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Crocin alleviates coronary atherosclerosis via inhibiting lipid synthesis and inducing M2 macrophage polarization

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ABSTRACT

Atherosclerosis is a chronic inflammatory disease arising from an imbalance in lipid levels and the accumulation of cholesterol-laden macrophages in the artery wall. Crocin is an active ingredient of *Crocus sativus* L. This study established a rat coronary atherosclerosis model induced by vitamin D3 (VD3), to explore the effect of Crocin on lipid metabolism, macrophage polarization and the activity of inflammatory proteins. The results revealed that Crocin decreased blood lipid levels by decreasing the levels of endothelin (ET), total cholesterol (TC), triglyceridelow (TG) and low-density lipoprotein cholesterol (LDL-c), elevating the level of high-density lipoprotein cholesterin (HDL-c). Crocin also inhibited lipogenesis by suppressing the expression of lipogenesis-related proteins and elevating lipid catabolism-related proteins. Moreover, Crocin effectively alleviated inflammation by suppressing the expression of pro-inflammatory cytokines and increasing levels of anti-inflammatory cytokines. We further found that Crocin promoted macrophage polarization to the M2 phenotype by reducing M1 markers ($CD40^+$ and $CD11c^+$) and elevating M2 markers ($CD68^+$ and $CD206^+$). Finally, Crocin strongly inhibited the expression of NF-κB p65 and its translocation into the nucleus. Crocin partially counteracted nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 expression and the nuclei accumulation caused by NF-κB p65 overexpression. Taken together, our research indicated that Crocin inhibited lipogenesis and alleviated the inflammation in a VD3-induced rat coronary atherosclerosis model by promoting M2 macrophage polarization and maybe by inhibiting NF-κB p65 nuclear translocation. This study implicates Crocin as a potential therapeutic strategy for coronary atherosclerosis.

1. Introduction

Atherosclerosis is an inflammatory disease of the arteries associated with lipid and other metabolic alterations [1]. It is an important contributing factor for cardiovascular diseases such as heart attack and stroke, with increasing morbidity and mortality worldwide [2]. Atherosclerosis is mainly caused by lipid accumulation and immune cell infiltration, as well as molecular interactions, resulting in plaque development, rupture and thrombosis [3]. Although the major steps of atherosclerotic plaque formation and development have been well researched, the exploration of newer molecules and identifying the mechanism of atherosclerosis is crucial for the development of new therapeutic strategies.

The pathologic process of atherosclerosis is commonly related to increased concentrations of low-density lipoprotein cholesterol (LDL-c), which promotes the migration of circulating monocytes to the subendothelial space [4]. Monocytes are then converted to foamy macrophages rich in cholesterol esters and free fatty acids that infiltrate the arterial walls and cause pathological intimal thickening, which triggers conversion of the lipid pool to a necrotic core [5]. Report indicated that lipid accumulation and oxidative tissue damage are hallmarks of atherosclerotic lesions, macrophages sense a variety of lipid classes, which induces phenotypic changes [6]. A continuum of pro- and anti-inflammatory macrophages, with extreme polarization phenotypes M1 and M2, can be found in atherosclerotic lesions. Macrophages can alter their phenotypes and functions accordingly in response to changes in the microenvironment [7]. Thus, the control of macrophage polarization could regulate the progression of atherosclerosis.

Certain cytokines and microbial moieties have been demonstrated in vitro to profoundly skew macrophage expression patterns and functions [8]. Specifically, the molecules that induce M1 polarization are characterized by pro-inflammatory capacity. These molecules

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include inducible nitric oxide synthase (iNOS), interleukin (IL)-6 and tumor necrosis factor (TNF). Conversely, micro-environmental factors that contribute to M2 polarization in atherosclerotic lesions are primarily thought to be anti-inflammatory mediators, like IL-4, IL-10 and transforming growth factor-β (TGF-β) [9,10]. Given the contrasting properties of pro-inflammatory M1 and anti-inflammatory M2 macrophages in homeostasis and disease, the balance between these populations has been deemed crucial in the progression of atherosclerosis.

Saffron, the dried stigmas of *Crocus sativus L.*, (Iridaceae family), has been used as a color and seasoning in food traditionally. Crocin is one of the active constituents of saffron. The well-known pharmacological effects of Crocin are anti-oxidant [11], anti-cancer [12] and anti-inflammatory properties [13]. Crocin has been reported to exert anti-atherosclerotic effects through decreasing the level of Ox-LDL [14], thus Crocin may play an important role in the initiation and progression of atherosclerosis.

This study explored the function of Crocin in a coronary atherosclerosis rat model. We found that Crocin decreased the blood lipid levels by promoting fatty acid catabolism. Besides, Crocin induced M2 macrophage polarization and inhibits the nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 to alleviate inflammatory response in a vitamin D3 (VD3)-induced rat coronary atherosclerosis model. These findings suggest that Crocin may function as a potential therapeutic strategy for the treatment of atherosclerosis.

2. Materials and methods

2.1. Animal models

Sixty healthy Wistar rats (male, 210–260 g, 8 weeks) of clean grade were purchased from the animal center of Xi'an Jiaotong University. Rats were reared at room temperature (25 ± 1 °C) in (55 ± 5)% relative humidity. The coronary atherosclerosis model was established as previous report [15]. Briefly, 40 rats were fed with high-fat diet and intraperitoneal injection of 2 mL/kg VD3 (600,000 IU/kg) every day for 3 days. As a negative control, the remaining 20 rats were fed with basal feed and injected intraperitoneally with 2 mL/kg normal saline. For the VD3 + Crocin group, after 12 weeks, half of the rats in the Model group received gavage of 100 mg/(kg·d) Crocin for 4 weeks. Rats in Model group and Control group received gavage of 5 mL/(kg·d) distilled water.

2.2. Enzyme-linked immunosorbent assay (ELISA)

The venous blood in each group was collected and the concentrations were measured using endothelin (ET), total cholesterol (TC), triglyceridelow (TG), low density lipoprotein cholesterin (LDL-c), high density lipoprotein cholesterin (HDL-c), IL-6, iNOS, TNF-α, IL-4, IL-10 and TGF-β ELISA kits (R&D, Minneapolis, MN, USA), according to the manufacturer's protocols. Optical density (OD value) was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA). The concentration of samples was calculated according to the corresponding OD value and the concentration of the standard substance.

2.3. Western blotting

Rats were anesthetized by intraperitoneal injection of 3% chloral hydrate (3 mL/kg) and then sacrificed. The coronary artery tissue (100 mg) was collected; 25 μg of total protein in each sample was separated by 12% SDS-PAGE and electro-transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA) for immunoblotting. The following primary antibodies were used: anti-IL-6, anti-iNOS, anti-TNF-α, anti-IL-4, anti-IL-10, anti-TGF-β, anti-AMP-activated protein kinase α1 (AMPKα1), anti-Aacyl coenzyme A(ACO), anti-carnitine palmitoyltransferase-1(CPT-1), anti-sterol regulatory element binding proteins-1c (SREBP-1c), anti-fatty acid synthetase (FAS) and anti-GAPDH, which was used as the internal reference. After incubation with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody, proteins were detected using a ChemiDoc XRS imaging system and Quantity One analysis software (Bio-Rad, San Francisco, California, USA).

2.4. Flow cytometry

Suspensions of mononuclear cells (MNCs) from coronary artery tissue were prepared by grinding the organ through a 40-mm nylon mesh in medium. For cell surface staining, MNCs were stained for 20 min at RT in 1% BSA-PBS buffer with the following panel of antibodies: Alexa Fluor 488-anti-F4/80 (Serotec, UK) and PE-CD11c, PE-CD40, PE-CD206 and PECD68 (eBioscience).

2.5. Preparation of Macrophages and NF-κB p65 overexpression

Macrophages were prepared as reported previously [16]. MNC from coronary artery tissue was seeded in six-well tissue culture plates (Corning, 2×10^6 cells/mL) and allowed to adhere for 1 h. Adherent cells were purified by positive selection using CD11b microbeads (Miltenyi Biotec, Leiden, The Netherlands). The purity of $CD11b^+$ cells was evaluated by flow cytometry ($CD11b^+F4/80^+$ cells > 96%). In addition, purified macrophages from healthy rats were isolated and used for in vitro experiments. NF-κB p65 overexpression was achieved by inserting its cDNA into pcDNA 3.1 vector (Invitrogen, Carlsbad, CA, USA). The recombinant plasmids and other agents were co-transfected into 3×10^6 macrophage cells using a Nucleofector instrument. Forty-eight hours later, subsequent experiments were performed. The experiment was replicated thrice for data calculation.

2.6. Immunofluorescence staining

Purified macrophages were cultured on an 8-well chamber CultureSlides (Becton Dickinson, Bedford, MA). After 8 h, cells were fixed in 3% paraformaldehyde in PBS at room temperature for 8 min, then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. After washing in PBS, the cells were incubated with primary mouse anti-NF-κB p65 monoclonal antibody (1 mg/mL; Transduction Laboratories, Lexington, KY) at 4 °C overnight. After washing, cells were incubated with biotinylated goat anti-mouse IgG (Pierce, Rockford, IL) at room temperature for 1 h. The immunoreactivity was revealed using Alexa568-conjugated streptavidin (Molecular Probes, Eugene, OR), and cells were counterstained with 10 mg/mL DAPI. The cells were examined under a Nikon fluorescence microscope (Image Systems, Columbia, MD).

2.7. Statistical analysis

All results were presented as mean ± SD from a minimum of three replicates. Differences between groups were evaluated by SPSS version 15.0 statistical software with Student's *t*-test when comparing only two groups or assessed by one-way ANOVA when more than two groups were compared. For the comparison of paired tissues, a paired Student's *t*-test was used to determine statistical significance. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Crocin decreases the blood lipid levels in a VD3-induced rat coronary atherosclerosis model

As indicated by ELISA analysis, the levels of ET, TC, TG and LDL-c were increased in the VD3 group compared with the Control group, but strongly decreased with Crocin treatment in VD3-induced rat (Fig. 1A,