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Differential effects of AT_1 receptor and Ca^{2+} channel blockade on atherosclerosis, inflammatory gene expression, and production of reactive oxygen species

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Abstract

Angiotensin II receptor blockade has been shown to inhibit atherosclerosis in several different animal models. We sought to determine if this effect was the result of blood pressure reduction per se or a result of the anti-inflammatory effects of receptor blockade. ApoE-deficient mice were fed a high fat diet and treated with either an angiotensin II receptor antagonist, candesartan (0.5 mg/kg/day, SC) or a calcium channel blocker, amlodipine (7.5 mg/kg/day, mixed with food). Atherosclerotic lesion area, aortic inflammatory gene expression as well as aortic H_2O_2 and superoxide production were assayed. We found that candesartan but not amlodipine treatment dramatically attenuated the development of atherosclerosis despite a similar reduction in blood pressure. Similarly, candesartan treatment inhibited aortic expression of inflammatory genes and production of reactive oxygen species, effects not seen with amlodipine. These data demonstrate that angiotensin II receptor blockade inhibits atherosclerosis by reducing vascular oxidative stress and inflammatory gene production independent of blood pressure reduction.

Keywords: Atherosclerosis; Angiotensin II; Angiotensin II receptor antagonists; Oxidative stress; Vascular biology

1. Introduction

Both humoral and mechanical factors appear to be involved in the pathogenesis of atherosclerosis. The renin–angiotensin system has been implicated as a major contributing factor to the progression of atherosclerosis in apoE-deficient mice [1–13] Smooth muscle cells exposed to angiotensin II also demonstrate an increase in MAP kinase activation, upregulation of NAD(P)H oxidase components, and increased expression of inflammatory markers such as MCP-1, VCAM-1, and M-CSF [6,14,15]. Hypertension and the biomechanical effects can lead to endothelial dysfunction [16], increased MMP and inflammatory gene expression [17–19], and accelerated atherosclerosis [2]. The magnitude of the relative contributions of humoral and mechanical factors to atherosclerosis remain unclear.

The renin–angiotensin system had been implicated in the pathogenesis of atherosclerosis based on both clinical and experimental studies [1,3,4,20–24]. Thus, it has been proposed that inhibition of the renin–angiotensin system may have anti-atherosclerotic effects independent of blood pressure reduction. This hypothesis remains controversial as there are data available that both support and refute this concept [25–29]. Therefore, we attempted to compare the relative effects of blood pressure reduction with an angiotensin II type-I receptor (AT1) blocker and a calcium channel blocker on atherosclerosis, inflammatory gene expression, and reactive oxygen species (ROS) generation in apoE-deficient mice while controlling for an equivalent degree of blood pressure reduction.

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2. Materials and methods

2.1. Animals, drugs, and diets

Male apolipoprotein E-deficient mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed individually in ventilated microisolator units on a 12 h light/dark schedule. The mice were given free access to water and food. The animals were housed and cared for according to the guidelines proposed by the National Institutes of Health for the care and use of experimental animals. All experiments in the present study were conducted on mice beginning at 6 and 8 weeks of age.

Candesartan was a kind gift from Astra-Zeneca. Dosages of candesartan and amlodipine used were determined by preliminary studies such that systolic blood pressure was reduced by approximately 30 mmHg. Candesartan was delivered via subcutaneous mini-osmotic pumps (Alzet, model 1002) implanted in a dorsal subcutaneous pocket after the mice were anaesthetized with 375 mg/kg 2,2,2-tribromoethanol (Avertin, Sigma Chemical Co.). The soluble and bioavailable form of candesartan (CV-11974) was used for all experiments, which was dissolved in 0.9% NaCl and 50 mM Na_2CO_3. Amlodipine was mixed with the powdered high fat diet using a food blender (Fisher Scientific). The final dose of amlodipine administered to the mice was 7.5 mg/kg/day.

The Western-type or saturated fat enriched diet (total caloric content 0.15% cholesterol, 42% fat) used in all experiments was purchased from Teklad, Inc. (TD 88137) in either pellet or powder form. The components per kilogram as listed by the manufacturer are as follows: 195 g high protein casein, 3 g DL-methionine, 341.46 g sucrose, 150 g corn starch, 210 g anhydrous milkfat, 1.5 g cholesterol, 50 g cellulose, 35 g mineral mix (AIN-76), 4 g calcium carbonate, 10 g vitamin mix, and 0.04 g ethoxyquin.

Systolic blood pressure was measured before the start of treatment, once a month thereafter, and just prior to sacrifice using a computerized, non-invasive, tail-cuff method (BP2000, Visitech). One set of 10 measurements was acquired for all animals and the mean blood pressure was calculated. All animals were acclimated to the machine before taking measurements to ensure accuracy.

2.2. Morphological evaluation

For the morphological endpoint we divided 50 apoE-deficient mice into five weight-matched groups. The first three groups were treated for 4 months as follows: (1) standard chow diet (Purina, Certified Rodent Diet), (2) pelleted high fat diet, and (3) pelleted high fat diet with candesartan treatment (0.5 mg/kg/day SC). The remaining 20 mice were split into two groups and treated for 6 months as follows: (4) pelleted high fat diet, (5) pelleted high fat diet with candesartan treatment (0.5 mg/kg/day SC) for the last 2 months only. In a similar experiment we divided 30 apoE-deficient mice into three groups as follows: (1) standard chow diet, (2) pulverized high fat diet fed through individual feeder/mesh cylinders (Allentown Caging Equipment Co., Allentown, NJ), and (3) pulverized high fat diet blended with amlodipine (7.5 mg/kg/day) using individual feeders.

Animals were euthanized by CO_2 inhalation at each designated time point. The animals were then perfused with 0.9% saline by cardiac puncture, followed by pressure fixation at 100 mm Hg with a 4% phosphate buffered formalin solution. Atherosclerotic plaque area in the descending aortas was measured as previously described [2]. Briefly, the aorta was opened longitudinally from the origin of the descending thoracic aorta to the renal arteries. Digital images were acquired and the extent of atherosclerosis was expressed as the percentage of the lumen surface area covered with atherosclerotic plaque.

2.3. Gene expression

Using the same experimental groups described above, mice were treated for 2 weeks, with blood pressures assessed at 0, 7 and 14 days. The mice were euthanized by CO_2 and their aortas were quickly harvested, snap frozen in liquid nitrogen, homogenized in Buffer RLT (Qiagen) and the homogenates were passed through a QiaShredder spin column to ensure complete homogenization of the samples. RNA was extracted and purified using the Qiagen Rneasy Mini protocol for animal tissue. All gene expression was quantified using real-time PCR (Roche light-cycler). The primers used for real-time PCR experiments were obtained from Sigma/Genosys and are as follows: MCP1-upstream primer: 5′-ACTCTCACTGAAGCCAGCTC-3′; MCP-1 downstream primer: 5′-CAGAGAGGGAAAAATGGATC-3′; PAI-1 upstream, 5′-ACAGCCAACAAGAGCCAATC-3′, downstream primer, 5′-GACACGCCATAGGGAGAGAA-3′; 18S rRNA PCR upstream primer, 5′-GAACGTCTGCCCTATCAACT-3′; downstream primer, 5′-CCAAGATCCAACTACGAGCT-3′. Superscript II, Platinum Taq polymerase, RnaseOut, and all other PCR components were purchased from Invitrogen/Life Technologies. cDNA was purified using the QIAquick PCR Purification kit (Qiagen), with an additional ethanol washing step added. Standards for each gene were created using conventional PCR and tested for purity by gel electrophoresis. All gene expression was normalized to 18s mRNA levels and all samples were run in triplicate and averaged.

2.4. ROS production in aortic ring segments

H_2O_2 production was measured using an Amplex Red assay kit purchased from Molecular Probes, Inc using the manufacturer's instructions except that Krebs–Ringers–Phosphate–Glucose (KRPG) buffer (145 mM NaCl, 5.7 mM NaH_2PO_4, 4.9 mM KCl, 0.5 mM $CaCl_2$, 1.2 mM