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Fumigaclavine C activates PPARγ pathway and attenuates atherogenesis in ApoE-deficient mice

Rong Hui Du [a,b,1], Si Yuan Qin [a,1], Lu Sen Shi [a], Zi Qian Zhou [a], Xi Yu Zhu [a], Juan Liu [a], Ren Xiang Tan [b], Wangsen Cao [a,c,*]

[a] Nanjing University School of Medicine, Jiangsu Key Laboratory of Molecular Medicine, Nanjing 210093, China
[b] State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, China
[c] Jinling Hospital Research Institute of Nephrology, Nanjing University School of Medicine, Nanjing 210008, China

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ABSTRACT

Objective: To develop alternative therapeutic strategy that reduces hypercholesterolemia, inflammation and atherosclerosis, we investigate if fumigaclavine C (FC), an indole alkaloid in structure, has anti-atherosclerosis function, and if so, what is the mechanism involved.
Methods and results: We used ApoE-deficient (ApoE$^{-/-}$) mice as an atherosclerosis model to examine if FC reduced aorta lesion size and improved serum lipid profiles. ApoE$^{-/-}$ mice at 6 weeks of age were fed on a western diet for 10 weeks before FC was administrated (5, 10 and 20 mg/kg) by gavage daily for additional 4 weeks. The mice were sacrificed at 20 weeks of age for examination. The atherosclerotic lesions were assessed with Oil Red O staining in the whole aorta and aortic sinus. Serum levels of triglycerides (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-c) and low density lipoprotein cholesterol (LDL-c) were determined enzymatically. Mouse macrophages were examined for lipid droplets inside cells. FC's effect on PPARγ and PPARγ signaling pathway were further investigated by western blot and luciferase assay. We found that FC decreased atherosclerotic lesion formation in ApoE$^{-/-}$ mice in a dose-dependent manner. Also FC improved lipid profiles in ApoE$^{-/-}$ mice and reduced the foam cell numbers of peritoneal macrophages. FC stimulated PPARγ signaling pathway proteins both in vitro and in vivo. FC enhanced PPARγ transactivation activity assayed by a PPRE reporter system.
Conclusion: Our data indicated that FC activated PPARγ signaling pathway as well as its downstream proteins and had an effective role of anti-atherosclerosis.
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1. Introduction

Atherosclerosis is usually characterized by progressive inflammation and abnormal lipid metabolism. Macrophages play a key role in both lipid metabolism and immune responses in atherosclerogenesis [1]. Especially, macrophage-derived foam cell formation is the hallmark of early atherosclerosis [2]. Important steps in foam cell formation include excessive cholesterol uptake from high levels of serum low density protein (LDL), accumulation of intracellular triglycerides (TG) and total cholesterol (TC), and reduction of cholesterol efflux. The intracellular cholesterol homeostasis in macrophages is dynamically regulated by cholesterol uptake and cholesterol efflux [3]. Regulation of cholesterol metabolism by macrophages is also critical in the initiation and progression of atherosclerotic lesions.

Several types of macrophage scavenger receptors (SRs), including SR-A and CD36, are responsible for the internalization of oxidized LDL (oxLDL) and promote cholesterol accumulation in macrophages [4]. CD36, the multiligand class B scavenger receptor expressed by monocytes/macrophages, has been reported to be a pivotal player in atherosclerosis development through oxLDL binding and internalization, thereby leading to macrophage-derived foam cell formation and to their accumulation into fatty streaks [5]. In contrast, the efflux of intracellular cholesterol is mediated by reverse cholesterol transporters (RCTs). Two major potential cholesterol efflux pathways from macrophages have been described: SR-BI-mediated cholesterol efflux and active cholesterol efflux mediated by the ATP-binding cassette transporter sub-family A member 1(ABCA1) and G member 1(ABCG1). ABCA1 promotes efflux of phospholipids and cholesterol to lipid-poor ApoA-I in a process that involves the direct binding of ApoA-I to the transporter

* Corresponding author. 22 Hankou Road, Nanjing University School of Medicine, Nanjing 210093, China. Tel.: +86 025 83594790.
E-mail address: wangsencao@nju.edu.cn (W. Cao).
[1] Equal contributions.

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[6]. SR-BI and ABCG1 were identified as the key mediators of macrophage cholesterol efflux to mature HDL [7].

Growing evidence suggests that peroxisome proliferator activated receptors (PPARs) exert antiatherogenic effects by enhancing cholesterol efflux via activation of the liver X receptor (LXR)–ABCA1 pathway [8,9]. PPARγ is a member of the nuclear receptor superfamily and highly expressed in macrophages including the foam cells of atherosclerotic lesions [10,11].

Recent studies show that a transcriptional cascade in the PPARγ and liver X receptor (LXR) pathways is important for maintaining cellular cholesterol homeostasis in macrophages [8,9]. It has been reported that PPARγ activation results in increased LXRα expression which in turn transactivates the target genes and reduces cell cholesterol levels [9].

Fumigaclavine C (FC, 5-(1,1-Dimethyl-allyl)-7,9-dimethy1-4,6,6a,7,8,9,10,10aoctahydro-indolo[4,3-fg]quinoline-10-carboxylic acid methyl ester) is an indole alkaloid, isolated from endophytic fungus identified as Aspergillus fumigatus CY018 in Cynodon dactylon. It was reported that FC exerted vasorelaxant effect in rat aorta, suggesting that FC might have potential protection against cardiovascular disease [12]. Furthermore, it was reported that FC had an anti-inflammatory effect [13] and inhibited the production of TNFα in mouse macrophage RAW264.7 cells [14].

In this study, we aimed to examine FC's anti-atherosclerogenesis role and to investigate the underlined mechanism. We showed that FC attenuated the formation of foam cells in mouse peritoneal macrophages and alleviated atherosclerotic lesions in ApoE$^{-/-}$ mice. Our data also showed that FC activated PPARγ, facilitated the cellular cholesterol efflux and improved cholesterol metabolism.

2. Materials and methods

2.1. Chemicals and reagents

FC was isolated as previously described [15]. Moderately oxidized LDL was purchased from Yiyuan Biochemical Corporation (Guangzhou, Guangdong, China). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). Oil Red O, GW9662, Triton X-100 and PMSF were purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). PPARγ agonist 15d-PGJ$_2$ was obtained from Tocris (Bristol, BS, UK). Antibodies against CD36 were from Gene Tex (GTX100642S, Irvine, CA, USA), PPARγ (sc-7273), LXRα (sc-13068), AdipoR2(sc-46755) and CYP7A1(sc-25536) antibodies were from Santa Cruz (Santa Cruz, CA, USA), β-actin (ab6227) and HMGR(ab174830) antibodies were from Abcam (Cambridge, MA, UK). ABCA1 (NB400-105) and ABCG1 (NB400-132) antibodies were from Novus Biological (Littleton, CO, USA), LDLR (AP8960c) antibody was from Abgent (San Diego, CA, USA).

2.2. Cell culture

RAW264.7 cell, a murine macrophage cell line and Thp1, a human monocytic cell line (ATCC, Rockville, MD), were cultured in DMEM and RPMI1640 media supplemented with 100 U/ml of penicillin G, 100 μg/ml streptomycin and 10% (vol/vol) FBS, respectively. The cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. For foam cell formation assay, cells were serum starved (1% FBS) for 24 h then treated with oxLDL for the indicated dose and time period.

2.3. Animal experiments

The animal protocol was approved by the Animal Research Committee at Nanjing University. C57BL/6 (B_6, control) and ApoE$^{-/-}$ male mice (Laboratory Animal Center Peking University, Beijing, China) at 6 weeks of age were fed with western diets containing 20% fat and 0.15% cholesterol (Qinglongshan, Jiangsu, China) for 10 weeks first according to a previously established procedure [16]. Then ApoE$^{-/-}$ mice were divided into four groups (10 animals in each group): the model group (vehicle; 0.5% sodium carboxymethylcellulose, CMC-Na) and three FC treated groups with different FC dosages (FC, 5 mg/kg, 10 mg/kg and 20 mg/kg, respectively). FC or CMC-Na was administered by oral gavage once a day for an additional 4 weeks. Then blood samples were taken to determine serum total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-c) and low density lipoprotein cholesterol (LDL-c) contents. Aortas were reserved for atherosclerotic lesion analysis. Liver samples were used for histological assessments or protein expression by western blot.

2.4. Atherosclerotic lesion analysis

At the end of the experiment, the whole aorta was prepared free of periadventitial fat by surgical procedure. The en face samples of aorta and the OCT-embedded frozen aortic valves, sectioned serially at 8 μm thickness, were stained with Oil Red O [17]. Atherosclerosis lesion in aortic valves was measured in aortic sinus spanning the region from the very proximal aorta to the point that contains 3 complete leaflets according to the method of Paigen et al. [18]. The lesion area in cross-sectional sinus was expressed as an absolute area (in μm^2) and the extent of atherosclerotic lesion in aorta was presented as the percentage of the total aortic by measuring the stained surface area using Image J software (National Institutes of Health, Bethesda, MD).

2.5. Lipid accumulation assessment

Serum TC, TG, HDL-c and LDL-c contents were determined enzymatically using a kit (Beijing BHKT Clinical Reagent Co.,Ltd, Beijing, China). The results were analyzed by an automated analyzer. The mouse livers were sequentially fixed, dehydrated, infiltrated first, and then cut into 5-mm paraffin-embedded tissue sections. The samples were stained with hematoxylin–eosin (HE) [19].

2.6. Isolation of mouse peritoneal macrophages and quantification of foam cell formation

HFHC-fed B6, B6 treated with FC (10 mg/kg), ApoE$^{-/-}$, and ApoE$^{-/-}$ mice treated with FC (10 mg/kg) for 4 weeks were used for foam cell formation assay. In brief, mouse peritoneal macrophages were collected as described before [20]. The harvested cells were cultured in RPMI1640 medium containing 10% FBS. After 2 h, the medium was changed according to different experimental procedures.

The extent of foam cell formation was determined by monitoring cell's lipid uptake using two different methods: Oil Red O staining and measurement of the internalized cholesterol level. Foam cells were quantified by counting the presence of lipid droplets inside the cells identified by Oil Red O staining [21]. Foam cells were defined as cells with ≥10 Oil Red O-positive droplets. Cholesterol content was determined by Amplex Red enzyme assay kit (Invitrogen, A12216, CA, USA) according to manufacturer's recommendation [22]. Cholesterol ester (CE) content was calculated by subtracting free cholesterol from total cholesterol for each sample. CE levels under different treatments were normalized to total cellular protein content. Cell total protein was determined by the BCA protein assay (Pierce, Rockford, USA).

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