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Angiotensin IV-evoked vasoprotection is conserved in advanced atheroma

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Abstract

Background: We have previously demonstrated that chronic treatment with the hexapeptide angiotensin (Ang) IV significantly improved endothelium-dependent vasorelaxation in isolated aorta obtained from young adult apolipoprotein E-deficient ($ApoE^{-/-}$) mice. The current study aimed to investigate whether this effect is evident using $ApoE^{-/-}$ mice with established atheroma.

Materials and results: $ApoE^{-/-}$ mice fed a high-fat diet for a period of 26 weeks displayed significantly impaired endothelial function compared to wild-type mice. Importantly, 2-week treatment with Ang IV, significantly improved endothelial function despite the abundance of atherosclerotic lesions. Endothelial nitric oxide synthase immunoreactivity was significantly increased, whereas superoxide levels assessed using dihydroethidium staining were concomitantly decreased, in aortic cross-sections taken from Ang IV treated mice compared with vehicle treated $ApoE^{-/-}$ mice. [^{125}I] Ang IV autoradiographic analysis revealed an upregulation of AT_4Rs in $ApoE^{-/-}$ mice fed a high-fat diet for 26 weeks compared to 8 weeks. Co-treatment with an AT_4R antagonist, divalinal-Ang IV or an AT_2R antagonist, PD123319 significantly abrogated these Ang IV-induced vasoprotective effects.

Conclusions: We have demonstrated that the improvement in endothelial function following chronic Ang IV treatment is conserved at an advanced stage of atherosclerosis. Consistent with previous findings using an early-stage model, this vasoprotective effect of Ang IV was AT_4R- and/or AT_2R-mediated, involving increased nitric oxide bioavailability.

Keywords: Endothelial dysfunction; Angiotensin IV; AT_4 receptor; AT_2 receptor; Nitric oxide bioavailability; Superoxide

1. Introduction

Atherosclerosis is a major cause of morbidity and mortality in developed countries. It is now recognized as a chronic inflammatory disease and is thought that endothelial dysfunction may be crucial in the initiation of lesion development [1]. Indeed endothelial dysfunction is considered a surrogate marker for cardiovascular diseases including atherosclerosis [2]. Disruption of the endothelium results in reduced synthesis and/or bioavailability of the potent vasodilator nitric oxide (NO) and subsequently vasoconstriction, enhanced migration of monocytes and lipids into the intima, inflammation, as well as, vascular smooth muscle cell (VSMC) proliferation and migration [3]. Furthermore, in disease states where reactive oxygen species (ROS) such as superoxide are increased, NO is rapidly scavenged and bioavailability is further decreased.

Angiotensin (Ang) II is the main effector hormone of the renin–angiotensin system (RAS), and is well recognized as a pro-atherogenic mediator by initiating cellular proliferation and migration, activating inflammatory markers as well as increasing oxidative stress [4–7]. In hypercholesterolemia and atherosclerosis, components of the RAS are upregulated, and importantly Ang II levels are increased [8–10]. Furthermore, the beneficial effects of AT_1-receptor (AT_1R) antagonists and angiotensin converting enzyme (ACE) inhibitors in experimental and clinical settings rein-

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force the prominent role of Ang II in development of atherosclerosis. However, there are a number of other Ang peptide fragments that can bind to non-AT_1Rs which are thought to counter-regulate the detrimental effects of Ang II [8,11–14]. One such peptide is Ang (3–8) also known as Ang IV. This hexapeptide is reported to exert a range of effects (for review, see Ref. [15]). It is now known that Ang IV acts on its own receptor, the AT_4-receptor (AT_4R), which was subsequently identified as the enzyme insulin regulated aminopeptidase (IRAP) [16].

In a recent study, we demonstrated that chronic Ang IV treatment of young adult ApoE$^{-/-}$ mice fed a high-fat diet, significantly potentiated endothelium-dependent vasorelaxation and thus reversed endothelial dysfunction [8]. This improvement in endothelial function was related to an increase in NO bioavailability as there was a concomitant increase in endothelial nitric oxide synthase (eNOS) protein expression and decreased vascular superoxide production in Ang IV-treated ApoE$^{-/-}$ mice. Moreover, the effect of Ang IV was markedly different to that produced by Ang II and was mediated via stimulation of the AT_4R and AT_2 receptor (AT_2R) [8]. In this previous study, ApoE$^{-/-}$ mice were only 14 weeks of age, fed a high-fat diet for 8 weeks and hence, exhibited only a modest level of atherosclerotic lesion development. By contrast, clinical diagnosis of atherosclerosis is often when the disease state is quite advanced given the insidious nature of progression. Therefore, as an extension of our previous study, it was important to determine if Ang IV could exert a similar vasoprotective effect at a more advanced stage of atherosclerosis. Thus, the current study investigated the influence of Ang IV on endothelial dysfunction in an advanced model of atherosclerosis with well-established lesions to more closely mimic the clinical setting.

2. Methods

2.1. Animals

Male B6 Apolipoprotein E-deficient (ApoE$^{-/-}$) mice with >99% C57BL/6J background (weighing 30–35 g), were purchased from the Animal Resource Centre, Western Australia. Male C57BL/6J wild-type (WT) mice (weighing 30–35 g) were obtained from Central Animal Services, Monash University. 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 either a period 6 or 24 weeks prior to, as well as during the subsequent treatment period. All procedures were approved by Monash University Animal Ethics Committee.

2.2. Treatments

Mice received 2-week subcutaneous infusions of the following drugs: vehicle (NaCl, 0.15 M), Ang II or Ang IV at a dose of 0.72 mg/kg/day. Given that Ang II can readily evoke abdominal aortic aneurysm (AAA) in ApoE$^{-/-}$ mice at this age and with existing atherosclerosis [17,18], we used half the effective dose of these peptides that evoked divergent effects on endothelial function in our previous study [8]. Two further groups of ApoE$^{-/-}$ mice were co-treated with Ang IV and either the AT_4R antagonist, divalinal-Ang IV (1.44 mg/kg/day), or the AT_2R antagonist, PD123319 (10 mg/kg/day) via osmotic mini pump. Systolic blood pressure (SBP) was measured before and at the end of treatment using non-invasive tail-cuff apparatus (ADInstruments, Sydney). SBP was averaged from three to five consecutive measurements taken at intervals of 1–2 min.

2.3. Assessment of endothelial vasodilator function

At the end of the treatment, animals were anaesthetised by isoflurane inhalation and decapitated. The whole aorta was cleared and placed in ice-cold Krebs-bicarbonate buffer (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4{\cdot}7H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.7. The aorta was then sectioned into thoracic and abdominal lengths, leaving the aortic arch attached to the heart. Abdominal aortic rings were mounted in water-jacketed organ baths and bubbled with carbogen (95% O_2 and 5% CO_2). Concentration response curves to the endothelium-dependent vasodilator acetylcholine (ACh) were constructed in tissues preconstricted with the thromboxane A_2 analogue, U46619 ([1,5,5-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid]), as previously described [8]. At the end of the acetylcholine curve, 10 μM of the endothelium-independent vasodilator, sodium nitroprusside (SNP), was added to the bath to test vascular smooth muscle integrity.

2.4. Localization of eNOS by immunohistochemistry

Immunohistochemical analysis of eNOS was conducted as previously described [8]. In brief, acetone fixed 10 μm thick aortic sections were washed in 10 mM Tris buffer (Sigma) prior to 4 h incubation with a 1:500 dilution of primary rabbit polyclonal anti-eNOS IgG antibody (Transduction Laboratories). Using sections from the same mouse, a rabbit immunoglobulin fraction (DAKO) was used at the same primary antibody protein concentration as a negative control. Sections were then washed in Tris buffer three times and stained using 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 1–5 (lowest to highest). Scores were averaged for each aortic section to allow comparison of eNOS levels between treatment groups.