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Cortistatin attenuates angiotensin II-induced abdominal aortic aneurysm through inactivation of the ERK1/2 signaling pathways

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ABSTRACT

Abdominal aortic aneurysm (AAA) is a fatal disease that is associated with chronic inflammation in the vessel wall. Cortistatin is implicated in inflammation, vascular smooth muscle cell migration and other cardiovascular pathologies. But, the hypothetical effect of cortistatin on AAA remains uncertain. We investigated the effect of cortistatin administration to angiotensin (Ang) II-induced AAA formation in apolipoprotein E deficient (Apoe$^{-/-}$) mice. We showed that cortistatin administration significantly suppresses incidence and severity of AAA in Apoe$^{-/-}$ mice. A significant increase in macrophage infiltration, excretion of inflammatory cytokines, activities and expression levels of MMP2 and MMP9, reactive oxygen species levels and cell apoptosis in aneurysmal aortic wall of Apoe$^{-/-}$ mice infused with Ang-II, and these events were significantly alleviated by co-treatment with cortistatin. Mechanistic studies showed that the protective effects of cortistatin were related to the blocking of ERK1/2 signaling pathways, while does not was not actually affect JNK, P38 phosphorylation.

In conclusion, cortistatin appears to play an essential role in the formation of AAA and indicate cortistatin may as novel therapeutic option for AAA.

1. Introduction

Abdominal aortic aneurysm (AAA) is an age-related fatal cardiovascular disease that characterized by vascular chronic inflammatory condition [1]. Effective pharmacological treatments to limit aneurysm initiation and progression is an attractive approach [2]. Hence, it is imperative for elucidation the underlying mechanisms of formation and progression of AAA. Notably, recent studies have demonstrated that vascular inflammation is a central process of AAA [3], indicating that get command of correlative malgenic inflammatory mechanism could be a possible therapeutic strategies for preventing AAA.

Cortistatin is a recently discovered cyclic neuropeptide and predominantly cortical expression that has been detected in various types of tissues and organs in the body including the heart, arterial blood vessels, and immune system [4]. However, several recent studies have indicated how cortistatin played an important role in the cardiovascular system. Cortistatin reduce the incidence of vascular occlusive disease by inhibiting the migration and proliferation of smooth muscle cell (SMC) in the intima and medial layer [5]. Furthermore, recent research has shown that cortistatin can reduce the formation of atherosclerotic plaques by inhibiting the infiltration of macrophages in endothelial cells [6]. These findings suggested that cortistatin has a potential role in the cardiovascular system. However, there is little is known about the potential role of cortistatin in the AAA. The present study were sought to explore the possible effects of cortistatin on AAA pathobiology.

2. Materials and methods

2.1. Establishment of the angiotensin (Ang) II-induced mouse AAA model

Approval for animal studies was obtained from the local ethics committee and experimental work performed in accordance with the institutional and ethical guidelines of Institutional Animal Care and Use Committee of Nanjing Medical University. Male apolipoprotein E deficient (Apoe$^{-/-}$) mice (8 weeks of age) received 1000 ng/kg/min of AngII (Sigma-Aldrich) vehicle via implanted with subcutaneous osmotic mini-pumps (Alzet Model 2004, Durect

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Corp.) for persisted up to 4 weeks as previously described [7]. Cortistatin or control saline vehicle was administered intraperitoneally every two days at a dose of 1 nmol starting 1 week before AngII infusion commenced and lasted for 4 weeks.

2.2. Measurements of blood pressure and plasma cholesterol

Systolic blood pressure (SBP) was measured by using a non-preheating MK-2000ST system (Muromachi Kikai Corp.). Conscious mice were placed in warming chamber and acclimated to the instrument before measurement. Total plasma total cholesterol (TC) levels were assayed by using a biochemistry automatic analyzer (HITACHI 7170A, Hitachi, Tokyo, Japan).

2.3. Analysis and quantification of AAA

Ultrasound imaging of the aorta were acquired in mice anesthetized with sufficiently isoflurane, using a small animal ultrasonography system (Vevo 770, Fujifilm VisualSonics Inc.) equipped with a highfrequency ultrasound device (RMV 704; Visual Sonics, Toronto, Canada). Abdominal aortas with external diameters $\geq$1.5 mm were considered as an aneurysm. For AAA incidence, the maximum diameter of the abdominal aorta was measured by Image Pro Plus software (Media Cybernetics, Bethesda, MD).

2.4. Elastin staining

Paraffin sections (5 μm) were prepared and subsequently stained with Verhoeff-Van Gieson (EVG) according to manufacturer's instruction. As described previously [8], for determination of elastin degradation, we used a scoring system for grades: Grade 1, no degradation; Grade 2, mild elastin degradation; Grade 3, severe elastin degradation; Grade 4, Aortic rupture.

2.5. Gelatin zymography and in situ zymography

Measurement of MMP2 and MMP9 in mouse aortic tissue using gelatin zymography was performed as previously described [8]. Extracted protein from mice aortas was separated on a 10% acrylamide-SDS gel containing 1 mg/ml of gelatin at room temperature. Proteins were renatured in 2.5% (vol/vol) Triton X-100, subsequently gels was incubated for 48 h in 50 m MTris (pH 8), 5 mM $CaCl_2$ at 37 °C. Following staining with 0.5% Coomassie brilliant blue, bands were visualized in destaining with a 10% ethanol/10% acetic acid solution. Band intensities were quantified by Image-Pro Plus software (Media Cybernetics, USA). For in situ zymography, freshly cut frozen sections (10 lm) were incubated with a fluorogenic gelatin substrate (DQ gelatin; Invitrogen) at 37 °C. Proteolytic activity was detected as green fluorescence with microscopy (IX70; Olympus, Japan).

2.6. Immunofluorescence staining

The frozen tissues were sliced to 5 μm section for immunofluorescence staining. Sections were fixed in 4% phosphate-buffered paraformaldehyde at −20 °C for 20 min and then blocked with 5% normal goat serum in PBS for 60 min at room temperature. Subsequently incubated for 16 h under 4 °C with applied the following primary antibodies respective: mouse anti-CD68 (1:500, Abcam), mouse anti-SM α-actin (1:400; Sigma-Aldrich), rabbit anti-mmp2 (1:500, Abcam), mouse anti-MCP-1(1:500, Santa Cruz Biotechnology), rabbit anti-mmp9 (1:500, Abcam), rabbit anti-il-6 (1:500, Abcam), rabbit anti-il-6 antibody (1:300, Abcam), rabbit anti-TNF-α (1:500, Abcam). And then the sections were incubated in secondary antibodies: Alexa Fluor 488 goat anti-mouse IgG (1:500; Invitrogen), Alexa Fluor 594 goat anti-rabbit IgG (1:500; Invitrogen) for 1 h. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, 1:30 in PBS, Sigma).

2.7. Immunohistochemistry

Paraffin-embedded aortae were cut into 6-μm sections. After deparaffinization, incubated with 3% H_2O_2 for 10 min at room temperature, then the sections were blocked with 5% normal goat serum for 30 min and incubated with primary antibodies against SM α-actin (1:400; Sigma-Aldrich), rabbit anti-cleaved caspase-3 (1:200; CST), and rabbit anti-p-ERK (1:200; CST), The sections were then incubated with MaxVisionTM IHC Kit (Fuzhou Maixin Biotech.) All sections were colorized with diaminobenzidine (DAB, Vector Laboratories) and counterstained with Mayer's hematoxylin (Histolab Products, Goteborg, Sweden).

2.8. TUNEL assay

TUNEL staining was assessed by a detection kit according to the manufacturer's instruction (Roche Applied Science, Indianapolis, USA). The tissue sections were incubated with proteinase K for 15 min at room temperature. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature. Sections were immersed in terminal deoxynucleotidyl transferase (TdT) buffer incubated in a humid atmosphere at 37 °C for 90 min. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody and the signals were visualized with diaminobenzidine.

2.9. Detection of reactive oxygen species (ROS)

ROS generation was assessed in fresh-frozen sections of the aortas by exposing to dihydroethidium (DHE) (Wako Chemicals). Freshly prepared frozen aortic sections (10 μm) were incubated with DHE (5 mmol/L) at 37 °C for 30 min. The DHE fluorescence intensity were observed by fluorescence inverted microscope (model DP71; Olympus America Inc.) to reveal the presence of ROS as red fluorescence (585 nm). The red fluorescence intensity was quantified by using NIH ImageJ software.

2.10. Western blot analysis

Tissue samples and lysing cells were extracted as per the manufacturer's protocol (Boster Biological). Briefly, equal amounts of extracts were separated by electrophoresed on 10% SDS-PAGE, transferred onto PVDF membranes. After blocking with 5% (w/v) bovine serum albumin (BSA), the membranes were exposed to with a primary antibody at 4 °C overnight. After incubation with peroxidase-conjugated secondary antibodies at room temperature for 1 h. Blots were developed with enhanced chemiluminescence detection reagent. Signals were quantified by using Quantity One software (Bio-Rad, Hercules). The antibodies for ERK (1:1000; CST), p-ERK (1:1000; CST), p38 (1:1000; CST), p-p38 (1:1000; CST), JNK (1:1000; CST), p-JNK (1:1000; CST), GAPDH (1:5000, Abcam).

2.11. Cell lines and culture

The mouse SMC-line mouse vascular smooth muscle (MOVAS) cells were cultured in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% carbon dioxide atmosphere. For the current experiments, cells were incubated with or not cortistatin (100 nM) followed by 1 μM AngII stimulation for 24 h [9].

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