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Artesunate attenuated progression of atherosclerosis lesion formation alone or combined with rosuvastatin through inhibition of pro-inflammatory cytokines and pro-inflammatory chemokines

Weiwei Jiang[a,1], Yanyan Cen[a,1], Yi Song[a], Pan Li[a], Rongxin Qin[a], Chao Liu[a], Yibo Zhao[a], Jiang Zheng[b], Hong Zhou[a,*]

[a] Department of Pharmacology, College of Medicine, The Third Military Medical University, Chongqing 400038, P. R. China
[b] Medical Research Center, Southwestern Hospital, The Third Military Medical University, Chongqing 400038, P. R. China

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ABSTRACT

Backgrounds: Inflammation plays an important role in all stages of atherosclerosis, but little is known about the therapeutic effects of quenching inflammation in atherosclerotic lesions formation.
Purpose: Herein, the effect of artesunate, a derivant from artemisinin from the traditional Chinese herb sweet wormwood, could attenuate the progression of atherosclerosis lesion formation alone or combined with rosuvastatin in Western-type diet (WD) fed ApoE$^{-/-}$ mice, and explored its possible mechanisms.
Methods: The methods such as ELISA for plasma lipids and cytokines analyses, qRT-PCR and western blot for mRNA and protein expressions, and MTT assay for human umbilical vein endothelial cells (HUVECs) viability were used for in vivo and in vitro experiments.
Results: Artesunate could attenuate the progression of atherosclerosis lesion formation alone or combined with rosuvastatin in WD fed ApoE$^{-/-}$ mice without changes in food uptake, body weight and plasma lipids level, but with a significant reduction of pro-inflammatory cytokine, such as TNF-α and IL-6. Furthermore, artesunate could down-regulate the pro-inflammatory chemokines such as IL-8 and MCP-1 in aorta of mice. Besides, artesunate didn't influence IL-8 and MCP-1 secretion in HUVECs up-regulated by TNF-α, but inhibited IL-8 and MCP-1 secretion up-regulated by LPS.
Conclusion: AS attenuated progression of atherosclerosis lesion formation alone or combined with rosuvastatin through anti-inflammatory effect, resulting in down-regulation of TNF-α and IL-6, and further down-regulating IL-8 and MCP-1 expressions in aorta of WD fed ApoE$^{-/-}$ mice. Rosuvastatin combined with artesunate could more effectively attenuate the progression of atherosclerosis lesions than when treated by one of them, demonstrating that lipid-lowering agents combined with anti-inflammatory agents could provide the greater benefit for cardiovascular disease patients. Artesunate is worth further investigating as a candidate drug for the treatment of atherosclerosis.

Introduction

Atherosclerosis is a chronic vascular disease which involves the progressive occlusion of blood vessels. It is characterized by sub-endothelial accumulation of inflammatory cells and lipids, which collectively contribute to occlusive disease or to less occlusive plaques at high risk for disruption (Ross R, 1999; Joana V and Oliver S, 2015).

Hypercholesterolemia is widely recognized as an important risk factor for atherosclerosis development. Animal studies have shown that reduced hypercholesterolemia strongly decreases the number of lesion macrophage foam cells within a few days. Later in time, a reduction of plaque size is observed (Kapourchali FR, et al, 2014). In humans, non-invasive imaging confirmed that plasma cholesterol-lowering alone can promote atherosclerosis regression (Bruckert E.et al, 2014). Statins, widely used for the treatment of atherosclerosis, are a group of 3-hydroxy-3-methylglutaryl

Abbreviations: ApoE, apolipoprotein e; WD, western-type diet; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; LPS, Lipopolysaccharide.

* Corresponding author: Department of Pharmacology, College of Medicine, The Third Military Medical University, Gaotanyan Street 30, Shapingba District, Chongqing, P. R. China., Tel./fax: +86 23 6875 2366.
E-mail address: zhouh64@163.com (H. Zhou).
[1] They equally contribute to this work.

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coenzyme A reductase inhibitors based on their lipid-lowering properties via blocking cholesterol biosynthesis.

It is also confirmed that atherosclerotic plaque development is an inflammation-driven condition (Buttari B, et al. 2015). The host inflammatory response and resultant cellular and soluble mediator mobilization is critical in innate immune responses and crucial for host defense against infections. However, in the face of unrelenting persistent inflammation, the initiation, progression, and degenerative features of chronic diseases like native atherosclerosis or the vasculopathy of autoimmune disorders like rheumatoid arthritis may appear (Galkina E and Ley K, 2009; Tall AR, et al, 2015). One of the key early events in the pathogenesis of atherosclerosis is inflammation-triggered endothelial activation that leads to the attraction and adhesion of monocytes to the endothelium followed by their transmigration into the subendothelial space. This process is primarily mediated by several intracellular signaling events, leading to the elevated expression of a number of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and several pro-inflammatory chemokines such as interleukin-8 (IL-8) and monocyte chemoattractant rotein-1 (MCP-1) (Ramji DP and Davies TS, 2015; Drechsler M, et al. 2015).

In recent years, there has been growing interest on the anti-inflammatory effects of natural components existed in commonly used traditional herbs. Artemisinin is an active ingredient in sweet wormwood, a Traditional Chinese Medicine (Tu Y, 2011). Artemisinin and its derivates such as artesunate (AS), hydroartemisinin, artemether and arteether have been clinically used to treat malaria. AS is a water-soluble hemisuccinate derivative of dihydroartemisinin, and also possesses anti-cancer, anti-angiogenesis and anti-inflammatory activities in human rheumatoid arthritis fibroblast-like synoviocytes (Lai HC, et al, 2014; Wang Z, et al, 2012; Wang HY, et al, 2014.) In our previous experiments, we found AS could protect sepsis model mice challenged with a heat-killed *E. coli* and *staphylococcus aureus* via reduction of inflammation (Jiang W, et al, 2011; Li B, et al, 2010).On the other hand, the modulate atherosclerosis effects of AS have been reported previously such as via inhibition of type I IFN in systemic lupus erythematosus patients (Gu F, et al, 2014) or inhibition of STAT1 (Feng XB, et al, 2015).

As mentioned above, unrelenting persistent inflammation may result in the initiation and progression of atherosclerosis. Therefore, was considered whether it could attenuate the progression of atherosclerosis lesion formation through its anti-inflammatory effect. With these considerations in mind, we undertook the current study to investigate the effects of alone or combined with rosuvastatin in WD fed ApoE$^{-/-}$ mice, and its possible mechanisms.

Materials and methods

Materials

Injectable artesunate (AS) was purchased from Guilin Nanyao Ltd (Guangxi, China), no endotoxin was detected. Rosuvastatin calcium (Rosuvastatin) was purchased from AstraZeneca UK limited. Oil red stain and Movat's stain were purchased from Senbeijia Biological Inc (Nanjing, China). Enhanced chemiluminescence reagents were purchased from Pierce, Inc. (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Mice ELISA kits for plasma lipids were purchased from Senbeijia Biological Inc (Nanjing, China). Mouse ELISA kits for TNF-α and IL-6 and human ELISA kits for MCP-1 and IL-8 were purchased from Boster Ltd (Wuhan, China). Avian myeloblastosis virus (AMV) reverse transcriptase and T4 polynucleotide kinase were purchased from Promega (Madison, WI, USA). Quantitative real-time PCR (qRT-PCR) Master Mix was purchased from ToYoBo Ltd (Osaka, Japan). Total Protein Extraction Kit was purchased from BestBio Ltd (Shanghai, China). Antibodies for western blot were all purchased from Santa Cruz Biotechnology, Inc (CA, US).

All primers were synthesized by Invitrogene Ltd (Shanghai, China). Cell culture medium and fetal bovine plasma were obtained from Invitrogen (Life Technologies Corporation, Carlsbad, CA, USA).

Mice

ApoE$^{-/-}$ mice lack the gene encoding apolipoprotein E (ApoE) and then spontaneously develops hypercholesterolemia, and atherosclerotic lesions similar to those found in human (Zhang S, 1992). Herein, C57BL/6 J ApoE$^{-/-}$ mice were purchased from the Model Animal Research Center MARC at Nanjing University (body weight 16 – 20 g, 4 – 6 weeks old). All procedures were approved by the committee on Ethics of Animal Experiment of The Third Military University of Medicine, in accordance with the Guide for the Care and Use of Laboratory Animals.

Mice were housed individually in wire-bottomed cages in a temperature controlled room (22 ± 0.8 °C) with a 12 h light-dark cycle and a relative humidity of 55% ± 10%. All mice were fed with an atherogenic WD containing 21% calorie from fat and 0.2% cholesterol. Mice had an ad libitum access to food and water.

Mice treatment

Mice were randomly divided into six groups (6 mice/group): control group, rosuvastatin treatment group (as a positive control, 10 mg/kg/day via intragastric administration), three treatment groups (1.5, 5 and 15 mg/kg/day, intramuscular injection), and combination group (5 mg/kg/day AS combined with 10 mg/kg/day rosuvastatin). The food intake, body weight were measured every day. Six months later, after an overnight abrosia, mice were anesthetized with an intraperitoneal injection of pentobarbital, plasma samples were collected from the orbital vein.

Herein, the doses of AS and rosuvastatin were converted from the clinical dosage regimens. 60 mg of AS is proposed to treat malaria in adult patients according to the drug conversion principle applied in pharmacology studies, conversion the dose of AS used from adult to mouse, 60 mg of AS in adults is the equivalence dose of about 15 mg/kg/day in mice. Therefore, 1.5, 5 and 15 mg/kg/day of AS were used in the present experiment. Similarly, the dose of rosuvastatin is also converted from clinical dose. Commonly, the dose of 10 – 20 mg/60 kg/day is proposed for rosuvastatin to treat atherosclerosis in adult. Therefore, the equivalence dose is about 10 – 20 mg/kg/day of rosuvastatin in mouse, 10 mg/kg/day of rosuvastatin was used in the present experiment.

Tissue preparation

The aortas were immediately frozen in liquid nitrogen for RNA isolation and Western blot analysis. For RNA isolation, thoracic aortas were additionally perfused with RNA Later to prevent RNA degradation. Frozen samples of aortas were crashed on liquid nitrogen and total RNA was prepared in accordance with the manufacturer's instruction.

The plaque area determination

The aortas of three mice of each group were opened longitudinally, stained with Oil Red O, and pinned out on a white surface. The percentage of the plaque area stained by Oil Red O and the total luminal surface area was determined by Image Pro Plus analysis software (Version 6.0). Lesions were reported as percentage of the plaque area consisting of aortas.

The other three aortas of each group were used to pathological section since they were the area of greatest plaque formation