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Vasoprotective and Atheroprotective Effects of Angiotensin (1-7) in Apolipoprotein E-Deficient Mice

Sonja Tesanovic, Antony Vinh, Tracey A. Gaspari, David Casley, Robert E. Widdop

Objective—To evaluate the effectiveness of long-term angiotensin (Ang) (1-7) treatment in inhibiting the proatherogenic effects of Ang for 16 weeks.

Methods and Results—Ang (1-7) is a heptapeptide fragment that has been proposed to counterregulate the Ang II proatherogenic effects. The effect of long-term 4-week Ang (1-7) treatment on both inhibition of atherosclerotic lesion development and improvement of endothelial function was examined in apolipoprotein E^{-/-} mice that had been fed an atherogenic high-fat (21%) diet for reversed with either angiotensin type 2 (AT₂) or *Mas* receptor blockade. In these vessels, Ang (1-7) treatment significantly decreased superoxide production and increased endothelial nitric oxide synthase immunoreactivity when compared with vehicle treatment. These effects were blocked by both AT₂ and *Mas* receptor antagonists. Lesion development, assessed as both fatty deposits (oil red O) and intima to media ratio, was also significantly decreased with Ang (1-7) treatment compared with respective controls. Cotreatment with either AT₂ or *Mas* receptor antagonists reversed Ang (1-7)-mediated reduction in lesion development.

Conclusion—Long-term Ang (1-7) treatment caused both vasoprotection, via improvement in endothelial function, and atheroprotection, with a reduction in lesion progression in a model of atherosclerosis. These effects appear to be mediated by the restoration of nitric oxide bioavailability and involve a complex interaction of both *Mas* and AT₂ receptors. (*Arterioscler Thromb Vasc Biol.* 2010;30:00-00.)

Key Words: atherosclerosis ■ endothelial dysfunction ■ angiotensin (1-7) ■ angiotensin receptors ■ nitric oxide

The renin-angiotensin (Ang) system is a major player in atherogenesis, as evidenced by the fact that the main hormone of the renin-Ang system, Ang II, is 1 of the key factors contributing to the development of atherosclerosis.¹⁻³ Atherosclerosis itself is a complex and incompletely understood progressive disease of the large arteries, characterized by endothelial dysfunction, vascular inflammation, and the accumulation of lipids and cellular debris within the intima of the vessel wall, resulting in arterial stiffness.⁴⁻⁶

Ang II is well-known to exert its physiological functions via 2 receptor subtypes, the angiotensin type 1 receptor (AT₁R) and the angiotensin type 2 receptor (AT₂R). It is widely accepted that the AT₁R accounts for most cardiovascular effects evoked by Ang II, such as vasoconstriction, stimulation of NADPH oxidase in vascular smooth muscle and endothelial cells, cell proliferation, and growth-promoting effects. In contrast, activation of the AT₂R generally produces counterregulatory effects that are mediated by the AT₁R, including vasodilation, apoptosis, and inhibitory proliferation of smooth muscle cells.^{7,8} It is known that upregulation of the AT₂R occurs under certain pathological conditions, such as heart failure, hypertension, vascular injury, atherosclerosis, and aging,⁹⁻¹³ indicating a potential role for this receptor in cardiovascular disease.

There is evidence that other Ang peptide fragments may bind to non-AT₁R-binding sites to produce effects that

counterregulate those mediated by the AT₁R. One such peptide fragment is the heptapeptide Ang (1-7), which may contribute to the antiatherogenic effects mediated by AT₁R antagonists and angiotensin-converting enzyme (ACE) inhibitors, with Ang (1-7) plasma levels reported to increase in the presence of these drugs.¹⁴⁻¹⁷ Formed from either Ang 1 or Ang 2 through endopeptidases and carboxypeptidase P, and via ACE2, a homolog of ACE,^{18,19} Ang (1-7) inhibits neointimal formation in stent-implanted rats,²⁰ improves endothelial function in Wistar rats,²¹ and activates endothelial nitric oxide synthase (eNOS) in Chinese hamster ovary cells.²²

Recently, it has been suggested that Ang (1-7) mediates its effects by acting as an agonist at the Ang (1-7)/*Mas* receptor (*MasR*),²¹⁻²³ although other data suggest that Ang (1-7) may also act at the AT₂R²⁴ or by antagonizing the AT₁R.²⁵ These protective effects of Ang (1-7) appear to involve stimulation of NO, bradykinin, and prostaglandin I₂.^{21,22,24} However, the long-term effects of Ang (1-7) in the setting of atherosclerosis are largely unknown. Therefore, this study aimed to investigate the effect of long-term Ang (1-7) infusion on endothelial dysfunction and the development of atherosclerotic lesions using the apolipoprotein E-deficient (ApoE^{-/-}) mouse model of atherosclerosis.

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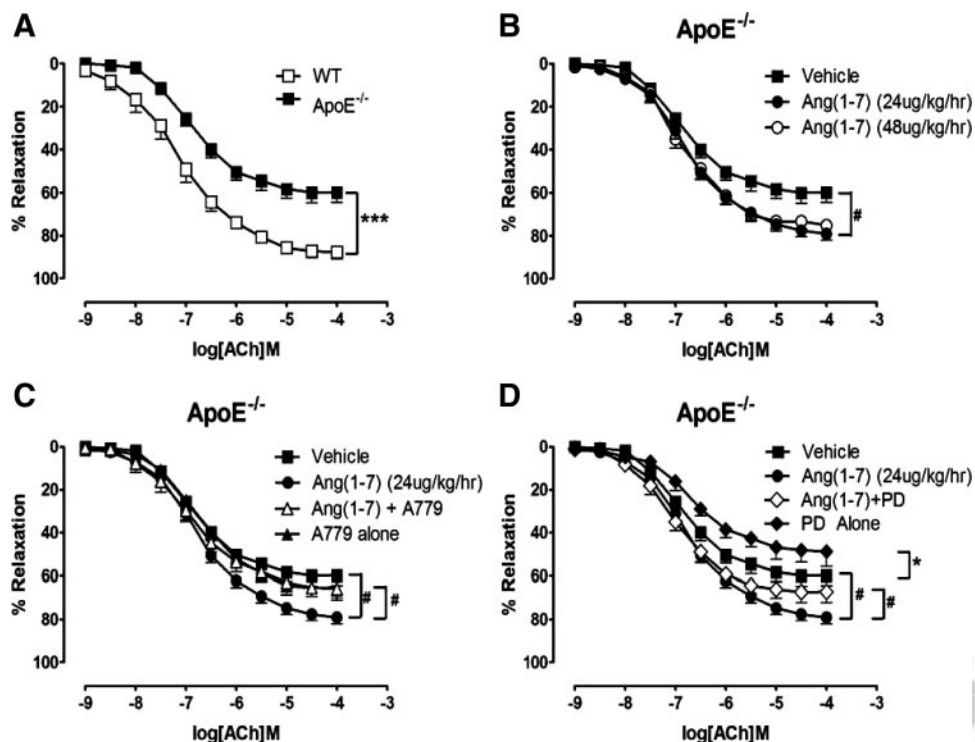


Figure 1. Concentration-response curve to the endothelium-dependent vasodilator ACh in abdominal aortic sections. A, Aorta from vehicle-treated ApoE^{-/-} mice (n=14) demonstrated significant endothelial dysfunction compared with aorta from vehicle-treated WT mice (n=6) (**P<0.001). B, Long-term Ang (1-7) treatment at both 24 μ g/kg (n=12) and 48 μ g/kg (n=8) per hour doses significantly improved endothelial function compared with vehicle-treated ApoE^{-/-} mice (n=14) (#P<0.01). C, The vasoprotective effect of 24 μ g/kg per hour of Ang (1-7) (n=12) was significantly attenuated in the presence of the MasR antagonist, A779 (n=9) at 48 μ g/kg per hour (#P<0.01). A779 alone (n=7) had no effect on endothelial function. D, The vasoprotective effect of 24 μ g/kg per hour of Ang (1-7) (n=12) was significantly attenuated in the presence of the AT₂R antagonist, PD123319 (n=9), at 10 mg/kg per day (#P<0.01). PD123319 alone (n=8) further exacerbated endothelial dysfunction (*P<0.05; all statistical testes were 2-way RM ANOVA).

Methods

Male B6 ApoE^{-/-} mice were obtained at the age of 5 weeks and weighed between 25 and 35 g when the experiments were performed. At the age of 6 to 8 weeks, the mice began receiving a high-fat diet containing 22% fat and 0.15% cholesterol for 12 weeks before treatment and during the subsequent 4-week treatment period. Mice were treated subcutaneously via osmotic minipumps with 1 of the following: vehicle (NaCl, 0.15 mol/L); Ang (1-7) alone (24 or 48 μ g/kg per hour); Ang (1-7) (24 μ g/kg per hour), in combination with either an AT₂R antagonist, PD123319 (10 mg/kg per day), or an MasR antagonist, A779 (48 μ g/kg per hour); or PD123319 (10 mg/kg per day) or A779 (48 μ g/kg per hour) alone. A group of age-/diet-controlled wild-type (WT) C57BL/6J mice were included and received vehicle treatment only.

Systolic blood pressure was measured by tail-cuff plethysmography. The heart and the whole aorta were removed, which was used to assess the effects of Ang (1-7) on vascular reactivity, atherosclerotic lesion development, and NO bioavailability (eNOS and superoxide). Detailed methods are described in the supplemental data (available online at <http://atvb.ahajournals.org>).

Data are given as mean \pm SD unless otherwise indicated.

Results

Ang (1-7) Improved Endothelial-Dependent Vasorelaxation in ApoE^{-/-} Mice

Systolic blood pressure was unaltered by any of the various treatment groups (supplemental Table I). Vehicle-treated ApoE^{-/-} mice demonstrated an attenuated maximal acetylcholine (ACh)-induced vasorelaxation response (R_{max} =59.80 \pm 4.55%; n=14) compared with vehicle-treated WT mice (R_{max} =87.87 \pm 7.44%;

n=6) (Figure 1A). Long-term infusion of Ang (1-7) in ApoE^{-/-} mice at both 24 μ g/kg per hour (R_{max} =79.15 \pm 2.90%; n=12) and 48 μ g/kg per hour (R_{max} =75.28 \pm 3.81%; n=8) significantly improved endothelial function to a similar extent when compared with vehicle-treated ApoE^{-/-} mice (Figure 1B); therefore, the results from the lower dose of Ang (1-7) (24 μ g/kg per hour) are reported. The improved endothelial response was significantly attenuated when ApoE^{-/-} mice were cotreated with the MasR antagonist, A779 (R_{max} =65.82 \pm 3.92%; n=9). Long-term A779 infusion alone had no effect on endothelial function (R_{max} =65.23 \pm 5.86%; n=7) (Figure 1C). Coinfusion of the AT₂R antagonist, PD123319, also significantly attenuated the Ang (1-7)-induced vasoprotective effect (R_{max} =67.19 \pm 4.88%; n=9). PD123319 treatment alone further exacerbated endothelial function (R_{max} =48.95 \pm 6.69%; n=8) (Figure 1D). Endothelium-independent vessel relaxation by the NO donor, SNP, remained unaltered in all the treatment groups (supplemental Table II).

In addition, the Ca²⁺ ionophore A23187-mediated vasorelaxation in the isolated aorta of Ang (1-7)-treated ApoE^{-/-} mice (R_{max} =80.98 \pm 6.89%; n=5) demonstrated similar responses to those seen with ACh-induced vasorelaxation, indicating that Ang (1-7) treatment significantly improved endothelial function via a mechanism independent of any effect on muscarinic receptors per se (supplemental Figure IA).

N^G-nitro-L-arginine methyl ester completely abolished ACh-mediated vascular relaxation (supplemental Figure IB)

in aorta taken from both vehicle-treated ($R_{\max}=0.57\pm 0.56\%$; $n=3$) and Ang (1-7)-treated ($R_{\max}=12.33\pm 7.34\%$; $n=5$) ApoE^{-/-} mice. In addition, short-term Ang (1-7) treatment of aortic rings taken from age-/diet-matched ApoE^{-/-} mice showed no improvement in ACh-mediated vasorelaxation ($R_{\max}=32.87\pm 6.42\%$; $n=3$) (supplemental Figure IC), unlike that seen with long-term Ang (1-7) treatment (Figure 1B and supplemental Figure IB). Ang (1-7) did not immediately relax isolated abdominal aortic rings (supplemental Figure IC).

Ang (1-7) Treatment Increased eNOS Immunoreactivity

eNOS expression in aortic cross sections of the thoracoabdominal aorta (the section of aorta immediately adjacent to the aortic sections, used for vascular reactivity studies) was examined using immunohistochemical methods in WT and ApoE^{-/-}-treated mice (Figure 2). eNOS immunoreactivity was significantly higher in vehicle-treated WT mice ($n=6$) compared with vehicle-treated ApoE^{-/-} mice ($n=12$). Long-term Ang (1-7) treatment ($n=6$) significantly increased eNOS immunoreactivity when compared with vehicle-treated ApoE^{-/-} mouse aorta (Figure 2A and B). Also, there was no difference in endothelial cell integrity as determined by von Willebrand factor staining ($n=4$) (Figure 2A). When Ang (1-7) was coinjected for a long time with either the AT₂R ($n=10$) or the MasR ($n=9$) antagonists, eNOS staining intensity was significantly attenuated. Neither PD123319 ($n=9$) nor A779 ($n=6$) had any effect on eNOS expression when used alone (Figure 2B).

Ang (1-7) Treatment Increased eNOS Protein Levels

When Western blot analysis is used, WT mice show greater immunoreactive bands for eNOS (approximately 140 kDa) ($n=3$) when compared with ApoE^{-/-} mice ($n=4$). However, long-term Ang (1-7) treatment in ApoE^{-/-} mice ($n=4$) significantly increased the density of the immunoreactive bands for eNOS when compared with vehicle treatment (Figure 2C), thus indicating a significant increase in eNOS protein expression compared with vehicle treatment (Figure 2C).

Ang (1-7) Decreased Superoxide Production

Superoxide was localized and quantified using dihydroethidium staining in the aortic cross sections from the thoracoabdominal region taken from ApoE^{-/-} mice from all long-term treatment groups and their age-/diet-matched WT control mice. Intracellular formation of superoxide attained by dihydroethidium oxidation is represented in red and is evident in the endothelium, media, and adventitia (Figure 3A). The intensity of fluorescence was quantified and expressed as total fluorescence intensity per area (arbitrary units per square micrometer). Superoxide levels were significantly lower in vehicle-treated WT mice ($n=7$) when compared with vehicle-treated ApoE^{-/-} mice ($n=7$). Ang (1-7) treatment in ApoE^{-/-} mice ($n=15$) significantly lowered the intensity of fluorescence when compared with vehicle-treated ApoE^{-/-} mice. This effect was reversed by cotreatment with either the AT₂R antagonist ($n=8$) or the MasR antagonist

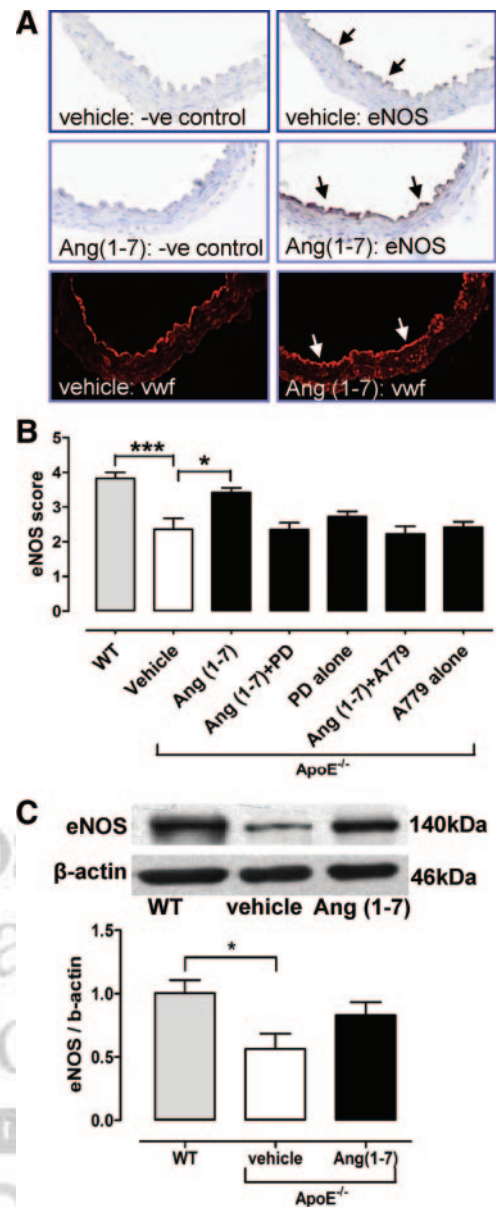


Figure 2. A, Immunohistochemical analysis of eNOS expression in the thoracoabdominal aortic cross sections taken from the area adjacent to aortic sections used for vascular function studies in ApoE^{-/-}-treated mice. In addition, analogous sections were also stained for von Willebrand factor (vwf) (representative of $n=4$ vehicle and $n=4$ Ang (1-7)-treated ApoE^{-/-} mice). The arrows represent positive staining for eNOS and vwf ($\times 20$ magnification). B, Mean data for eNOS intensity scores from the various treatment groups compared with vehicle-treated ApoE^{-/-} mice ($n=6$ to 12) ($*P<0.05$). C, Western blot of eNOS and β -actin probed in aortic homogenates taken from WT and ApoE^{-/-} mice that have been treated for a long time with either vehicle or Ang (1-7). The densities of the bands were quantified and expressed as a ratio of β -actin density ($*P<0.05$; $n=3$ to 4).

($n=8$). Neither PD123319 ($n=6$) nor A779 ($n=7$) had any effect on superoxide levels when used alone (Figure 3B).

Ang (1-7) Decreased Superoxide Basal Levels

The L-012 chemiluminescence method was also used to detect superoxide basal levels in abdominal aortic rings taken from ApoE^{-/-} mice. Long-term Ang (1-7) treatment ($n=7$)

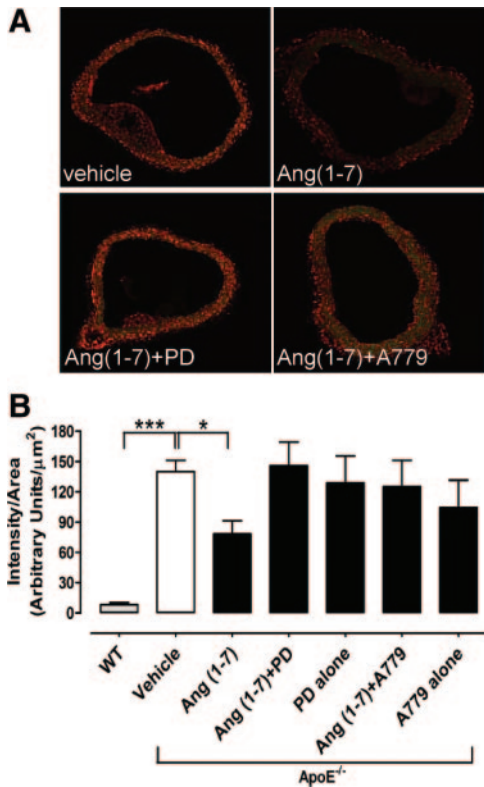


Figure 3. A, Dihydroethidium staining for superoxide in thoracoabdominal aortic sections taken adjacent to aorta used for vascular reactivity studies. Red staining represents 2-hydroxyethidium fluorescence and superoxide localization; and green staining, autofluorescence in elastin layers in the medial wall. Polyethylene glycol superoxide dismutase, 500 U/mL, was applied topically during the 30-minute incubation with dihydroethidium in an adjacent section; and abolished 2-hydroxyethidium fluorescence, confirming specificity of the fluorescent signal for superoxide (data not shown) ($\times 40$ magnification). B, Mean data ($n=7$ to 15) for superoxide quantification of fluorescence expressed as total fluorescence per area in various treatment groups compared with vehicle-treated ApoE^{-/-} mice ($*P<0.05$).

tended to lower superoxide levels when compared with vehicle-treated animals ($n=6$) (Figure 4A). Long-term coinfusion of Ang (1-7) with either the AT₂R ($n=8$) or the MasR ($n=8$) antagonist resulted in superoxide levels that were similar to the vehicle-treated group. To confirm the specificity and source of superoxide, parallel aortic rings were incubated with either superoxide dismutase, 300 U/mL, or the NADPH oxidase inhibitor, diphenyliodonium, 10 μ mol/L, for a short time. This incubation attenuated the signal in tissues from all treatment groups, indicating that the main source of superoxide was the enzyme NADPH oxidase (Figure 4B and C).

Ang (1-7) Decreased En Face Lesion Development

The aortic arch and the thoracic aorta were longitudinally cut open and stained en face with oil red O for lipid deposition, after which the lesions were measured as percentage lesion area. WT mice had no lesions; thus, the data were not included. Long-term Ang (1-7) treatment significantly decreased longitudinal lesion development in ApoE^{-/-} mice (lesion area, $15.33 \pm 1.13\%$; $n=12$) compared with vehicle-treated ApoE^{-/-} mice (lesion area, $28.62 \pm 2.83\%$; $n=12$) (Figure 5A and C).

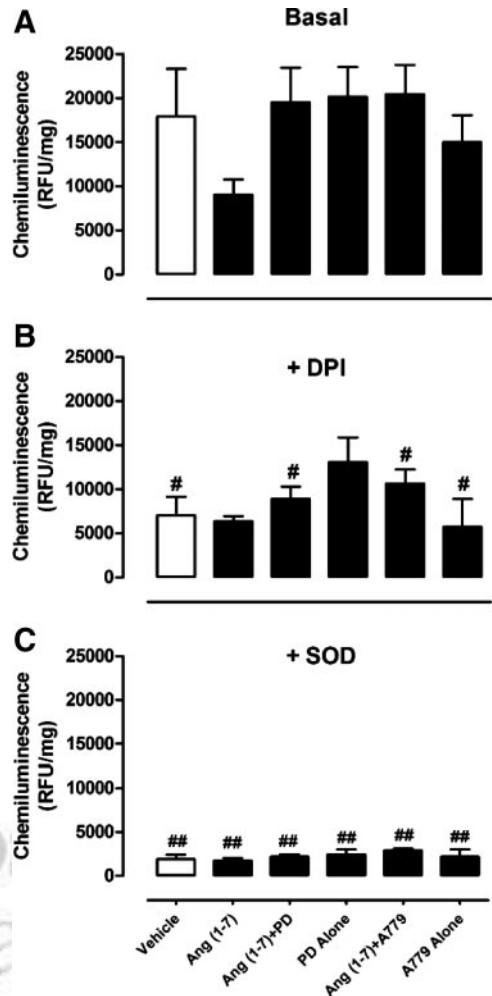


Figure 4. L-012 chemiluminescence was used to detect superoxide levels in abdominal aortic rings from ApoE^{-/-} treated mice ($n=6$ to 8). A, Histogram representing mean values for basal superoxide levels obtained from groups treated for a long time, as indicated. B and C, In all of the groups treated for a long time, superoxide levels were measured in parallel tissues that were incubated for only 30 minutes with either the NADPH oxidase inhibitor, diphenyliodonium, 10 μ mol/L (B), or superoxide dismutase, 300 U/mL (C) ($\#P<0.05$ and $\#\#P<0.01$ vs the corresponding value from the basal count).

When Ang (1-7) was coinfused with either the AT₂R antagonist (lesion area, $20.82 \pm 1.82\%$; $n=9$) or the MasR antagonist (lesion area, $22.91 \pm 1.09\%$; $n=9$), the antiatherogenic effect of Ang (1-7) was attenuated. Neither the AT₂R antagonist (lesion area, $24.01 \pm 1.07\%$; $n=6$) nor the MasR antagonist (lesion area, $22.11 \pm 2.22\%$; $n=6$) had any effect on lesion development when infused for a long time (Figure 5A and C).

Ang (1-7) Decreased Cross-Sectional Lesion Development

WT mice had no lesions present; therefore, data were not included. Long-term Ang (1-7) treatment significantly decreased cross-sectional lesion development (intima to media ratio [IMR], 0.45 ± 0.05 ; $n=12$) in ApoE^{-/-} mice when compared with vehicle-treated ApoE^{-/-} mice (IMR, 1.19 ± 0.17 ; $n=8$; Figure 5B and D), which is consistent with the longitudinal lesion results obtained using the oil red O

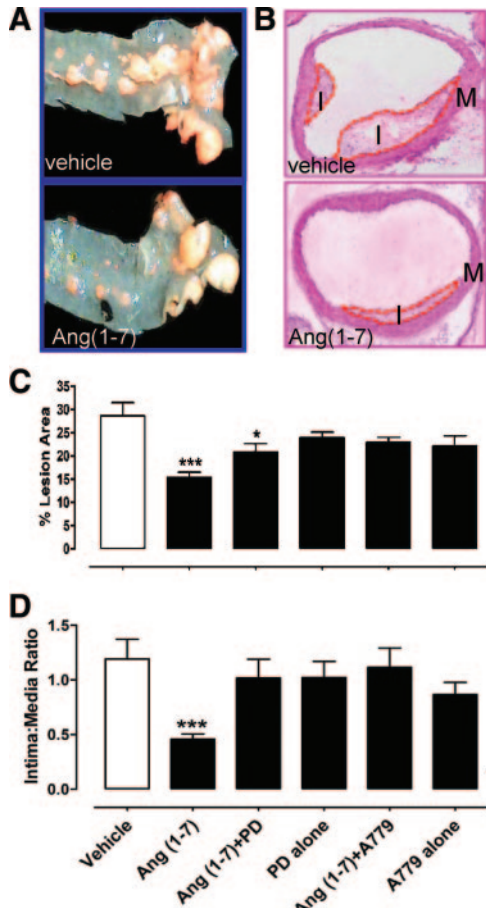


Figure 5. A and B, Representative photographs of en face oil red O staining of the aortic arch and thoracic aorta ($\times 10$ magnification) (A) and cross-sectional hematoxylin-eosin staining of the aortic arch from a vehicle- and Ang (1-7)-treated ApoE^{-/-} mouse (B). The intima (I) and media (M) areas (border identified by a dotted line) were represented as IMR ($\times 20$ magnification). C, Histogram comparing percentage lesion area from all treatment groups (n=6 to 12). D, Histogram representing mean IMR data from all treatment groups (n=6 to 12) (* $P < 0.05$ and *** $P < 0.001$ vs vehicle).

method. This antiatherogenic effect of Ang (1-7) was reversed by cotreatment with either the AT₂R antagonist (IMR, 1.02 ± 0.17 ; n=10) or the MasR antagonist (IMR, 1.11 ± 0.18 ; n=6) (Figure 5B and D). Again, neither antagonist had any effect on lesion development when infused alone.

Lipid Analysis

ApoE^{-/-} mice (n=5) eating a high-fat diet exhibited significantly elevated plasma levels of total cholesterol when compared with either WT mice eating a normal diet (n=6) or a high-fat diet (n=6) at the same age. In ApoE^{-/-} mice, long-term Ang (1-7) treatment (n=5) had no effect on these lipid levels when compared with vehicle treatment (n=5) (supplemental Table III).

The plasma Ang II and Ang (1-7) levels were unchanged after long-term Ang (1-7) (24 $\mu\text{g}/\text{kg}$ per hour) treatment (supplemental Table IV).

Discussion

To our knowledge, we demonstrated for the first time that long-term Ang (1-7) treatment produces vasoprotective and

atheroprotective effects in the ApoE^{-/-} mouse model of atherosclerosis. This was evidenced by improved endothelial function as the result of an increase in NO bioavailability as Ang (1-7) increased eNOS expression and protein levels and decreased superoxide levels; these results translated into antiatherosclerotic effects, mediated by both the Ang (1-7)/MasR and the AT₂R.

By using an infusion dose based on previous studies^{20,21,26,27} that showed Ang (1-7) improved endothelial function in other settings, we report that Ang (1-7) significantly improved endothelial function in ApoE^{-/-} mice when it was administered for a long time. Moreover, this effect was independent of agonist used to elicit endothelium-dependent relaxation (ACh or A23187), and long-term Ang (1-7) treatment was obligatory for improved endothelial function because short-term treatment with this peptide was ineffective. We performed these functional measurements using abdominal aortas because they were previously shown to exhibit greater endothelial dysfunction than thoracic aortas from ApoE-deficient mice.²⁸ However, we did not determine if similar Ang (1-7)-induced changes occur in other vascular regions.

The effects mediated by Ang (1-7) do not involve any direct effect on systolic blood pressure. Researchers^{10,29,30} have previously reported an improvement in endothelial function in ApoE^{-/-} mice with a variety of treatments that have not had any effect on systolic blood pressure; this may reflect that their normotensive status and basal blood pressure is less sensitive to vasodilator activity. Endothelial dysfunction is associated with the early stages of atherosclerosis and is considered an initiator of its progression.³¹ In this context, we have also demonstrated striking inhibition of further lesion development measured via 2 methods. Both lipid deposition and luminal encroachment were significantly inhibited, correlating with the reversal of endothelial dysfunction. This antiatherosclerotic effect of Ang (1-7) most likely reflects prevention of lesion development, which occurs over this time line, rather than regression of existing lesions; thus, proof of principle for an important role for Ang (1-7) in the setting of atherosclerosis exists.

One potential mechanism accounting for the observed effects is an increase in NO bioavailability. Long-term Ang (1-7) treatment increased eNOS protein levels and immunoreactivity and reduced superoxide production, thus suggesting an overall increase in NO bioavailability. To further support this hypothesis, the potentiation by Ang (1-7) of vascular relaxation was completely abolished in the presence of the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester, which is consistent with previous findings.^{24,32} More important, increased eNOS immunoreactivity was not the result of differences in endothelium integrity between treatments because staining using the endothelial cell marker von Willebrand factor was similar in untreated and Ang (1-7)-treated tissue; however, eNOS immunoreactivity was not performed in the same region as atherosclerosis measurements because it was considered more relevant to correlate changes in vascular function with changes in eNOS expression. The reduction in reactive oxygen species in long-term Ang (1-7)-treated ApoE^{-/-} mice (measured via both dihydroethidium staining

and lucigenin-enhanced chemiluminescence) appeared to be predominantly superoxide derived from NADPH oxidase because both superoxide dismutase and diphenyliodonium generally reduced basal levels. In *Mas*^{-/-} mice, there is increased blood pressure, evidence of endothelial dysfunction, and increased expression of Nox2, a subunit of the phagocyte-derived NADPH oxidase, when compared with WT mice.³³ This previous study³³ also showed reductions in superoxide dismutase and catalase activity, demonstrating impaired antioxidant ability in *Mas*^{-/-} mice. The current study indicates the functional importance of the *MasR* in the regulation of oxidative stress; future studies should be aimed at addressing this relationship, including whether Ang (1-7) counterregulates Ang II–driven superoxide production in this model.

There is much speculation as to which receptor Ang (1-7) activates to mediate its effects. Ang (1-7) has been reported to produce cardioprotective effects by binding to the AT₁R, the AT₂R,^{24,34–37} or both³²; and by inhibiting ACE.³⁸ However, with the discovery of the G-protein–coupled receptor *Mas*, suggested to be a functional Ang (1-7) receptor,²³ there are increasing studies showing that Ang (1-7) mediates its effects via the *MasR*.²³ Ang (1-7) has been shown to have a 13-fold greater binding affinity than Ang II for a non-AT₁R/AT₂R in bovine aortic endothelial cells, which was sensitive to blockade by the *MasR* antagonist, A779.³⁹ This was reflected in a recent study⁴⁰ in which Ang (1-7)–mediated relaxation was equally impaired in WT arteries both pretreated with A779 and isolated from *Mas*^{-/-} mice, indicating an *MasR*–sensitive effect. On the other hand, Ang (1-7) reportedly has a modest affinity for AT₂R_s in COS-7 cells.⁴¹

In the current study, we have shown that Ang (1-7) can act via both the AT₂R and the *MasR* because both PD123319 and A779 abrogated the Ang (1-7)–evoked vasoprotection and atheroprotection and the reciprocal changes in eNOS and superoxide.

Researchers^{24,42,43} have demonstrated that Ang (1-7) can mediate a number of functional responses via the AT₂R. In particular, we have demonstrated an AT₂R–sensitive, but not an *MasR*–sensitive, vasodepressor response to Ang (1-7) in conscious and spontaneously hypertensive rats.²⁴ Furthermore, Ang (1-7)–induced vasorelaxation in pig coronary arteries³⁵ and NO release in bovine aortic endothelial cells³² were attenuated by the addition of the AT₂R antagonist, PD123319. In rabbit vascular smooth muscle cells, Ang (1-7)–stimulated arachidonic acid release was partially attenuated by either A779 or PD123319 alone, but was abolished with combined AT₂R and *MasR* inhibition.⁴⁴ In addition, 2 independent studies,^{45,46} using either Ang (1-7) or the Ang (1-7) nonpeptide mimetic AVE 0991, demonstrated that Ang (1-7)–stimulated NO release in bovine aortic endothelial cells was more sensitive to AT₂R inhibition (approximately 90%) than *MasR* inhibition with A779 (approximately 50%), again implicating multiple receptor functions. Moreover, AT₁R blockade also caused partial inhibition of Ang (1-7)–mediated NO release.^{32,45} In this context, the *MasR* may act as a physiological antagonist of the AT₁R in mammalian transfected cells via hetero-oligomerization of the *MasR* and the AT₁R.²⁵

Thus, there is much precedence that Ang (1-7) is able to stimulate multiple Ang receptor subtypes, although the exact mechanism of how this occurs is not entirely clear. Interestingly, although Ang (1-7) appears to act via the *MasR* in many in vitro/ex vivo settings,²³ this selectivity is lost in vivo.⁴⁶ In the current study, PD123319 alone further exacerbated the endothelial dysfunction; although it did not modify eNOS. Thus, functional antagonism of AT₂R–mediated NO release may play a role in the attenuated effect of Ang (1-7) in the presence of the AT₂R antagonist. However, this possibility regarding endothelial dysfunction does not affect interpretation of PD123319 in subsequent experiments because it had no basal effect on all other indexes, which implies that endogenous activation of AT₂R was subthreshold.

Regarding the role of AT₂R in atherosclerosis, there are both positive and negative reports. AT₂R–mediated antiatherosclerotic effects have been reported because AT₂R^{-/-}/ApoE^{-/-} double-knockout mice eating a high-fat diet display exacerbated atherosclerotic lesions and/or lesion development.^{47,48} Conversely, there are also studies^{49–51} that have suggested no protective role for the AT₂R in atherosclerosis. Recently, Dandapat and colleagues⁵² reported that vascular overexpression of AT₂R in mice decreased atherosclerotic lesion formation by decreasing collagen accumulation and pro-oxidant signals. In the current study, it appears that Ang (1-7) is able to mediate at least some of its vasoprotective and atheroprotective effects via the AT₂R, thus suggesting that under some circumstances, Ang (1-7) may act as an endogenous AT₂R agonist, as previously reported.^{24,8} On the other hand, to our knowledge, the current study is the first demonstration of long-term Ang (1-7) treatment exerting atheroprotection in ApoE^{-/-} mice via the *MasR*, which is consistent with it inhibiting neointimal formation in stent-implanted rats²⁰ and improving endothelial function in Wistar rats.²¹ Interestingly, both AT₂R and *MasR* blockade attenuated the response mediated by Ang (1-7) to a similar extent. As already outlined, there are many instances^{32,34,36,45,46} of receptor promiscuity for Ang (1-7) in that this peptide was inhibited individually by either *MasR*, AT₂R, or AT₁R blockade or by combinations of these antagonists. On this latter point, the current study would suggest that the inhibition of either *MasR* or AT₂R was sufficient to dampen Ang (1-7) signaling, which implies some cross talk between these receptors, as previously noted.^{32,34,36,45,46} Alternatively, it is possible that AT₂R blockade by PD123319 has redirected endogenous Ang II toward further activation of the AT₁R, thus overcoming the protective effects mediated by Ang (1-7) acting at the *MasR*. Another possibility is that the effects of PD123319 were nonspecific against the *MasR*.

Researchers^{10,11,48,51,53} have demonstrated increases in AT₁R, AT₂R, and ACE2 immunoreactivity in atherosclerotic vessels. We confirmed these findings in the current study, as well as increased *MasR* immunoreactivity in thoracoabdominal aortic cross sections (data not shown). Given that ACE2 readily converts Ang II to Ang (1-7) and that there are increased tissue ACE2 levels, it is feasible that local production of Ang (1-7) contributes to the regulation of vascular disease. We did not measure tissue Ang (1-7) levels; basal

plasma Ang (1-7) levels were similar to those recently reported in rats, in which measurements occurred by the same method.⁵⁴ Plasma peptide levels in our study did not change after long-term subcutaneous Ang (1-7) treatment. In contrast, there was a marked elevation of the plasma Ang (1-7) level after intravenous administration in rats,²⁶ which suggests that route of administration and/or species may contribute to this discrepancy. Alternatively, unchanged plasma levels may reflect greater metabolism of infused peptides in mice than in rats. In any case, systemic Ang (1-7) may be atheroprotective by increasing local tissue levels of Ang (1-7); however, this theory awaits confirmation.

In conclusion, we have reported novel Ang (1-7)-evoked vasoprotection and antiatherosclerotic effects. These effects appear to be dependent on elevated NO bioavailability because eNOS protein levels and immunoreactivity were increased and superoxide levels were concomitantly reduced. To our knowledge, this is the first study presenting the atheroprotective effects of Ang (1-7) involving complex interactions between AT₂R and MasR, which have previously been reported.^{32,34,36,45,46} The increased NO bioavailability mediated by the AT₂R/MasR axis is an important therapeutic target for vasoprotection and atheroprotection.

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Disclosures

None.

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Supplement Material

VASO- AND ATHERO- PROTECTIVE EFFECTS OF ANGIOTENSIN (1-7) IN APOLIPOPROTEIN E-DEFICIENT MICE

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Methods

Animals

Male B6 Apolipoprotein E-deficient (ApoE^{-/-}) mice with a >99% C57BL/6J background were obtained from Animal Resources Centre (WA), and male C57BL/6J wild-type (WT) mice were obtained from Central Animal Services (Monash University). All mice were obtained at 5 weeks of age and weighed between 25-35g at time of experiments. At 6-8 weeks of age mice were transferred to a high fat diet (HFD) containing 22% fat and 0.15% cholesterol (Specialty Feeds, Western Australia) for a period of 12 weeks prior to, as well as during the subsequent 4 week treatment period. We performed animal experiments in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health and these were approved by the Monash University Animal Ethics Committee.

Blood Pressure Measurement

Systolic blood pressure was measured prior to and at the end of treatment using non-invasive tail-cuff plethysmography (ADInstruments, Sydney). Following treatment mice were euthanized and their aortas were collected for functional, histopathological and biochemical analysis.

Surgical Procedure

ApoE^{-/-} mice were anaesthetised using inhaled isoflurane and an incision made in the midscapular region through which mini osmotic pumps (Alzet model 2004, Alza Corp) were inserted for subcutaneous drug administration. Mice received one of the following treatments: vehicle (NaCl 0.15M); Ang (1-7) alone (24 or 48 µg/kg/hr; Auspep, Australia); Ang (1-7) (24µg/kg/hr) in combination with either an AT₂R antagonist, PD123319 (10mg/kg/day; a gift from Pfizer, USA), or a MasR antagonist, A779 (48µg/kg/hr; Auspep, Australia); PD123319 (10mg/kg/day) or A779 (48µg/kg/hr) alone. A group of age/diet-controlled WT C57BL/6J mice were included and received vehicle treatment only.

At the end of treatment, animals were anaesthetized by isoflurane inhalation and decapitated. Heart and the whole aorta were removed and placed in ice-cold Krebs-bicarbonate buffer (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, CaCl₂ 2.5, NaHCO₃ 25 and glucose 11.7. The connective tissue surrounding the aorta was carefully removed under a binocular dissection light microscope (Olympus SZ40) and the abdominal aorta was dissected for vascular reactivity studies, leaving the aortic arch and thoracic aorta attached to the heart.

Vascular Reactivity Studies

Four aortic rings per mouse (~3mm long) were obtained from the abdominal aorta and each ring was suspended between two stainless steel wires connected to an isometric force transducer (FT-03, Grass Instruments). Concentration responses curves to the

endothelium-dependent vasodilator acetylcholine (ACh) were constructed in tissues precontracted with the thromboxane A₂ analogue, U46619 ([1,5,5-hydroxy-11 α , 9 α -(epoxymethano)prosta-5Z, 13E-dienoic acid]), as previously described.¹ At the end of the acetylcholine curve, 10 μ M of the endothelium-independent vasodilator, sodium nitroprusside (SNP), was added to the bath to test vascular smooth muscle integrity.

In addition, concentration-response curves to the Ca²⁺-ionophore A23187 were performed to test endothelial function using a different endothelial dependent vasodilator agent.

Furthermore, concentration-response curves to Ang (1-7) were constructed to assess the direct vascular effect of Ang (1-7), as well as separate vehicle treated aortic rings incubated with either Ang (1-7) (10 μ M) or the NO synthase inhibitor N ω -nitro-L-arginine methyl ester (L-NAME; 10 μ M) for 20 mins, after which a concentration-response curve to ACh was constructed to assess the effect of acute Ang (1-7) treatment on endothelial function, and determine whether the effect is NO-mediated.

Localization of eNOS and Von Willebrand factor

Immunohistochemical analysis of eNOS was assessed as previously described,¹ using frozen 10 μ m aortic cross-sections taken from thoracoabdominal aorta immediately adjacent to segments used for vascular reactivity studies in WT and ApoE^{-/-} mice. The measurements of 2 sections (20 μ m apart) for each mouse were averaged and analysed. In brief, aortic sections were immunostained using a rabbit polyclonal anti-eNOS antibody (BD Biosciences) and the DAKO EnVision+ System (DAKO). Staining was analysed by two blinded observers who scored the intensity of positive staining using an arbitrary

grading system based on a scale of 1 to 5 (lowest to highest). Scores were averaged for each aortic section to allow comparison of eNOS levels between treatment groups.

Localization of Von Willebrand factor using immunofluorescence was performed in aortic sections of untreated ApoE^{-/-} mice, as well as chronically treated Ang (1-7) mice, to determine endothelial cell integrity. Sections were incubated overnight at 4°C with a 1:1000 dilution of rabbit polyclonal antibodies against von-Willebrand factor (AbCAM). Negative controls consisted of substitution of primary antibody with rabbit IgG immunoglobulin at the same protein concentration as that of the primary antibody. Following washing with Tris-buffer, sections were incubated with a 1:500 dilution of secondary goat-anti rabbit Alexa568 antibody (Invitrogen, USA) for 2.5 hrs at room temperature. After washing in Tris buffer, sections were mounted with anti-fade medium (Vectrosshield) and cover slipped. Slides were stored flat at 4°C until visualized on an Olympus Fluoview 500 confocal microscope equipped with a krypton/argon laser. Laser settings had an excitation and emission spectra of 488 and 610 nm, respectively.

Quantification of eNOS by western blotting

A separate set of chronically treated ApoE^{-/-} mice were used for western blot analysis. Whole mouse aorta was dissected free of connective tissue and homogenized in laemmli lysis buffer (5% glycerine, 2.5% mercaptoethanol, 1.5% SDS, 50mM Tris-HCl, pH=8.0, 0.05mg/ml bromphenol blue). Cell bodies were lysed and cells removed by centrifugation at 15000 g for 15 mins at 4°C. Proteins were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes, which were blocked in 5% skim milk powder

for 2 h at room temperature, and then incubated with a mouse monoclonal anti-eNOS antibody (BD Biosciences) at 1:2500 dilution overnight at -4°C. Membranes were washed in Tris buffered saline-tween (TBS-T) solution three times at 15 min intervals, and then incubated with a goat anti-mouse secondary antibody at 1:10000 dilution for 1 hr at room temperature. Membranes were then washed in TBS-T solution three times at 15 min intervals and immunoreactive bands were visualized by ECL (Amersham) and film exposure for 5 mins. Immunoreactive bands were quantified using chemiDoc XRS imager using Quantity One software (BioRad). Immunoblotting with a monoclonal anti- β -actin antibody (1:1000, Sigma) was performed to ensure equal protein loading.

Localization of Superoxide by Dihydroethidium Staining

Aortic cross-sections (30 μ m) were cryocut from the same segments as those used for immunohistochemistry as described above and were mounted on gelatin chromalumin coated slides. Dihydroethidium (DHE) (2 μ M) was used as previously described¹ to detect superoxide in frozen sections of both wild type and ApoE^{-/-} mouse aorta. PEG-SOD (500 U/mL) was applied topically during the 30-minute incubation with dihydroethidium in an adjacent section to confirm the specificity of the fluorescent signal for superoxide. Fluorescence of 2-hydroxyethidium, the specific product of the reaction, was imaged with an Olympus Fluoview 500 confocal microscope equipped with a krypton/argon laser. Laser settings were identical for each image acquired (excitation and emission spectra of 488 and 568 nm, respectively). Fluorescence was quantified using analySIS software (Soft Imaging System, Singapore) as previously described.¹

Superoxide Detection using L-012 Enhanced Chemiluminescence

In a separate set of chronically-treated ApoE^{-/-} mice, abdominal aortic rings (~2mm in length) dissected free of connective tissue, were used for L-012 enhanced chemiluminescence as previously described.¹ Aortic segments were incubated with either: control (krebs-HEPES) solution, superoxide dismutase (SOD, 300U/mL) or an NADPH oxidase inhibitor diphenyliodonium (DPI, 10µmol/L) in a 96 well plate. Each treatment group was performed in duplicate and photon emission was recorded for 20 cycles at 2 minute intervals. Photon emissions (relative light units/sec – RLU/s) were then averaged over the 20 cycles and normalized to the dry weight of the aortic ring.

Quantification of Atherosclerotic Lesion Development

Lesion development was measured using two methods: (1) cross sectionally-using frozen 10µm thick aortic sections that were taken at the aortic root on the proximal side of brachiocephalic artery and then stained with haematoxylin and eosin before the medial and neointimal areas were measured. The measurements of 5 sections (50µm apart) for each mouse were averaged and analysed before being expressed as an intima:media ratio (IMR), and (2) *en face* method- using oil red O staining for lipid deposition in the aorta, immediately distal to the brachiocephalic artery through to the thoracoabdominal aorta at the level of the diaphragm, as previously described,¹ given that this is the area of the aorta where the lesions are most advanced.²

Plasma Lipid Levels

Plasma samples (200µl) from ApoE^{-/-} mice chronically treated with either vehicle or Ang (1-7) (24µg/kg/hr), as well as plasma samples from C57BL/6J mice on both normal diet (ND) and HFD, were collected. Total cholesterol and triglyceride levels were measured as part of a Lipid Panel Analysis (Gribbles Veterinary Pathology Lab, Clayton, Victoria).

Plasma Ang II and Ang (1-7) Measurements

Plasma Ang II and Ang (1-7) levels were measured in ApoE^{-/-} mice chronically treated with either vehicle or Ang (1-7) (24µg/kg/hr) by radioimmunoassay (RIA) according to recently published methods.³ Briefly, antibodies used to measure Ang II and Ang (1-7) levels were raised in rabbit and guinea-pig, respectively, by immunizing animals against the natural sequences and then these were NH-terminally conjugated to either bovine (Ang II) or porcine (Ang (1-7)) thyroglobulin.

Angiotensin II was measured by direct RIA in plasma using delayed tracer addition. Plasma (100µL) was equilibrated with antibody raised in rabbit against angiotensin II n-terminally conjugated to bovine thyroglobulin for 20 hrs, 4°C, total volume 300µL. Monoiodinated ¹²⁵I- Angiotensin II tracer, 10 000 cpm in 100µL was added and allowed to equilibrate for a further 16 hrs at 4°C whereupon bound and free phase was separated using Dextran 10 coated charcoal and centrifugation. Sensitivity was 3.5 pg/mL. Intra and inter-assay variabilities were 6.4 and 12%. Cross reactivity to other angiotensins were:- Ang I = 0.52%, Ang (1-7) = 0.0128% and to all other pertinent hormones less than 0.1%. However, cross reactivity to Ang III and Ang IV are 98% and 100% as these peptides have the same c-terminal as Ang II.

Ang (1-7) was measured directly in an RIA using antibody raised in guineapig to Ang (1-7) n-terminally conjugated to porcine thyroglobulin and Ang (1-7) monoiodinated with ¹²⁵I. Antibody bound ¹²⁵I-Ang (1-7) was separated from free by Dextran 10 coated charcoal and counts free were compared to serially diluted standard amounts of Ang (1-7). Sensitivity was 14 pg/mL. Cross reactivity to Ang I, Ang II, Ang III and Ang IV were 0.108%, 0.036%, 0.526% and 0.025% respectively. Intra- and inter-assay variabilities were 4.5% and 10%.

Data Analysis

All results are expressed as mean \pm standard error of the mean (SEM). Statistical comparisons were by 1- or 2-way repeated measures ANOVA with Bonferroni corrections where appropriate using GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA). The non-parametric statistical Kruskal-Wallis test was used for analysis of the eNOS immunohistochemistry. $P < 0.05$ was deemed statistically significant.

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Supplementary data**Table I.** Systolic Blood Pressure (mmHg) in WT and ApoE^{-/-} mice before and after diverse treatments

Treatment	Before	After
WT +vehicle (n=6)	95±3	98±1
ApoE ^{-/-} + vehicle (n=14)	98±2	96±3
+ Ang (1-7) (24µg/kg/hr) (n=12)	99±3	100±4
+ Ang (1-7) (24µg/kg/hr) (n=8)	98±1	100±7
+ Ang (1-7)/PD123319 (n=9)	102±2	98±1
+ PD123319 (n=8)	97±2	98±2
+ Ang (1-7)/A779 (n=9)	102±1	102±1
+ A779 (n=7)	97±1	98±1

Supplementary data**Table II.** Endothelium-independent vessel relaxation induced by SNP in all treatment groups

Treatment	SNP Max % Relaxation
WT + vehicle (n=6)	95.01±1.72
ApoE ^{-/-} + vehicle (n=14)	90.76±2.78
+ Ang (1-7) (n=12)	93.98±5.76
+ Ang (1-7)/PD123319 (n=9)	96.53±2.39
+ PD123319 (n=8)	89.73±5.38
+ Ang (1-7)/A779 (n=9)	97.29±2.75
+ A779 (n=7)	94.29±4.78

Supplementary data**Table III.** Lipid Panel analysis (mmol/L) in WT and ApoE^{-/-} mice

Treatment	Total Cholesterol	Triglyceride Level
WT (ND) (n=6)	2.4±0.04	1.38±0.15
WT (HFD) (n=6)	5.7±0.33	0.72±0.05*
ApoE ^{-/-} + vehicle (n=5)	24.12±3.02** [#]	0.96±0.11
ApoE ^{-/-} + Ang (1-7) (n=5)	24.86±1.34** [#]	0.96±0.04

ND, normal diet; HFD, high fat diet; * $P < 0.01$; ** $P < 0.001$ vs WT (ND); # $P < 0.001$ vs WT (HFD).

Supplementary data**Table IV.** Plasma Ang II and Ang (1-7) levels (pg/mL) in ApoE^{-/-} mice

Treatment	Ang II (pg/mL)	Ang (1-7) (pg/mL)
Vehicle	370±128 (n=5)	84±35 (n=3)
Ang (1-7)	356±63 (n=8)	50±24 (n=4)

Supplementary Data
Fig. I

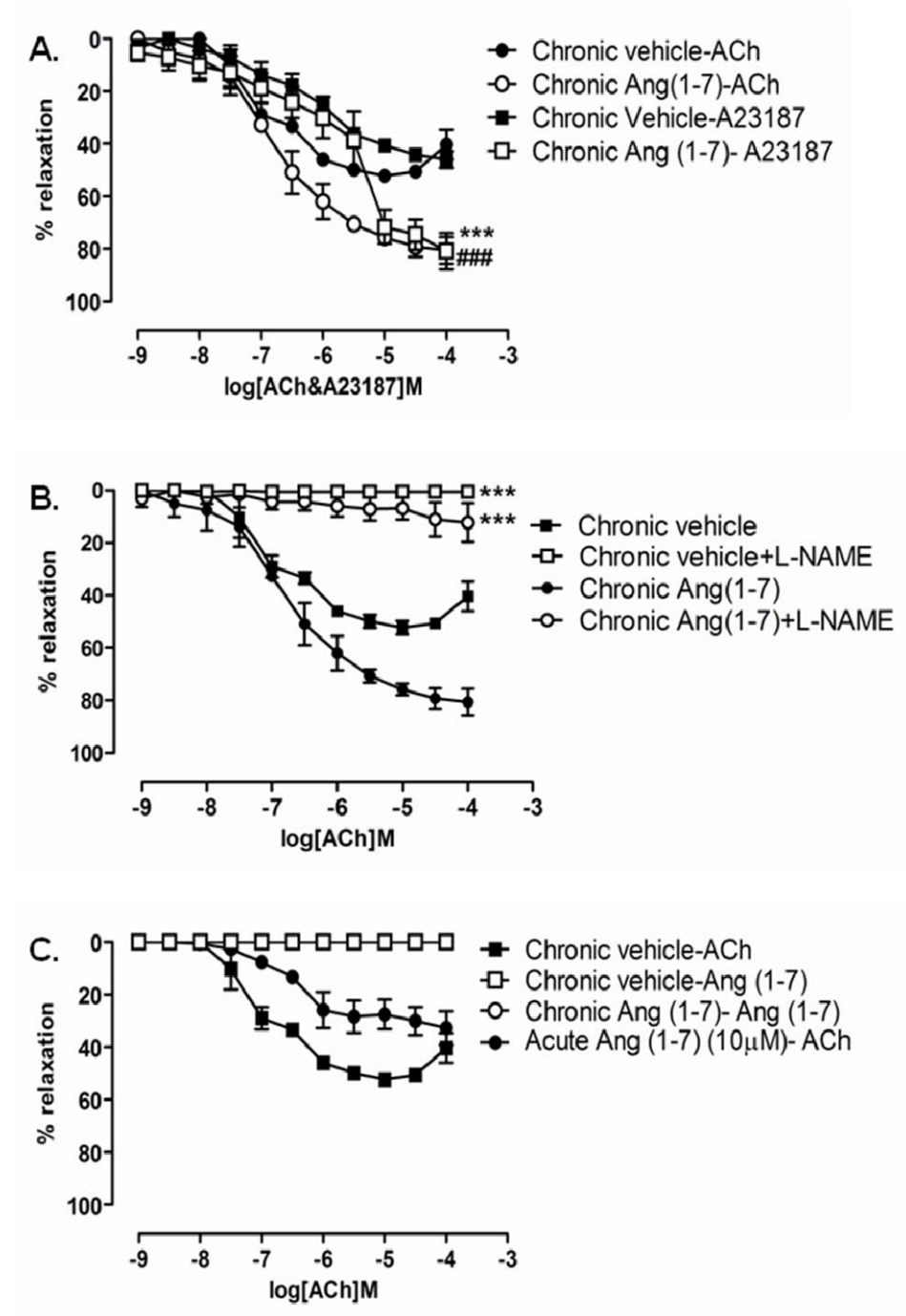


Figure I. **A)** Concentration-response curve to the endothelium-dependent vasodilators acetylcholine and Ca^{2+} ionophore A23187 in abdominal aorta taken from ApoE^{-/-} mice that received either vehicle ($n=3$) or Ang (1-7) ($n=5$) treatments chronically for 4 weeks. **A)** Chronic Ang (1-7) treatment significantly improved endothelial function compared to vehicle treatment when measured with both ACh and A23187; *** $P<0.001$ Ang (1-7) vs vehicle – ACh; ### $P<0.001$ Ang (1-7) vs vehicle – A23187 (two-way RM ANOVA). **B)** L-NAME significantly blocked ACh-induced vasorelaxation in both Ang (1-7) and vehicle treated ApoE^{-/-} mice; *** $P<0.001$ vehicle vs. vehicle/L-NAME; Ang (1-7) vs Ang (1-7)/L-NAME. **C)** Acute incubation with Ang (1-7) (10 μ M) did not improve ACh-induced vasorelaxation. Ang (1-7) does not induce vasorelaxation when used acutely, instead of ACh in groups chronically treated with either vehicle or Ang (1-7) (superimposed open squares and open circles).