Chronic angiotensin IV treatment reverses endothelial dysfunction in ApoE-deficient mice

Antony Vinh, Robert E. Widdop*, Grant R. Drummond, and Tracey A. Gaspari

Department of Pharmacology, Monash University, Building 13E, Clayton, Victoria 3800, Australia

Received 15 June 2007; revised 11 August 2007; accepted 3 September 2007; online publish-ahead-of-print 19 September 2007

Time for primary review 18 days

KEYWORDS
Angiotensin IV; AT4 receptor; AT2 receptor; Endothelial dysfunction; Nitric oxide bioavailability; Superoxide; Angiotensin II

Aims Endothelial dysfunction is considered a surrogate marker for cardiovascular disease. Angiotensin II, the principal hormone of the renin angiotensin system, is known to promote atherogenesis. However, other angiotensin peptide fragments such as angiotensin IV possess biological activity that may in fact counter-regulate the actions of angiotensin II. Therefore, we investigated the role of angiotensin IV on the development of endothelial dysfunction in apolipoprotein E-deficient (ApoE−/−) mice.

Methods and results In contrast to their wild-type control, ApoE−/− mice that were fed a high-fat diet had exacerbated endothelial dysfunction, evidenced by impaired endothelium-dependent vasodilation. Chronic infusion of angiotensin IV (1.44 mg/kg per day) in ApoE−/− mice for 2 weeks resulted in significant improvements in endothelial function. Angiotensin IV treatment markedly decreased superoxide levels (dihydroethidium staining fluorescence and L-012 chemiluminescence) and increased endothelial nitric oxide synthase expression (immunoreactivity and western blotting) in aortic tissue. Co-treatment of angiotensin IV with either AT4 receptor antagonist divalinal-Ang IV or AT2 receptor antagonist PD123319 attenuated these changes, indicating involvement of both the AT4 and the AT2 receptors.

Conclusion Chronic angiotensin IV treatment in ApoE−/− mice evoked a marked vasoprotective effect that appeared to be mediated by improved NO bioavailability as a result of AT4 and/or AT2 receptor stimulation.

1. Introduction

The octapeptide angiotensin (Ang) II is considered the major bioactive peptide of the renin angiotensin system (RAS) involved in the control and regulation of blood pressure. RAS, and in particular the role of Ang II, has emerged as a crucial pathophysiological factor in many cardiovascular diseases, with Ang II being a potent mediator of oxidative stress, stimulates the release of cytokines and adhesion molecule expression that mediate vessel wall inflammation, and acts as a direct growth factor for vascular smooth muscle cells (VSMCs).1–4 Logically, the RAS has been targeted in therapeutic strategies aimed at reducing the incidence of cardiovascular disease. AT1 receptor antagonists and angiotensin converting enzyme inhibitors have been proved to be very effective in preventing neointimal formation and the occurrence of endothelial dysfunction in animal studies.5–7 and significantly lowering the risk of cardiovascular endpoints and total mortality in clinical studies.8,9

There is also a growing body of evidence which suggests that other angiotensin peptide fragments [e.g. Ang (1–7) and Ang IV (Ang (3–8))] can bind to non-AT1 receptor subtypes (e.g. AT2, AT4, and Ang (1–7)/mas receptor) to counter-regulate the effects mediated via the AT1 receptor.10–14 Ang IV is a hexapeptide fragment of Ang II (amino acids 3–8 of parent peptide) formed from Ang III via the action of aminopeptidase N and binds with high affinity to the designated AT4 receptor, which, recent evidence suggests, is the enzyme insulin-regulated aminopeptidase (IRAP), with Ang IV functioning to inhibit this enzyme.15 Ang IV binding sites have been detected in the brain,16 as well as in coronary and aortic endothelial cells,17,18 consistent with Ang IV inducing vasodilatation and enhancing learning and memory retention.19,20

The AT4 receptor has been shown to be highly concentrated on endothelial cells17 and Ang IV-mediated vasodilatory responses have been well documented.19,21,22 Ang IV has been shown to activate eNOS via post-transcriptional modulation, increase NO release, and stimulate cGMP accumulation in porcine pulmonary artery endothelial cells.19,23 Importantly, the AT4 receptor has been shown to be up-regulated in carotid arteries of rabbits that had been fed a high-cholesterol diet and then underwent balloon angioplasty, indicating that Ang IV may play a role in vascular repair and remodelling.24 However, as yet, there is no report on the effect of chronic angiotensin IV

*Corresponding author. Tel: +61 3 9905 4858; fax: +61 3 9905 5851. E-mail address: robert.widdop@med.monash.edu.au

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2007.

For permissions please email: journals.permissions@oxfordjournals.org
treatment on vascular function, particularly in ApoE\(^{-/-}\) mice that exhibit endothelial dysfunction.\(^{25}\)

Given the diverse roles reported for Ang IV in vascular function,\(^{18,19}\) in the present study we determined the ability of chronic Ang IV administration to modulate endothelial function in the ApoE\(^{-/-}\) mouse model fed a high-fat diet which is known to exacerbate the endothelial dysfunction.\(^{25-27}\) The potential importance of Ang IV is underscored by reports that levels of this peptide are persistently elevated during chronic AT\(_1\) receptor blockade in hypertensive patients.\(^{28}\)

Indeed, our findings provide the first evidence that Ang IV may play a protective role in vascular disease by improving vascular function by increasing the NO bioavailability.

2. Methods

2.1. Animals

Male B6 apolipoprotein E-deficient (ApoE\(^{-/-}\)) mice with >99% C57BL/6J background (weighing 25–35 g) were purchased from the Animal Resource Centre, Western Australia. Male C57BL/6J wild-type (WT) mice (weighing 25–35 g) were obtained from Central Animal Services, Monash University. At 6 weeks of age mice were fed a high-fat diet containing 22% fat and 0.15% cholesterol (Speciality Feeds, Western Australia) for a period of 6 weeks prior to as well as during the subsequent treatment period. We performed animal experiments in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and these experiments were approved by the Monash University Animal Ethics Committee.

2.2. Surgical procedure

WT and ApoE\(^{-/-}\) mice were anaesthetized using isoflurane and an incision was made in the midscapular region through which mini osmotic pumps (Alzet model 2002, Alza Corp) were inserted for subcutaneous drug administration. Mice received one of the following treatments: vehicle (NaCl 0.15 M), Ang II, or Ang IV (0.72 mg/kg per day; a kind gift from Pfizer, USA), both administered via mini osmotic pumps (Alzet model 2002, Alza Corp) were inserted for subcutaneous drug administration. Mice received one of the following treatments: vehicle (NaCl 0.15 M), Ang II, or Ang IV (0.72 mg/kg per day; a kind gift from Pfizer, USA), both administered via minipumps. In addition, minipumps were implanted into the subcutaneous tissue under a dissecting microscope (Olympus SZ40) and either the AT\(_4\) receptor antagonist, divalinal-Ang IV (1.44 mg/kg per day), or the AT\(_2\) receptor antagonist, PD123319 (10 mg/kg per day; a kind gift from AstraZeneca, Sweden) was added to the bath to test vascular smooth muscle integrity.

In addition, the acute effect of angiotensin IV on vascular function was tested on abdominal aortic rings taken from age- and diet-matched ApoE\(^{-/-}\) mice (14 weeks of age). Concentration-response curves to Ang IV were constructed to assess the direct vascular effect of Ang IV, and separate aortic rings were pre-treated with either Ang IV (10 μM) or vehicle 15 min prior to construction of an acute angiotensin concentration-response curve to assess the acute effects of Ang IV on endothelium-dependent vasodilatation.

2.4. Localization of eNOS and AT receptors by immunohistochemistry

Sections were taken from the same part of the aorta as that used for dihydroethidium (DHE) staining that was adjacent to the aortic sections taken for vascular reactivity studies. Sections were immersed in Tissue-Tek (Sakura), snap frozen in liquid nitrogen and stored at −80°C until they were cytocut into 10 μm thick sections mounted on gelatin chromatulin-coated slides. Acetone-fixed sections were then washed in Tris buffer (Sigma, pH 7.4) before incubation in a humidified chamber in 10% goat serum in Tris buffer for 30 min to block non-specific binding. Sections were then incubated for 4 h in 1:500 dilutions of primary rabbit polyclonal anti-eNOS (BD Biosciences) IgG antibody. Adjacent sections were incubated with a rabbit immunoglobulin fraction (DAKO), and used at the same primary antibody protein concentration, as a negative control. Sections were then washed three times in Tris buffer and stained using the DAKO EnVision\(^{+}\) system (DAKO). Briefly, sections were incubated for 2 h with a peroxidase-labelled polymer conjugated to goat anti-rabbit IgGs, washed in Tris buffer, followed by incubation with diaminobenzidine for 5 min. Sections were then rinsed in distilled water and counter-stained with haematoxylin and cover slipped. Staining was analysed by two blinded observers who scored the intensity of positive staining using an arbitrary grading system based on a scale of 1–5 (lowest to highest). Scores were averaged for each aortic section to allow for the comparison of eNOS levels between treatment groups.

Localization of AT\(_1\), AT\(_2\), and AT\(_{4}\) receptors was performed in aortic sections of untreated WT and ApoE\(^{-/-}\) mice to examine the receptor expression in normal and diseased states. Sections were incubated for 4 h in 1:500 dilutions of primary rabbit polyclonal anti-AT\(_1\) receptor, anti-AT\(_2\) receptor (Santa Cruz Biotechnology Inc.), and anti-AT\(_{4}\) receptor/IRAP (Alpha Diagnostic, TX, USA) IgG antibodies. Visualization of Ang receptors was conducted using the DAKO envision\(^{+}\) System as detailed above.

2.5. Quantification of eNOS by western blotting

In a separate set of chronically treated ApoE\(^{-/-}\) mice, aortae dissected free of connective tissue were homogenized in laemmlly lysis buffer (5% glycerin, 2.5% mercaptoethanol, 1.5% SDS, 50 mM
Tris-HCl, pH = 8.0, 0.05 mg/mL bromphenol blue). Cell bodies and unlysed cells were removed by low-speed centrifugation at 10,000 g for 15 min at 4°C. Proteins were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes, which were blocked in 3% skim milk powder for 1 h at room temperature, and then incubated with a rabbit polyclonal anti-eNOS antibody (BD Biosciences) at a 1:1000 dilution overnight at 4°C. Following washing in Tris-buffered saline–Tween (TBS-T) solution three times at 10 min intervals, membranes were then incubated with a goat anti-rabbit secondary antibody conjugated with horse-radish peroxidase at a 1:5000 dilution (DAKO) for 2 h at room temperature. Membranes were then washed in TBS-T three times at 10 min intervals and immunoreactive bands were visualized by ECL (Millipore, MA, USA) and film exposure for 10 min. Immunoreactive bands were quantified using chemiDoc XR images using Quantity One software (BioRad). Immunoblotting with a monoclonal anti-β-actin antibody (1:1000, Sigma) was conducted to ensure equal protein loading.

2.6. Localization of superoxide by dihydroethidium staining

Sections taken from the aorta adjacent to the arterial rings used in the vascular reactivity studies were immersed in Tissue-Tek (Sakura), snap frozen in liquid nitrogen, and stored at −80°C until they were cryocut into 30 μm thick sections mounted on gelatin chromalumin coated slides. Dihydroethidium (2 μM/l) was used to detect superoxide in frozen sections of both wild type and ApoE−/− mouse aorta. 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. The intensity of fluorescence was quantified in two whole aortic sections from each mouse at two optical levels ~3 μm apart, using analySIS software (Soft Imaging System, Singapore) with identical measurement settings.

2.7. Superoxide detection using L-012 enhanced chemiluminescence

In a separate set of chronically treated ApoE−/− mice also used for western blotting experiments, aortic rings (~2 mm in length) dissected free of connective tissue were incubated for 30 min at 37°C in HEPES-buffered Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 7H₂O 1.2, CaCl₂ 2.5, NaHCO₃ 25, glucose 11.7, and HEPES, with either no inhibitors, superoxide dismutase (SOD; 300 U/mL), an NADPH oxidase inhibitor diphenyldiaminonaphthalene (DPI, 10 μM/l) or an inhibitor of eNOS, N⁷-nitro-L-arginine methyl ester (L-NAME, 100 μM/l) in a 96-well plate. Following the 30 min incubation, L-012 (100 μM/l) was added to each well, which was then loaded into a HideX Chameleon Luminescence detector. Duplicates were done for each treatment group and photon emission was recorded for 20 cycles at 2 min intervals. Photon emissions (relative light units per second) were then averaged over the 20 cycles and normalized to the dry weight of the aortic ring.

2.8. Data analysis

All results are expressed as mean ± SEM. Statistical comparisons were done by paired t-tests or by one- or two-way repeated measures ANOVA with Bonferroni corrections where appropriate using GraphPad Prism version 4.00 (GraphPad Software, San Diego CA, USA). The non-parametric statistical Kruskal-Wallis test was used for the analysis of the eNOS immunohistochemistry. P < 0.05 was deemed statistically significant.

3. Results

The lower dose (0.72 mg/kg per day) of both Ang IV and Ang II failed to evoke changes in endothelial function (data not shown), therefore only the effects of the higher dose (1.44 mg/kg per day) of each peptide are reported here. The higher dose of Ang II induced abdominal aortic aneurysms in three ApoE−/− mice, which were excluded from further analysis. Ang II-induced aneurysm formation is well described in this model, albeit after longer treatment time; however, Ang IV did not induce aneurysms over this period. Atherosclerotic lesions were not present in WT mice. In ApoE−/− mice treated with Ang II, there was a significant increase in cross-sectional area compared with vehicle-treated group (intima: media ratio media ratio 0.40 ± 0.03, P < 0.05). Co-treatment with the AT₁ receptor antagonist abolished the Ang II-induced lesion formation. Chronic Ang IV treatment in ApoE−/− mice did not significantly increase lesion formation (intima: media ratio 0.27 ± 0.10).

3.1. Effect of treatments on systolic blood pressure

In ApoE−/− mice, Ang II (1.44 mg/kg per day) increased SBP (Table 1) and this pressor response was attenuated with co-administration of candesartan cilexetil, the AT₁ receptor antagonist. As expected, a depressor effect was also evident with the use of candesartan alone. Ang IV had no effect on SBP alone or in combination with either the AT₂ receptor antagonist, divalinal Ang IV, or the AT₂ receptor antagonist, PD123319. Co-treatment with candesartan cilexetil induced a significant depressor response in ApoE−/− mice (Table 1), similar to the depressor response seen with candesartan alone.

3.2. Ang IV treatment improved endothelium-dependent vasorelaxation

Acetylcholine caused concentration-dependent vasorelaxation in pre-constricted aortic rings from WT mice (R_max, 95.07 ± 4.33%; n = 5), with the maximum relaxation significantly reduced in aortic rings taken from ApoE−/− mice (R_max, 72.72 ± 5.95%, n = 8) (Figure 1A).

Chronic infusion of Ang II in WT mice induced significantly impaired vasorelaxation responses to acetylcholine (Figure 1B), whereas chronic treatment of ApoE−/− mice with Ang II did not further exacerbate endothelial dysfunction in response to acetylcholine (Figure 1C). Co-administration of Ang II with the AT₁ receptor antagonist, candesartan

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wild type (WT)</th>
<th>ApoE−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>After treatment</td>
</tr>
<tr>
<td>Vehicle</td>
<td>115 ± 4</td>
<td>122 ± 3</td>
</tr>
<tr>
<td>Ang II</td>
<td>114 ± 1</td>
<td>151 ± 7**</td>
</tr>
<tr>
<td>Ang II + Cand</td>
<td>115 ± 3</td>
<td>119 ± 5</td>
</tr>
<tr>
<td>Ang IV</td>
<td>120 ± 5</td>
<td>119 ± 5</td>
</tr>
<tr>
<td>Ang IV + Cand</td>
<td>123 ± 7</td>
<td>116 ± 6</td>
</tr>
<tr>
<td>Ang IV + PD</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ang IV + DV</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cand alone</td>
<td>116 ± 6</td>
<td>100 ± 5</td>
</tr>
</tbody>
</table>

**P < 0.05, ***P < 0.01, and ** **P < 0.001 vs. corresponding SBP before treatment (n = 6–12). ND, not determined.
Acute incubation with Ang IV (10 μg/kg per day) treatment in ApoE/−/− mice resulted in a striking improvement in aortic endothelium-dependent vasorelaxation responses to acetylcholine in mouse aorta, indicative of improved endothelial function (Figure 1C). In contrast, chronic Ang IV treatment in ApoE/−/− mice resulted in a striking improvement in aortic endothelium-dependent vasorelaxation responses to acetylcholine in ApoE/−/− mice, indicative of improved endothelial function (Figure 1E). This improved response was not attenuated following co-treatment with candesartan cilexetil; yet it was abolished when Ang IV was co-administered with either the AT1 receptor antagonist, divalinal-Ang IV, or the AT2 receptor antagonist, PD123319 (Figure 1E). Acetylcholine-mediated vasorelaxation in isolated aorta of WT mice chronically infused with Ang IV was identical to that of vehicle-treated WT mice which already exhibited normal endothelium-dependent relaxation (Figure 1D). Responses to the NO donor SNP resulted in maximum relaxation in all tissues and were unchanged throughout all treatment groups (Supplementary material online, Figure S2).

Direct vasodilator effects of Ang IV in the abdominal aorta of ApoE/−/− mice were not observed (data not shown). Furthermore, acute Ang IV pre-treatment of aortic rings taken from age- and diet-matched ApoE/−/− mice showed no improvement in acetylcholine-mediated vasodilation (Figure 1F) unlike that observed in mice chronically treated with Ang IV (Figure 1E).

3.3. eNOS immunohistochemistry

Immunohistochemical analysis of eNOS expression was compared in aortic cross sections taken from the area adjacent to the aortic sections utilized for vascular reactivity studies in both WT and ApoE/−/− mice. eNOS immunoreactivity was slightly lower in ApoE/−/− mouse aorta compared with WT aorta (Figure 2). In WT mice, infusion of Ang II or Ang IV had no effect on eNOS immunoreactivity in the aorta (Supplementary material online, Figure S1A). Ang II treated ApoE/−/− mice showed a trend towards greater intensity of eNOS staining than vehicle treated animals (Figures 2A and B). However, chronic Ang IV treatment showed a significant increase in eNOS staining intensity which was attenuated when mice were co-treated with either the AT1, AT2 or AT4 receptor antagonists (Figure 2A and C).

3.4. eNOS western blotting

Utilizing tissue taken from ApoE/−/− mice chronically treated with either vehicle, Ang II, or Ang IV, immunoreactive bands for eNOS (~140 kDa) revealed greater immunoreactivity in Ang II and Ang IV treated mouse aorta compared with vehicle (Figure 3A). Densitometric analysis confirmed these observations although only Ang IV significantly increased eNOS protein expression when expressed as a ratio to β-actin density (Figure 3B), consistent with immunohistochemistry data.

3.5. Superoxide localization and quantification

Dihydroethidium staining was used to localize superoxide in cross-sections taken from WT and ApoE/−/− mice, with positive staining apparent in the endothelium, media, and adventitia (Figure 4A). The intensity of fluorescence was measured, normalized, and expressed as intensity (arbitrary units) per μm². Aortic sections from ApoE/−/− mice exhibited significantly greater intensity of fluorescence compared with WT mouse aorta, indicative of the oxidative state of the vasculature of ApoE/−/− mice (Figure 4). Infusion of Ang II in WT mice appeared to increase superoxide levels although this effect was not considered significant. Ang IV infusion had no effect on superoxide levels in WT mouse aortae (see Supplementary material online, Figure S1B). On the other hand, ApoE/−/− mice treated with Ang IV at 1.44 mg/kg per day had significantly decreased levels of superoxide compared with vehicle-treated controls.
This effect was not significantly attenuated by co-treatment with either an AT$_1$ or an AT$_4$ receptor antagonist, whereas co-treatment with the AT$_2$ receptor antagonist abolished the decrease in superoxide production mediated by Ang IV. Sections taken from Ang II-treated ApoE$^{-/-}$ mice showed intense fluorescence that was similar to vehicle treatment, whereas the AT$_1$ receptor antagonist candesartan cilexetil significantly reduced the levels of superoxide in the aorta (Figure 4B).

3.6. Superoxide detection using L-012-enhanced chemiluminescence

Basal levels of superoxide were measured in aortic rings from ApoE$^{-/-}$ mice using L-012-enhanced chemiluminescence. Chronic treatment with Ang II significantly increased basal superoxide levels compared with vehicle-treated mice. In contrast, Ang IV treatment significantly decreased basal superoxide levels, which is consistent with the DHE data (Figure 5A). To confirm the specificity and source of superoxide, parallel aortic rings were acutely incubated with SOD, DPI, and L-NAME. SOD quenched the signal in tissues from all treatment groups (Figure 5B), as did DPI (Figure 5C) indicating that the main source of superoxide was the enzyme NAD(P)H oxidase. L-NAME had no effect on basal superoxide levels in vessels taken from vehicle- or Ang IV-treated ApoE$^{-/-}$ mice. However, in aortic rings taken from Ang II-treated ApoE$^{-/-}$ mice, L-NAME significantly decreased the basal superoxide signal (Figure 5D vs. Figure 5A). Thus, some of the superoxide being produced in the Ang II treatment group may come from 'uncoupled' eNOS.

3.7 AT$_1$, AT$_2$, and AT$_4$ receptor immunohistochemistry

Immunohistochemical localization of Ang receptors in aortic sections from WT mice revealed strong immunoreactivity of AT$_1$ receptors in the medial smooth muscle layer, whereas negligible AT$_2$ and AT$_4$ receptor expression was observed at 1/500 dilution (Figure 6A, C, E and G). In ApoE$^{-/-}$ mouse aorta which exhibited atherosclerotic lesions, AT$_1$ receptor expression was also strongly localized to atherosclerotic plaques (Figure 6D). Furthermore,
4. Angiotensin IV improves endothelium-dependent vasodilation

**Figure 3** (A) Representative western blots of eNOS and β-actin probed in aortic homogenates taken from ApoE \( ^{-/-} \) mice that had received either vehicle, Ang II, or Ang IV. (B) The densities of the blots were quantified and were expressed as a ratio to β-actin density. \(^{**P < 0.01} \) vs. vehicle treated mice (n = 3–6).

Contrast to WT mouse aorta, AT2 and AT4 receptor immunoreactivity in ApoE \( ^{-/-} \) mouse aorta was greatly enhanced suggesting an up-regulation of these receptors, particularly in the endothelium and the atherosclerotic plaques (Figure 6F and H).

4. Discussion

We demonstrate here for the first time that chronic treatment of ApoE \( ^{-/-} \) mice with Ang IV produced a vasoprotective effect, as evidenced by improved aortic endothelial function that was associated with decreased superoxide levels and increased eNOS expression; all of which would be consistent with increased NO bioavailability. Strikingly, these vasoprotective effects of Ang IV were not evident with Ang II treatment and in fact Ang IV-induced changes in vascular function and signalling do not appear to be mediated via the AT1 receptor.

**4.1 Ang IV improves endothelium-dependent vasodilation**

In order to assess the chronic effects of the angiotensin peptides on vascular function, we utilized two doses based on the conventional dose of Ang II used to accelerate atherosclerotic lesion development in ApoE \( ^{-/-} \) mice.\(^{29}\) The chronic effects of Ang IV to improve vascular function were found to be dose-related since half of the hexapeptide dose did not alter vascular function (data not shown). At the higher dose of Ang IV (1.44 mg/kg per day), this equates to low (\(~15 \mu g/mL\) ) concentrations being infused subcutaneously that are in the range of plasma concentrations of Ang IV that have been measured in a limited number of studies.\(^{28,31}\) Divergent effects of the two angiotensin peptides were seen in WT mice since Ang II, but not Ang IV, caused marked endothelial dysfunction. A lack of further endothelial impairment evoked by chronic Ang II in ApoE \( ^{-/-} \) mice likely reflects existing endothelial dysfunction in this model. Interestingly, in ApoE \( ^{-/-} \) mice, Ang IV appeared to act via both the AT4 and the AT2 receptors to improve endothelial-dependent vasorelaxation since divalinal-Ang IV and PD123319, respectively, prevented the vascular improvement facilitated by Ang IV. These findings are consistent with a recent study in which acute treatment with Ang IV caused cerebral vasodilatation via mechanisms involving both AT4 and AT2 receptors.\(^{32}\) Chronic treatment with Ang IV produced a significant improvement in endothelium-dependent vasodilation, yet we were unable to induce any direct vasodilator (or vasoconstrictor) effects when Ang IV was acutely incubated with the tissue which implicates the involvement of downstream signalling mechanisms to increase the NO bioavailability.

**4.2 Ang IV increases NO bioavailability**

Potential mechanisms for increased relaxation may involve altered eNOS activity and/or production of reactive oxygen species. Indeed, we have demonstrated, for the first time, that at least some of the vasoprotective effects mediated by Ang IV involve a significant decrease in superoxide production as well as an increase in eNOS expression. Ang IV has previously been shown to activate eNOS in porcine pulmonary arterial endothelial cells, leading to activation of NO/cGMP-mediated signalling mechanisms.\(^{19}\) In the present study, we confirmed by western blot analysis that Ang IV treatment significantly increased eNOS protein levels. Moreover, Ang IV-mediated increase in eNOS immunoreactivity was attenuated by both divalinal-Ang IV and PD123319, indicating possible involvement of both the AT4 and AT2 receptor sub-types. Not surprisingly, Ang IV did not alter eNOS immunoreactivity in WT mice. At the same time, there was a marked increase in aortic superoxide levels, assessed by DHE staining, in ApoE \( ^{-/-} \) mice compared with WT mice. Strikingly, Ang IV markedly inhibited aortic superoxide levels, and this effect was selectively inhibited by PD123319, indicating that the AT2 receptor may play a major role in reducing oxidative stress and thus contribute to the Ang IV-mediated improvement in endothelial function. Many studies have previously demonstrated a primary role for Ang II in stimulating NAD(P)H oxidases and subsequent superoxide production.\(^{33,34}\) In this study using DHE staining, chronic Ang II treatment did not significantly increase superoxide production in ApoE \( ^{-/-} \) mice although this effect was more evident in Ang II-treated WT mice. We also measured basal superoxide production by L-012 chemiluminescence in intact vessels from the angiotensin peptide-treated ApoE \( ^{-/-} \) mice. In this instance, Ang II significantly increased superoxide levels using L-012-enhanced chemiluminescence, possibly indicating a greater sensitivity with the latter assay. Importantly, our novel finding of a marked inhibitory effect of Ang IV on vascular superoxide using DHE staining was also confirmed using L-012 chemiluminescence.

We also examined the likely source of superoxide production in the different treatment groups. The fact that both SOD and DPI markedly attenuated the basal signal in the vehicle-treated tissue suggests the formation of...
Figure 4  (A) Representative sections of dihydroethidium staining for superoxide in aortic sections from vehicle-treated WT mice and ApoE\(^{-/-}\) mice chronically treated with vehicle, Ang II, and Ang IV. Red staining represents 2-hydroxyethidium fluorescence and superoxide localization, and green staining represents auto-fluorescence in elastin layers in the medial wall. PEG-SOD (500 U/mL) was applied topically during the 30 min incubation with dihydroethidium in an adjacent section, and abolished 2-hydroxyethidium fluorescence, confirming specificity of the fluorescent signal for superoxide. (B) and (C) Mean data for the quantification of fluorescence expressed as intensity per \(\mu\text{m}^2\) (\(n = 6-8\)) in aortic sections from vehicle-treated WT mice and ApoE\(^{-/-}\) mice chronically treated with vehicle, Ang II, or Ang IV in the absence or presence of various antagonists. \#P < 0.01 vs. WT; **P < 0.01, ***P < 0.001 vs. vehicle; \&\&P < 0.01 vs. Ang IV alone.
Angiotensin IV improves endothelial dysfunction

4.3 Vessel morphology

While not the primary outcome of the current study, we also measured lesion formation following each treatment. Ang II significantly increased cross-sectional atherosclerotic lesions, consistent with previous studies. 

Endothelial dysfunction is a surrogate marker of early vascular damage and clinical efficacy of many drugs includes improved endothelial vasorelaxation. Thus, it is possible that the vasoprotective effects of Ang IV on endothelial function described in the present study limited the degree of superoxide via NAD(P)H oxidases. Interestingly, l-NAME had no significant effect on signals except in the chronic Ang II group where it inhibited basal vascular superoxide production in ApoE \(-/\) mice. However, of particular importance is the observation that l-NAME had no effect on superoxide formation in aorta taken from ApoE \(-/\) mice chronically treated with Ang IV, suggesting that the Ang IV-mediated increase in eNOS is likely to represent functional NOS producing NO. Our study indicated that Ang II treatment tended to increase eNOS expression as has been reported previously. However, Ang II is associated with ‘uncoupling’ of eNOS, leading to increased production of ROS, our finding that l-NAME attenuated ROS production in Ang II-treated vessels is consistent with eNOS being in an uncoupled state where it produces ROS rather than NO.

Collectively, the present data support the hypothesis of increased NO bioavailability as a result of increased eNOS expression and reduced superoxide production following Ang IV treatment, consistent with the observed improvement in endothelial-dependent vasorelaxation. These results are consistent with a recent report that Ang IV produces cerebroprotective effects via both AT1 and AT2 receptors, triggering an NO-dependent mechanism.

Figure 5 L-012 enhanced chemiluminescence detection of superoxide in intact aortic rings from ApoE \(-/\) mice. (A) Histograms represent mean values for basal superoxide levels obtained from chronically treated groups, as indicated. In addition, in all of the chronically treated groups, superoxide levels were measured in parallel tissues that were acutely incubated for 30 min with either (B) SOD (300 U/mL), (C) the NADPH oxidase inhibitor DPI (10 \(\mu\)mol/L), or (D) the eNOS inhibitor l-NAME (100 \(\mu\)mol/L). *P < 0.05, **P < 0.01 vs. vehicle, ##P < 0.05, ###P < 0.01 vs. corresponding value from basal counts (n = 3–6).

Figure 6 Representative immunohistochemical images showing AT receptor localization in aortic sections from untreated WT mice (A, C, E, and G) and untreated ApoE \(-/\) mice (B, D, F, and H). (C–D) AT1 receptor immunoreactivity was clearly evident in the normal and diseased vessel; (E–F) AT2 and (G–H) AT4 receptor immunoreactivity was more apparent in ApoE \(-/\) compared with WT mouse aorta, particularly in the endothelium and atherosclerotic lesion.
of lesion development via AT₄ receptors observed with Ang II. Recently, Esteban et al. reported that, in cultured VSMCs, Ang IV (1–100 nM) via the AT₄ receptor, activated the nuclear transcription factor-κB (NF-κB) pathway and up-regulated several pro-inflammatory mediators including MCP-1 and PAI-1, thereby implicating a possible pro-inflammatory role for the hexapeptide, at least on the plasma levels reported in another hyperlipidaemic mouse model. Moreover, these in vivo subcutaneous doses are not likely to be higher than those reported following direct application to cultured VSMCs. Clearly, future studies, using longer treatment regimes, will inform on whether Ang IV-mediated vasoprotection affects the extent of lesion formation.

The AT₄ receptor has been found to increase by ~220% in balloon-injured carotid arteries of rabbits, which is consistent with a role for the AT₄ receptor in vascular remodeling. We have also demonstrated marked up-regulation of both AT₂ and AT₄ receptors in atherosclerotic lesions, which were localized to the endothelium and plaque, which clearly identifies the AT receptor targets underpinning the vasoprotective effects of Ang IV that we have shown. Increased AT₂ receptor expression was also recently reported in a rabbit model of atherosclerosis. Thus, it is possible that the divergent effects of Ang IV could be attributed to the stimulation of multiple angiotensin receptor subtypes resulting in a complex pattern of activity. Clearly, there are precedents for Ang IV interacting with all Ang receptor subtypes despite the earlier reports that Ang IV has high affinity for the AT₄ receptor, at least in brain. Indeed, the hexapeptide evoked AT₄ receptor-mediated increased NO signalling in some studies while, in other studies, Ang IV caused both AT₁ receptor-mediated vasoconstriction and AT₃ receptor-mediated vasodilation in resistance arteries and rat isolated lung. Thus, it is possible that the diverse actions of Ang IV observed in the present study represent the effects of the hexapeptide interacting with different AT receptors in a tissue-specific manner caused by the vascular disease process. For example, a greater vasoprotective effect of Ang IV may be mediated by both AT₂ and AT₄ receptors whereas AT₄ and AT₁ receptors may mediate pro-inflammatory events, some of which were offset by opposing vascular effects of Ang IV. Furthermore, hypercholesterolemia itself may increase the levels of Ang peptides that then could potentially interact with multiple AT receptors, given that hypercholesterolemia stimulated the production of many angiotensin peptides in LDL receptor-deficient mice, with the greatest increases seen in Ang II and Ang IV levels. In addition, it is possible that the expression of AT receptors may be altered by the chronic effects of angiotensin peptide treatment per se, which will be the subject of future investigations.

In summary, we have shown for the first time the complex cardiovascular profile resulting from chronic treatment with the hexapeptide Ang IV, most likely interacting with multiple AT receptors. There was a marked enhancement of endothelium-dependent vasodilation in vessels that normally exhibit endothelial dysfunction, and this vasoprotective effect of Ang IV was accompanied by hallmarks of improved NO bioavailability (increased eNOS; decreased superoxide levels, both measured by two different assays), because of stimulation of AT₄ receptors and/or AT₂ receptors.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Funding**

This research was supported by National Health & Medical Research Council of Australia (436823) and the Monash University Small Grants scheme.

**Conflict of interest:** none declared.

**References**

Angiotensin IV improves endothelial dysfunction


