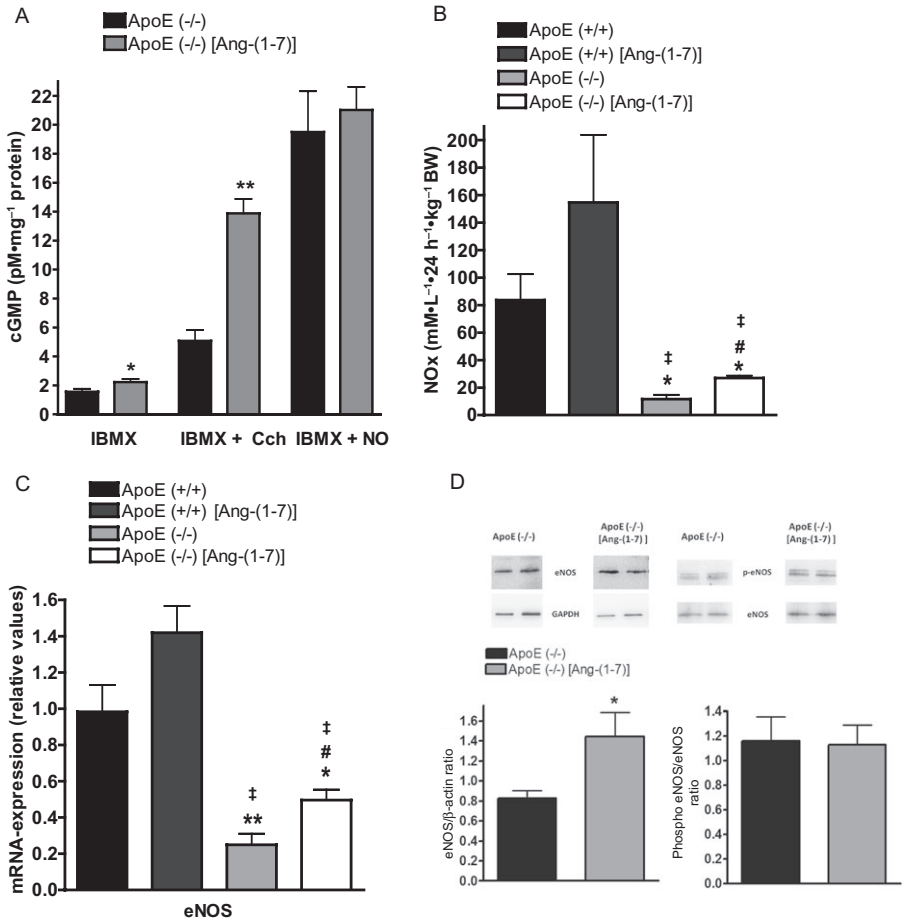

Figure 2

(A) cGMP production was measured, in the presence of IBMX, in cortical renal slices from apoE (–/–) mice, with or without Ang-(1-7) treatment. Basal and carbachol (CCh)-induced cGMP production was significantly increased in Ang-(1-7)-treated apoE (–/–) ($n = 4$) mice, compared with untreated apoE (–/–) mice ($n = 4$). Exogenous NO (IBMX + NO) was provided by DEA-NO (100 μM). Data represent means ± SEM; *$P < 0.05$, **$P < 0.01$ versus apoE (–/–), Student's *t*-test. (B) Urinary excretion (over 24 h) of nitric/nitrate (NO_x) in apoE (+/+) ($n = 14$), Ang-(1-7)-treated apoE (+/+) ($n = 8$), apoE (–/–) ($n = 7$) and Ang-(1-7)-treated apoE (–/–) ($n = 7$) mice. Ang-(1-7) treatment significantly increased NO_x excretion in apoE (–/–) mice. *$P < 0.05$ versus apoE (+/+), #$P < 0.01$, versus apoE (–/–). ‡$P < 0.05$ versus apoE (+/+) [Ang-(1-7)]. Kruskal–Wallis test followed by Mann–Whitney *U*-test. (C) Relative expression of eNOS mRNA, respectively, in relation to GAPDH mRNA in isolated preglomerular arteries of apoE (+/+) ($n = 12$), Ang-(1-7)-treated apoE (+/+) ($n = 4$), apoE (–/–) ($n = 9$) and Ang-(1-7)-treated apoE (–/–) ($n = 9$) measured by quantitative real-time PCR. *$P < 0.05$, **$P < 0.01$ versus apoE (+/+), #$P < 0.05$, versus apoE (–/–), ‡$P < 0.01$ versus apoE (+/+) [Ang-(1-7)]. Kruskal–Wallis test followed by Mann–Whitney *U*-test. (D) representative immunoblots of renal cortical eNOS and phosphorylated eNOS in samples from apo (–/–) and Ang-(1-7)-treated apoE (–/–) mice. Ang-(1-7) treatment significantly increased eNOS protein levels ($n = 4$), expressed as eNOS/β-actin levels, but did not change the ratio between phosphorylated eNOS and total eNOS levels in apoE (–/–) mice ($n = 4$). *$P < 0.05$ versus apoE (–/–). Kruskal–Wallis test followed by Mann–Whitney *U*-test. Ang, angiotensin; apoE, apolipoproteinE; IBMX, 3-isobutyl-1-methylxanthine; NO, nitric oxide.

animals or the tail-cuff method for measuring blood pressure which has its limitation in detecting small blood pressure differences.

The most striking finding of the present study was that chronic treatment with Ang-(1-7) restored endothelial function in isolated kidneys from apoE (–/–) mice. This effect seems to be exclusively mediated by activation of Mas receptors as D-Ala-Ang-(1-7), a specific Mas receptor antagonist, abolished the beneficial effect of Ang-(1-7) on endothelium-dependent vasorelaxation in kidneys of apoE (–/–) mice. This observation would discount effects of Ang-(1-7) on other possible targets such as ACE and the AT_1 receptor (Stegbauer *et al.*, 2003; Stegbauer *et al.*, 2004). Moreover, effects of Ang-(1-7) treatment on renal AT_{1A} receptor expression levels could also be excluded, although Mas receptor-deficient mice have increased renal AT_1 receptor expression levels (Pinheiro *et al.*, 2009). Additionally, chronic Ang-(1-7) treatment did not have any effects on blood pressure in apoE (–/–) mice and, in our study, we could not find any difference in blood pressure between any of the experimental groups. This observation is not surprising and might be related to the genetic background (Rabelo *et al.*, 2008). Furthermore, it should be noted that Ang-(1-7) seems to be effective in reducing blood pressures in animal models of severe hypertension (Rentzsch

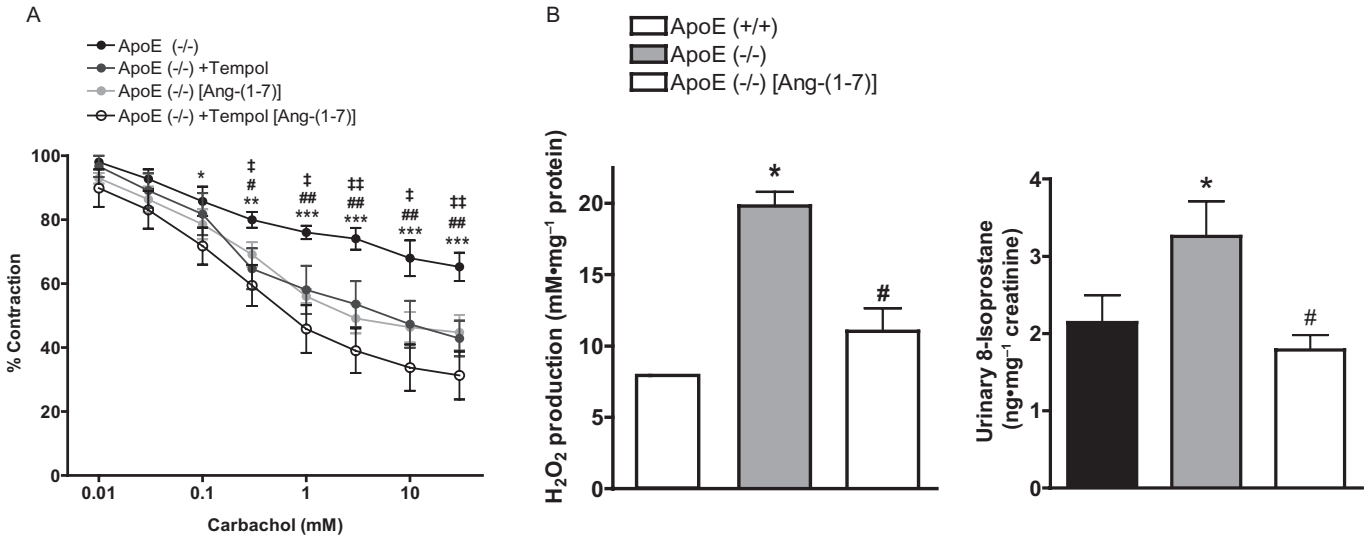

Figure 3

(A) carbachol-induced endothelial-dependent vasorelaxation was impaired in kidneys of apoE (–/–) mice (n = 7). In the presence of tempol (1 mM), endothelial-dependent vasorelaxation improved in kidneys of apoE (–/–) mice (n = 5). No additional effect of tempol on carbachol-induced vasorelaxation was observed in kidneys of Ang-(1-7)-treated apoE (–/–) mice (n = 6). Data represent means ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001 versus apoE (–/–) + tempol [Ang-(1-7)]. #P < 0.05, ##P < 0.01 versus apoE (–/–) [Ang-(1-7)]. ‡P < 0.05, ‡‡P < 0.01 versus apoE (–/–) + tempol. One-way ANOVA for repeated measurements followed by Student's t test. (B) H_2O_2 production in renal cortex of apoE (+/+) (n = 9), apoE (–/–) (n = 7) and Ang-(1-7)-treated apoE (–/–) (n = 9) was measured using the Amplex Red assay. H_2O_2 production was increased in renal cortex of apoE (–/–) compared with apoE (+/+) mice. Ang-(1-7) treatment significantly decreased H_2O_2 production in apoE (–/–) mice. The fluorescence values were normalized to protein content in the tissue probes. Urinary 8-isoprostane levels (over 24 h) were measured in apoE (+/+) (n = 16), apoE (–/–) (n = 10) and Ang-(1-7)-treated apoE (–/–) mice (n = 9). Urinary 8-isoprostane levels were increased in apoE (–/–) mice compared with apoE (+/+). Chronic Ang-(1-7) treatment reduced urinary 8-isoprostane levels significantly. Data represent means ± SEM; *P < 0.01 versus apoE (+/+), #P < 0.01 versus apoE (–/–). Kruskal–Wallis test followed by Mann–Whitney U-test. Ang, angiotensin; apoE, apolipoproteinE.

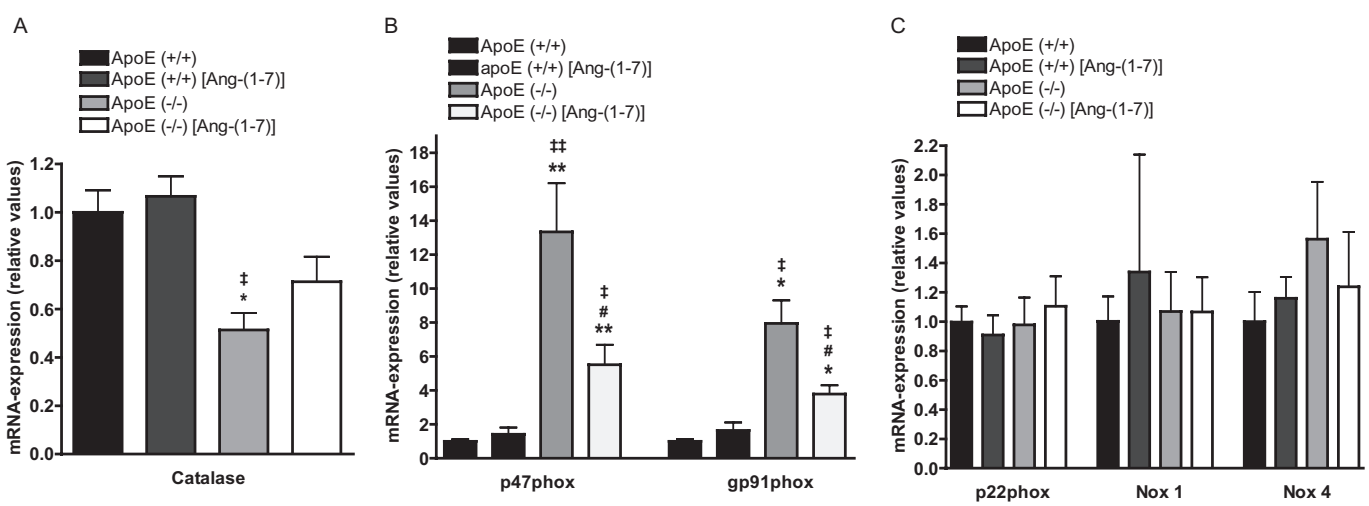

Figure 4

Relative expression of catalase (A), gp91phox (B), p47phox (B), Nox1 (C), Nox4 (C) and p22phox (C) mRNA, respectively, in relation to GAPDH mRNA in isolated preglomerular arteries of apoE (+/+) (n = 12), Ang-(1-7)-treated apoE (+/+) (n = 4), apoE (–/–) (n = 11) and Ang-(1-7)-treated apoE (–/–) (n = 11) measured by quantitative PCR. *P < 0.05, **P < 0.01 versus apoE (+/+). #P < 0.05 versus apoE (–/–), ‡P < 0.05, ‡P < 0.01 versus apoE (+/+) [Ang-(1-7)]. Kruskal–Wallis test followed by Mann–Whitney U-test. Ang, angiotensin; apoE, apolipoproteinE.

et al., 2008) but this effect appears to be limited in normotensive animals (Gomes *et al.*, 2010).

The beneficial effect of Ang-(1-7) treatment in our experiments seems to be restricted to endothelial cells, as a direct vasodilatory effect of Ang-(1-7) on smooth muscle cells could be ruled out as renal vasorelaxation induced by exogenous NO was similar in kidneys of wild type, apoE (–/–) and Ang-(1-7)-treated apoE (–/–) mice. In line with these results,

Lovren *et al*. (2008) demonstrated that ACE2 over-expression leading to increased Ang-(1-7) generation, protects the endothelium in apoE (–/–) mice and therefore attenuates the development of atherosclerosis. Additionally, improved endothelial function in Ang-(1-7)-treated apoE (–/–) seemed to be independent of lipid status, as no changes in the lipid profile were observed after Ang-(1-7) treatment in apoE (–/–) mice. However, Mas receptor-deficient mice on the FVB background do show increased serum triglyceride and cholesterol levels (Santos *et al*., 2008).

Endothelial dysfunction results from decreased levels of NO. Therefore, we investigated whether Ang-(1-7) increased NO production or decreased NO degradation. Renal basal cGMP levels as well as urinary excretion of NO metabolites were increased in Ang-(1-7)-treated mice, showing direct evidence for increased NO bioavailability after Ang-(1-7) treatment in apoE (–/–) mice. Recent studies showed that ROS are increased in the vascular bed of apoE (–/–) mice (Doran *et al*., 2007) and therefore are in part responsible for NO degradation. Tempol, a ROS scavenger, improved endothelium-dependent renal vasorelaxation in these mice. Interestingly, no additional effect of tempol was observed in Ang-(1-7)-treated mice, indicating that Ang-(1-7) administration similarly reduced ROS production (Polizio *et al*., 2007). However, tempol can decrease blood pressure and induce vasorelaxation in an O_2-/NO-independent mechanism (Xu *et al*., 2004). Thus, a direct impact of Ang-(1-7) treatment on ROS production was confirmed by H_2O_2 measurements in renal cortex, revealing a decreased H_2O_2 amount in Ang-(1-7)-treated mice compared with untreated apoE (–/–) mice. In line with these results, aortas of Mas receptor-deficient mice, which are characterized by pronounced endothelial dysfunction, showed increased ROS production (Peiro *et al*., 2007; Xu *et al*., 2008). ROS include superoxide anions, hydrogen peroxide or hydroxyl radicals, and they are generated by NAD(P)H oxidases which are detectable in all vascular layers. Membrane bound gp91phox (Nox2) and cytosolic p47phox NAD(P)H oxidase subunits were markedly increased in preglomerular arteries of apoE (–/–) mice and decreased by Ang-(1-7) treatment. However, there were no changes in Nox1, Nox4 and p22phox, under our conditions. Ang-(1-7)-mediated reduction of NAD(P)H activity and NAD(P)H oxidase expression was recently demonstrated in kidney homogenates of diabetic spontaneous hypertensive rats (Benter *et al*., 2008). Moreover, increased Gp91phox expression was also detected in isolated aortas of Mas receptor-deficient mice (Xu *et al*., 2008).

Beside an increased ROS production, ROS degradation seems to be diminished in apoE (–/–) mice (t Hoen *et al*., 2003). Thus, it has been shown that catalase expression, responsible for the conversion of H_2O_2 to H_2O, was decreased. In our experiments in preglomerular arteries, a reduced catalase expression was also detectable, but chronic Ang-(1-7) treatment did not significantly affect catalase expression levels. Hence, in apoE (–/–) mice, Ang-(1-7) seems to reduce ROS levels by decreasing expression of NAD(P)H subunits but not by increasing ROS clearance.

On the other hand, improved NO bioavailability observed after chronic Ang-(1-7) treatment could result not only from decreased NO degradation but also from increased NO synthesis compared with untreated apoE (–/–) mice. Therefore, we measured eNOS mRNA expression in preglomerular vessels. Endothelial NOS expression was decreased in apoE (–/–) mice and chronic administration of Ang-(1-7) led to a significant increase of eNOS mRNA expression in preglomerular vessels of apo (–/–) mice (Sampaio *et al*., 2007b). Concordant with mRNA measurements, chronic Ang-(1-7) treatment increased renal protein levels of total eNOS in apoE (–/–) mice. The present findings are consistent with findings in Mas receptor-deficient mice, showing decreased total eNOS expression levels. Moreover, activation of Mas receptors induced by Ang-(1-7) improved endothelial function through the facilitation of NO release *in vitro* (Peiro *et al*., 2007; Sampaio *et al*., 2007a). Interestingly, chronic Ang-(1-7) treatment did not influence the ratio between phosphorylated and total eNOS expression levels. These results indicate that chronic Ang-(1-7) treatment in apoE (–/–) mice increased total eNOS expression and, by the preservation of the ratio between phosphorylated and total eNOS, also increased the absolute amount of phosphorylated eNOS, leading to an increased NO/cGMP production in these mice. In contrast to the present study, Costa *et al*. found that acute Ang-(1-7) infusion increased both cardiac eNOS expression and the phosphorylation of eNOS in spontaneous hypertensive rats (Costa *et al*., 2010).

In summary, the present study clearly demonstrated that Ang-(1-7) application had a major effect on vascular function in a well-established mouse model of human cardiovascular disease. Ang-(1-7) increased NO bioavailability by sustained influence on the balance of NO generation and degradation. This beneficial action seems to be mediated by activation of Mas receptors. Thus, Ang-(1-7) may have the potential to be an effective therapeutic agent to reduce cardiovascular morbidity and mortality. Most likely, these effects are related to long-lasting beneficial effects on the endothelium rather than cute effects of Ang-(1-7) administration. The underlying molecular mechanism(s) of the Ang-(1-7)-mediated effects still remain to be established. Furthermore, it will be very interesting to investigate the effects of chronic Ang-(1-7) treatment on the progression of atherosclerosis in this animal model.

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Conflict of interest

None.

References

Alexander, SPH, Mathie, A, Peters, JA (2009). Guide to Receptors and Channels (GRAC), 4th Edition. Br J Pharmacol 158 (Suppl. 1): S1–254.

Arruda RM, Peotta VA, Meyrelles SS, Vasquez EC (2005). Evaluation of vascular function in apolipoprotein E knockout mice with angiotensin-dependent renovascular hypertension. Hypertension 46: 932–936.

Benter IF, Yousif MH, Dhaunsi GS, Kaur J, Chappell MC, Diz DI (2008). Angiotensin-(1-7) prevents activation of NADPH oxidase and renal vascular dysfunction in diabetic hypertensive rats. Am J Nephrol 28: 25–33.

Brooker G, Harper JF, Terasaki WL, Moylan RD (1979). Radioimmunoassay of cyclic AMP and cyclic GMP. Adv Cyclic Nucleotide Res 10: 1–33.

Cai H, Harrison DG (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 87: 840–844.

Castro CH, Santos RA, Ferreira AJ, Bader M, Alenina N, Almeida AP (2005). Evidence for a functional interaction of the angiotensin-(1-7) receptor Mas with AT1 and AT2 receptors in the mouse heart. Hypertension 46: 937–942.

Costa MA, Lopez Verrilli MA, Gomez KA, Nakagawa P, Pena C, Arranz C *et al.* (2010). Angiotensin-(1-7) upregulates cardiac nitric oxide synthase in spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 299: H1205–H1211.

Custodis F, Baumhakel M, Schlimmer N, List F, Gensch C, Bohm M *et al.* (2008). Heart rate reduction by ivabradine reduces oxidative stress, improves endothelial function, and prevents atherosclerosis in apolipoprotein E-deficient mice. Circulation 117: 2377–2387.

Doran DE, Weiss D, Zhang Y, Griendling KK, Taylor WR (2007). Differential effects of AT1 receptor and Ca2+ channel blockade on atherosclerosis, inflammatory gene expression, and production of reactive oxygen species. Atherosclerosis 195: 39–47.

d'Uscio LV, Baker TA, Mantilla CB, Smith L, Weiler D, Sieck GC *et al.* (2001). Mechanism of endothelial dysfunction in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 21: 1017–1022.

Faria-Silva R, Duarte FV, Santos RA (2005). Short-term angiotensin(1-7) receptor MAS stimulation improves endothelial function in normotensive rats. Hypertension 46: 948–952.

Ferrario CM, Chappell MC, Dean RH, Iyer SN (1998). Novel angiotensin peptides regulate blood pressure, endothelial function, and natriuresis. J Am Soc Nephrol 9: 1716–1722.

Gomes ER, Lara AA, Almeida PW, Guimaraes D, Resende RR, Campagnole-Santos MJ *et al.* (2010). Angiotensin-(1-7) prevents cardiomyocyte pathological remodeling through a nitric oxide/guanosine 3′,5′-cyclic monophosphate-dependent pathway. Hypertension 55: 153–160.

Grobe JL, Mecca AP, Lingis M, Shenoy V, Bolton TA, Machado JM *et al.* (2007). Prevention of angiotensin II-induced cardiac remodeling by angiotensin-(1-7). Am J Physiol Heart Circ Physiol 292: H736–H742.

Hartley CJ, Reddy AK, Madala S, Martin-McNulty B, Vergona R, Sullivan ME *et al.* (2000). Hemodynamic changes in apolipoprotein E-knockout mice. Am J Physiol Heart Circ Physiol 279: H2326–H2334.

t Hoen PA, Van der Lans CA, Van Eck M, Bijsterbosch MK, Van Berkel TJ, Twisk J (2003). Aorta of ApoE-deficient mice responds to atherogenic stimuli by a prelesional increase and subsequent decrease in the expression of antioxidant enzymes. Circ Res 93: 262–269.

Iusuf D, Henning RH, van Gilst WH, Roks AJ (2008). Angiotensin-(1-7): pharmacological properties and pharmacotherapeutic perspectives. Eur J Pharmacol 585: 303–312.

Kostenis E, Milligan G, Christopoulos A, Sanchez-Ferrer CF, Heringer-Walther S, Sexton PM *et al.* (2005). G-protein-coupled receptor Mas is a physiological antagonist of the angiotensin II type 1 receptor. Circulation 111: 1806–1813.

Laursen JB, Boesgaard S, Trautner S, Rubin I, Poulsen HE, Aldershvile J (2001). Endothelium-dependent vasorelaxation in inhibited by in vivo depletion of vascular thiol levels: role of endothelial nitric oxide synthase. Free Radic Res 35: 387–394.

Loot AE, Roks AJ, Henning RH, Tio RA, Suurmeijer AJ, Boomsma F *et al.* (2002). Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. Circulation 105: 1548–1550.

Lovren F, Pan Y, Quan A, Teoh H, Wang G, Shukla PC *et al.* (2008). Angiotensin converting enzyme-2 confers endothelial protection and attenuates atherosclerosis. Am J Physiol Heart Circ Physiol 295: H1377–H1384.

Mehta PK, Griendling KK (2007). Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am J Physiol Cell Physiol 292: C82–C97.

Mercure C, Yogi A, Callera GE, Aranha AB, Bader M, Ferreira AJ *et al.* (2008). Angiotensin(1-7) blunts hypertensive cardiac remodeling by a direct effect on the heart. Circ Res 103: 1319–1326.

Osada J, Joven J, Maeda N (2000). The value of apolipoprotein E knockout mice for studying the effects of dietary fat and cholesterol on atherogenesis. Curr Opin Lipidol 11: 25–29.

Patzak A, Steege A, Lai EY, Brinkmann JO, Kupsch E, Spielmann N *et al.* (2008). Angiotensin II response in afferent arterioles of mice lacking either the endothelial or neuronal isoform of nitric oxide synthase. Am J Physiol Regul Integr Comp Physiol 294: R429–R437.

Peiro C, Vallejo S, Gembardt F, Azcutia V, Heringer-Walther S, Rodriguez-Manas L *et al.* (2007). Endothelial dysfunction through genetic deletion or inhibition of the G protein-coupled receptor Mas: a new target to improve endothelial function. J Hypertens 25: 2421–2425.

Pinheiro SV, Ferreira AJ, Kitten GT, da Silveira KD, da Silva DA, Santos SH *et al.* (2009). Genetic deletion of the angiotensin-(1-7) receptor Mas leads to glomerular hyperfiltration and microalbuminuria. Kidney Int 75: 1184–1193.

Polizio AH, Gironacci MM, Tomaro ML, Pena C (2007). Angiotensin-(1-7) blocks the angiotensin II-stimulated superoxide production. Pharmacol Res 56: 86–90.

Rabelo LA, Xu P, Todiras M, Sampaio WO, Buttgereit J, Bader M *et al.* (2008). Ablation of angiotensin (1-7) receptor Mas in C57Bl/6 mice causes endothelial dysfunction. J Am Soc Hypertens 2: 418–424.

Rentzsch B, Todiras M, Iliescu R, Popova E, Campos LA, Oliveira ML *et al.* (2008). Transgenic angiotensin-converting enzyme 2 overexpression in vessels of SHRSP rats reduces blood pressure and improves endothelial function. Hypertension 52: 967–973.

Sampaio WO, Henrique de Castro C, Santos RA, Schiffrin EL, Touyz RM (2007a). Angiotensin-(1-7) counterregulates angiotensin II signaling in human endothelial cells. Hypertension 50: 1093–1098.

Sampaio WO, Souza dos Santos RA, Faria-Silva R, da Mata Machado LT, Schiffrin EL, Touyz RM (2007b). Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. Hypertension 49: 185–192.

Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I *et al*. (2003). Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc Natl Acad Sci U S A 100: 8258–8263.

Santos SH, Fernandes LR, Mario EG, Ferreira AV, Porto LC, Alvarez-Leite JI *et al*. (2008). Mas deficiency in FVB/N mice produces marked changes in lipid and glycemic metabolism. Diabetes 57: 340–347.

Stegbauer J, Vonend O, Oberhauser V, Rump LC (2003). Effects of angiotensin-(1-7) and other bioactive components of the renin-angiotensin system on vascular resistance and noradrenaline release in rat kidney. J Hypertens 21: 1391–1399.

Stegbauer J, Oberhauser V, Vonend O, Rump LC (2004). Angiotensin-(1-7) modulates vascular resistance and sympathetic neurotransmission in kidneys of spontaneously hypertensive rats. Cardiovasc Res 61: 352–359.

Stegbauer J, Vonend O, Habbel S, Quack I, Sellin L, Gross V *et al*. (2005). Angiotensin II modulates renal sympathetic neurotransmission through nitric oxide in AT2 receptor knockout mice. J Hypertens 23: 1691–1698.

Wassmann S, Czech T, van Eickels M, Fleming I, Bohm M, Nickenig G (2004). Inhibition of diet-induced atherosclerosis and endothelial dysfunction in apolipoprotein E/angiotensin II type 1A receptor double-knockout mice. Circulation 110: 3062–3067.

Xu H, Fink GD, Galligan JJ (2004). Tempol lowers blood pressure and sympathetic nerve activity but not vascular O2- in DOCA-salt rats. Hypertension 43: 329–334.

Xu P, Costa-Goncalves AC, Todiras M, Rabelo LA, Sampaio WO, Moura MM *et al*. (2008). Endothelial dysfunction and elevated blood pressure in MAS gene-deleted mice. Hypertension 51: 574–580.