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Endogenous activated angiotensin-(1-7) plays a protective effect against atherosclerotic plaques unstability in high fat diet fed ApoE knockout mice

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ABSTRACT

Background and objective: We recently found that exogenous angiotensin-(1-7) [Ang-(1-7)] inhibits Angiotensin II (Ang-II)-induced atherosclerotic lesion formation and enhances plaque stability. Our objective was to evaluate the role of endogenous activated Ang-(1-7) during atherosclerosis.

Methods and results: In mice, the effects of endogenous Ang-(1-7) on atherogenesis in early stage and plaque stability in late stage were observed in ApoE knockout (ApoE −/−) mice fed with a high fat diet. Blockage of endogenous Ang-(1-7) with A779, an Ang-(1-7) antagonist, did not increase early plaque lesion formation, however, it remarkably enhanced contents of lipids and macrophages and decreased contents of vascular smooth muscle cells (VSMCs) and collagens in late lesions. The expressions of proinflammatory cytokines, and the expressions and activities of matrix metalloproteinases were significantly elevated in A779-treated group than those in vehicle-treated group in late lesions. Exogenous Ang-(1-7) treatment attenuated early atherosclerotic plaque formation and enhanced late plaques stability in this model. The contents of Ang-II and Ang-(1-7) and activity of ACE2 in late atherosclerotic plaques were higher than those of early atherosclerotic lesions.

Conclusion: Endogenous activated Ang-(1-7) enhanced late atherosclerotic plaques stability but did not affect early atherosclerotic plaque formation. Therapies to elevate endogenous Ang-(1-7) may be a potentially effective approach to attenuate atherosclerotic plaques vulnerability.

The renin–angiotensin system (RAS) has been implicated in the pathogenesis of a number of vascular diseases such as hypertension, stroke, heart failure and atherosclerosis [1]. Among the peptides of the RAS family, angiotensin II (Ang-II) is the key cytokine contributing to the formation and progression of atherosclerosis [2]. Recent studies have found that angiotensin-converting enzyme-2 (ACE2) and angiotensin 1-7 [Ang-(1-7)], two new components of the RAS, exhibit potential therapeutic benefit for Ang-II-related cardiovascular diseases [3,4]. Sluimer JC et al. reported that ACE2 activity was higher in human ruptured atherosclerotic lesions, compared to stable atherosclerotic lesions [5]. We have previously reported that exogenous ACE2 gene overexpression effectively inhibited atherosclerotic lesion formation and stabilized plaques in two animal models [6,7]. Further studies in our laboratory found that chronic treatment with exogenous Ang-(1-7) dose-dependently inhibited Ang-II-induced atherosclerotic lesion formation and enhances plaque stability by targeting vascular smooth muscle cells (VSMCs) and macrophages.

Despite these research efforts, a series of problems should be clarified. First, whether the levels of endogenous Ang-(1-7) in early and late atherosclerotic plaques were similar in high fat diet ApoE −/− mice is still unclear; second, the effects of endogenous Ang-(1-7) on atherosclerotic plaque formation and plaque stability is unknown. The present study was designed to test the hypothesis that endogenous Ang-(1-7) may play a protective role in atherosclerotic lesion formation and plaque stability. In the present study, we performed a series experiments to determine whether blockage of endogenous Ang-(1-7) with A779, an Ang-(1-7) receptor antagonist, enhances atherosclerosis and reveals evidence for Ang-(1-7) as an endogenous protective factor during atherosclerosis, and whether treatment with exogenous Ang-(1-7) prevents atherosclerosis in high fat diet fed ApoE −/− mice. The effects of A779 and Ang-(1-7) on VSMCs proliferation activity, plaque inflammation and matrix metalloproteinases activities in vivo were also investigated as the potential mechanisms for the protection. Taken together, the results show for the first time whether endogenous Ang-(1-7) produces protection against atherosclerotic plaque formation and plaque stability in high fat diet fed ApoE −/− mice.

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1. Materials and methods

1.1. Animal model

Male ApoE−/− mice (n = 108), aged 8 weeks and weighing 20–22 g, were used in the present study. The mice were obtained from Peking University (Peking University, Beijing, China). All animals were conventionally housed in standard cages and kept on a 12-hour light/12-hour dark cycle with food and water freely available. All the animals received a high-fat diet (15% cocoa butter and 0.25% cholesterol) during the entire experimental period. In the first part (part I) of the study, after 4 weeks of high fat diet, 36 mice were randomly divided into 3 groups: vehicle-treated group, Ang-(1-7)-treated group and A779-treated group (n = 12 in each group). In the second part (part II) of the study, 72 mice were randomly divided into the aortic plaque group and the carotid plaque group (n = 36, each). Mice in the aortic plaque group were randomly divided into 3 subgroups: vehicle-treated group (n = 12), Ang-(1-7)-treated group (n = 12) and A779-treated group (n = 12). Mice in the carotid plaque group received a constrictive silastic tube around the right common carotid artery as previously described and were also randomly divided into 3 subgroups: vehicle-treated group (n = 12), Ang-(1-7)-treated group (n = 12) and A779-treated group (n = 12). The mice were implanted with a subcutaneous osmotic pump (Alzet model 2004, Alza Corp., Palo Alto, CA, USA) that delivered saline, Ang-(1-7) (400 $ng \cdot kg^{-1} \cdot min^{-1}$) or A779 (400 $ng \cdot kg^{-1} \cdot min^{-1}$) for 4 weeks. Thereafter these mice underwent euthanasia for pathological studies. The experimental procedure was demonstrated in Supplemental Fig. 1. The doses of Ang-(1-7) and A779 were chosen on the basis of our previous study [8]. The animal experimental protocol complied with the Animal Management Rules of the Chinese Ministry of Health and approved by Animal Care Committee of Qilu Hospital, Shandong University.

1.2. Measurement of body weight and blood pressure

Body weight and blood pressure were obtained at the end of the experiment and before euthanasia. Systolic and diastolic blood pressures were measured by use of a noninvasive tail-cuff system (Softron BP-98A, Tokyo, Japan).

1.3. Serum lipid assay

At the end of the experiment, serum levels of total cholesterol (TC) and triglyceride (TG) were measured by enzymatic assay. Low-density lipoprotein-cholesterol (LDL-C) was calculated according to Fredewald's formula. High-density lipoprotein-cholesterol (HDL-C) was determined after precipitation of apolipoprotein B by enzymatic methods.

1.4. Histopathology and immunohistochemistry

Histopathological slides were analyzed by use of the computer-assisted morphometric analysis system. The extent and the cross-sectional area of the aortic lesions were measured. The relative contents of lipids, VSMCs, collagen, macrophages, inflammatory cytokines and MMPs were quantitated and the vulnerability index was calculated. The vulnerable index was calculated by (macrophage staining % + lipid staining %)/(VSMCs staining % + collagen staining %). The primary antibodies included those against macrophage-specific antigen (MOMA-2, diluted 1:150; Abcam), α-smooth muscle cell actin (α-actin, diluted 1:200; Abcam), SM22α (diluted 1:200; Abcam), tumor necrosis factor-α (TNF-α, diluted 1:200; Abcam), interleukin-6 (IL-6, diluted 1:200; Abcam), monocyte chemotactic protein-1 (MCP-1, diluted 1:100; Abcam), matrix metalloproteinase-2 (MMP-2, diluted 1:150; Abcam), and matrix metalloproteinase-9 (MMP-9, diluted 1:150; Abcam). Histopathological slides were analyzed by use of the computer-assisted morphometric analysis system Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

1.5. Zymography

The activities of MMP-2 and MMP-9 in aortas were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) zymography as previously described [9]. In control groups, GM6001 (2 μmol/L) was added to the final incubations of the gels to inhibit MMPs activity.

1.6. Western blot analysis

The protein expression of signaling molecules was assessed using specific antibodies (anti-ERK1/2, 1:1000, Cell Signaling Technology; anti-P38,1:2000, Cell Signaling Technology; anti-JAK2, 1:2000, Cell Abcam; anti-STAT3, 1:2000, Abcam; anti-phospho-ERK1/2, 1:1000, Cell Signaling Technology; anti-phospho-P38,1:2000, Cell Signaling Technology; anti-phospho-JAK2,1:1000, Abcam; anti-phospho-STAT3,1:1000, Abcam; anti-PCNA, diluted 1:100, Abcam and anti-β-actin, 1:10 000, Jingmei Technology).

1.7. Measurement of Ang-II and Ang-(1-7) levels

The tissue levels of Ang-II and Ang-(1-7) in the aortic lesions of both vehicle-treated groups in the first and third parts of the in vivo study were determined using a previously described method [6]. In brief, vascular lesions were homogenized in ice-cold buffer containing a protease inhibitor cocktail (Sigma Chemical, St. Louis, MO, USA). Then the insoluble fraction was discarded after centrifugation and the soluble fraction was stored in aliquots at −80 °C. The protein concentration was determined using a Bio-Rad protein assay kit, with BSA as a standard. The Ang-II levels of the aortic lesions were measured by RIA (Uscnlife, Wuhan, China) and Ang-(1-7) levels were detected using an ELISA kit (Uscnlife, Wuhan, China).

1.8. Measurement of ACE2 activity

ACE2 activity in the aortic lesions of both vehicle-treated groups in the first and third part of the in vivo study was determined by a fluorometric method as described previously [8]. 7-Mca-YVADAPK (Dnp) (R&D Systems, Minneapolis, USA), which can be cleaved by ACE2, was used as a fluorogenic substrate. An amount of 10 μg total protein was incubated with 1.0 μmol/l 7-Mca-YVADAPK (Dnp) in a final volume of 100 μl reaction buffer at room temperature. EDTA (1 mmol/l) and mouse ACE2 (25 ng) (R&D Systems, Minneapolis, USA) were used as negative and positive controls, respectively. After 4 h incubation, fluorescence kinetics was measured by use of Varioskan Flash (Thermo Scientific, USA) at an excitation wavelength of 320 nm and an emission wavelength of 400 nm. The difference in fluorescence with or without the ACE2 inhibitor DX600 (1 μmol/l, Phoenix Pharmaceuticals, Belmont, CA, USA) was defined as ACE2 activity. Data was acquired from triplicate wells and shown as fluorescence unit per hour and normalized to milligram tissue protein.

1.9. Statistical analysis

SPSS 15.0 for windows (SPSS, Inc., Chicago, IL) was used for statistical analysis. All data were expressed as mean ± SD. Differences among multiple means were evaluated by ANOVA test with LSD post-hoc analysis.