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Lactones from *Ligusticum chuanxiong* Hort. reduces atherosclerotic lesions in apoE-deficient mice *via* inhibiting over expression of NF-kB -dependent adhesion molecules

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ABSTRACT

The present study aims to investigate the anti-atherosclerotic effects of lactones extracted from *Ligusticum chuanxiong* Hort (LLC) in apoE-deficient mice (ApoE$^{-/-}$ mice) and proclaim its underlying mechanisms. Expression of endothelial adhesion molecules and NF-κB around the atherosclerotic lesions was detected by immunohistochemistry (IHC). To further validate the mechanism, effect of LLC on the secretion of ICAM-1 and VCAM-1 of human umbilical vein endothelial cells (HUVECs) induced by tumor necrosis factor α (TNF-α) was measured by ELISA. And the activation of NF-κB was detected by western blot. Mice treated with LLC showed significant reduction in lesion sizes of thoracic segments of the aorta ($p < 0.01$). Meanwhile, LLC treatments lead to decreases of serum TG, TC and LDL-C contents, respectively. LLC also decreased the expression of CD31, intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1) and nuclear factor-kappa B (NF-κB) in the atherosclerotic plaque. Moreover, LLC at 3.125–25 μg/mL can dose-dependently attenuate the expression of ICAM-1 and VCAM-1 in TNF-α stimulated HUVECs. Western blot result indicated LLC inhibited activation of NF-κB. These results suggested that LLC could ameliorate atherosclerosis in ApoE$^{-/-}$ mice. The mechanism of action of LLC on anti-atherosclerotic effect may be attributed to the suppression of the production of NF-κB-dependent adhesion molecules.

1. Introduction

Atherosclerosis (AS) is the major form of cardiovascular and cerebrovascular diseases, which caused the highest incidence of mortality worldwide [1]. Endothelial cells, vascular smooth muscle cells, and leukocytes are major players in the development of atherosclerosis [2]. And it is a multi-factorial disease with an important inflammatory component, associated with changes in plasma lipid profile. Endothelial adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) are indicative of inflammatory processes [3]. These molecules are transcriptionally regulated by NF-κB [4]. In hypercholesterolemic animals, both ICAM-1 and VCAM-1 are up-regulated even before the atherosclerotic lesions are formed, and their contents increase proportionally to the extent of plaque [5].

Ligusticum chuanxiong Hort, belonging to *Umbelliferae* family, *Ligustrum* genus [6], has been widely used for many years to treat various cardiovascular and cerebrovascular diseases. The major chemical constituents of *L.chuanxiong*

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include ligustrazine, volatile oil, and ferulic acid [7]. Ligustrazine is the commonly used component for its strong effect of anti-vasoconstriction and scavenging oxygen free radicals [8]. The volatile oil, however, has been reported to have many significant effects on relieving spasm [9], neuroprotective effect [10,11] and some other cerebrovascular effects. In 1995, *Wang* and his colleague reported that extracts of *L.chuanxiong* could decrease cholesterol and low density lipoprotein content in experimental atherosclerotic rabbits [12], whereas the mechanism of action of lactones from *L.chuanxiong* on atherosclerosis is still uncertain.

In this study, we examined the therapeutic effect of lactones from *L.chuanxiong* (LLC) on atherosclerosis in apolipoprotein E-knockout mice. To investigate the mechanisms underlying the inhibition of atherogenesis by LLC, production of adhesion molecules both *in vitro* and *in vivo* were measured. According to the results, we concluded that LLC played anti-atherosclerotic effect through inhibition of NF-κB activation and then resulting in down regulation of adhesion molecules.

2. Materials and methods

2.1. Materials

Medium 199 (M199) and fetal bovine serum (FBS) were from GIBCO-BRL (USA). Anti-ICAM-1 pAb (Abnova), CD31 and Rb pAb NF-κB p65 (Abcam), MCP-1 (Rodent) Antibody (ABBIOTEC) were from Santa Cruz Biotechnology. Recombinant human TNF-α was purchased from Prospec (Israel). NF-κB p65 antibody used for western blot was purchased from Cell Signaling Technology (USA).

2.1.1. Preparation of LLC

LLC was extracted and separated in Zhejiang University. A detailed description on preparation and chemical characterization of LLC can be found in the Supplementary Materials. For animal experiment, LLC was dissolved into 0.5% CMC-Na. And as to the cell experiment, LLC was dissolved in dimethyl sulfoxide (DMSO) as stock solution; the final DMSO concentration in the experiment did not exceed 0.1% (v/v).

2.2. Animals and treatment

All animals were provided by Medical Sciences of Beijing University and housed in standard conditions. 6 week old $ApoE^{-/-}$ mice were fed with normal chow diet for 4 weeks, after which the diet was changed to high fat diet content (77.8% stock blend, 10% custard powder, 10% pork fat, 1.0% cholesterol and 0.2% pork chocolate) for 12 weeks. And the C57BL/6 J mice were fed with normal chow diet for 16 weeks. Then $ApoE^{-/-}$ mice were divided into 3 groups (Model, LLC high dose, and LLC low dose), while C57BL/6 J mice were used as control. For LLC treatment, the animals were orally given 30 mg/kg and 60 mg/kg LLC (dissolved in 0.5% CMC-Na) for 3 months successively. Mice in model and control group were treated orally with 0.5% CMC-Na. All of the protocols were approved by the Ethics Committee for Animal Experiments of Tianjin University of Traditional Chinese Medicine (TJUTCM).

2.3. Determination of serum lipids level and atherosclerotic lesions

After treated for 3 months, all the animals were sacrificed. The blood was collected and centrifuged at 3500 rpm/min for 10 min. Total cholesterol (TC), triglycerides (TG), cholesterol in low density lipoproteins (LDL-C) and high density lipoproteins (HDL-C) in the serum were measured according to the manuscript's instruction (BIOSINO BIO-TECHNOLOGY AND SCIENCE INC). In each group, arotas of five mice were isolated and used for histological analysis. The aortas were fixed using 10% formaldehyde, and HE staining was used to detect the plaque in aortic arch, thoracic aorta and abdominal aorta, respectively. Results were represented using the ratio of plaque area to total vessel lumen area.

2.4. Immunohistochemistry

After fixing the aortas by 10% formaldehyde, expression of CD31, intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1) and nuclear factor-kappa B (NF-κB) in aortas were detected by immunohistochemistry.

2.5. Cell culture

Primary human umbilical vein cells (HUVECs) were isolated and grown as previously described [13]. Cells with passages between 4 and 8 were used. HUVECs were cultured in medium 199 (M199) with 12.5% fetal bovine serum (FBS), 10 ng/ml epidermal growth factor (EGF), 30 μg/ml endothelial cell growth supplement (ECGS) and 100 U/ml penicillin and streptomycin. Cell culture medium was replaced every two days while cells were passaged every 3~4 days.

2.6. Cell proliferation assay

After grown confluence, cells were digested and cultured in 96-well plates at a density of 2.0×10^4 cells/well for 24 h. After the medium was replaced with 0.1 ml of M199 without FBS for 12 h, 10 ng/ml TNF-α together with 3.125–25 μg/ml LLC were added to the cells. Cells were cultured in a humidified incubator maintained at 5% CO_2/95% room air and 37 °C for 24 h. Cell culture medium was replaced with 0.1 ml MTT (0.5 mg/ml) solution, and cultured for another 4 h. After that, the medium was removed, and the formazan crystals were dissolved in 0.1 ml DMSO. Absorbance was measured at 550 nm

2.6.1. Measurement of sICAM-1 and sVCAM-1 level by enzyme-liked immunosorbent assay

Confluent HUVECs in 6-well plates were treated with TNF-α and LLC for 24 h. The cell supernatant was collected and sICAM-1 and sVCAM-1 were detected using enzyme linked immunosorbent assay according to the manufacturer's instructions.

2.7. Western blot

After treatment with TNF-α and LLC for 24 h, HUVECs (1.0×10^6 cells/well/3 ml) were washed with cold PBS and cell nucleus proteins were extracted according to the