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Original Research Article

Chrysophanol exerts protective effect against atherosclerosis via NFκB-mediated signaling in LDLR-/- mice model

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Abstract

Purpose: *To study the therapeutic effect of chrysophanol (CHR) on diet-induced atherogenesis in LDLR-/- mice.*
Methods: *Mice were fed atherogenic diet for 12 weeks after which some lipid profile markers such as total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c) and triglyceride (TG) were measured. The mRNA expression levels of lipid synthesis genes and lipid overload-related inflammatory indicator molecules were assayed with quantitative real time polymerase chain reaction (qRT-PCR), while the corresponding protein expressions were determined with western blotting assay. The therapeutic effect of CHR on atherogenesis was confirmed using H & E and Oil red O stainings of mice aortic sections.*
Results: *CHR administration significantly reduced levels of TC, LDL-c, HDL-c and TG ($p \leq 0.05$), and restored the mRNA and protein expressions of genes involved in lipid and glucose homeostasis, namely, AdipoR1, PPAR-Y and HMco-A ($p < 0.05$). Moreover, CHR potentially alleviated diet-induced inflammation, as is evident in reduced levels of molecular inflammatory signaling factors NF-κB and TLR-4, and significant down-regulations of the proinflammatory cytokines, TNF-α, IL-6 and IL-1β ($p < 0.05$). Furthermore, aorta histology revealed that CHR significantly reduced lipid storage in the arteries of mice fed atherogenic diet ($p < 0.05$).*
Conclusion: *These results indicate that CHR reduces diet-induced lipid storage in LDLR-/- mice and also controlled inflammation-associated lipid overload. These findings may provide a molecular basis for potential application of chrysophanol in the treatment of atherosclerosis.*

Keywords: *Chrysophanol, Inflammation, Atherosclerosis*

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INTRODUCTION

Atherosclerosis is a disease caused by deposition of passive lipids in the arterial wall, leading to their subsequent layering in the endothelial and smooth muscle cells, and prolonged active deposition of oxidized cholesterol [1]. This process constitutes the main abnormalities associated with myocardial infarction, stroke and coronary atherosclerosis which are leading causes of large number of mortalities worldwide [2]. Formation of lipid

droplets is an early indication of atherosclerosis. These droplets develop further into detectable lesions and fatty streaks in arteries. Atherosclerotic inflammation is recognized as a primary contributor to atherogenesis through its harmful effect on arterial wall structure and its impairment of lipid metabolism [3]. In this pathological process, monocyte-derived macrophages and T-cells are involved, with respect to innate and acquired immunological responses. Monocytes and T cells migrate to the arterial intima through the blood circulation and differentiate further into macrophages. These macrophages further take up lipoproteins, and the lipid-laden macrophages transform into foam cells, leading to formation of atherosclerotic plaques or plaque rupture and thrombosis, and eventually cause occlusion of the arteries [4].

Transcription factor NF-κB is the primary regulator of inflammation in response to various stimuli. Activation of NF-κB accelerates the transcription of several molecules such as cytokines, chemokines, matrix metalloproteinases (MMPs), and adhesion molecules [5]. Indeed, NF-κB activation is implicated in numerous pathological stages of atherogenic progression such as foam cell formation, and other events like vascular smooth muscle cell proliferation and inflammation, arterial calcification, and formation of arterial plaques [6]. In addition, during atherogenesis, Toll-like receptor (TLR) acts as receptor of ligand lipopolysaccharide (LPS). The activated macrophages utilize oxidized low-density lipoprotein (ox-LDL), resulting in formation of foam cells [7]. Reduction of lipid storage considerably decreases the formation of atherosclerotic coronary lesions, leading to reduction in the level of low-density lipoproteins, a strategy which has potential for preventing atherosclerosis-mediated cardiovascular events [8,9].

Chrysophanol (CHR) also known as chrysophanic acid (IUPAC: 1,8-dihydroxy-3-methyl-anthraquinone) (Figure 1) is an anthraquinone compound found naturally in many traditional Chinese medicines [10]. A large number of studies have reported the beneficial effects of CHR when used on asthma, diabetes, Alzheimer's disease (AD), and osteoarthritis complications. Moreover, CHR exerted significant protective effects against alcoholic liver disease and retinal degeneration [11,12]. However, the protective effect of CHR against atherosclerosis and inflammation remains uninvestigated. The present study was designed to provide insight into the potential of CHR in the attenuation of atherosclerosis in both inflammatory and lipid mechanistic pathways.

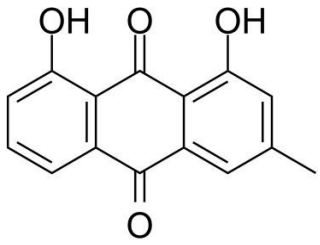

Figure 1: Molecular structure of chrysophanol

EXPERIMENTAL

Reagents and drugs

Chrysophanol was provided by Energy Chemical (Shanghai, China), while simvastatin was obtained from Sigma-Aldrich. Unless mentioned otherwise, analytical grade standard reagents and chemicals were used throughout this study.

Animals and experimental design

Low-density lipoprotein receptor knockout (LDLR−/−) mice weighing 18 – 22 g were obtained from Nanjing University affiliated Nanjing Biomedical Research Institute at Nanjing, P.R. China. The animals were housed under controlled temperature and humidity of 25 ± 2 °C and 70 %, respectively, in an environment with 12-h alternate light and dark periods. All mice were provided water and normal diet *ad libitum* for one week. The study strictly adhered to the guidelines of laboratory animal use and care issued by the National Institute of Health (NIH), USA (NIH publication No. 85-23, as revised in 1996). The experimental protocol was approved by the Ethics Committee of Hubei University of Medicine. All efforts were made to minimize suffering in the animals. After a week of acclimatization, the LDLR−/− mice were randomly divided into 4 groups, with 8 mice in each group (n = 8). All groups received atherogenic diet except the control group. Group-I (diet control) was fed with normal diet up to 12 weeks, while groups II, III and IV (treatment groups) received various treatments viz: group II (inducer group) was given experimental atherogenic diet, group IV was given simvastatin (10 mg/kg), while group III received CHR (25 mg/kg) [13]. The mice groups were subjected to the respective treatments for 12 weeks. Both vehicle (5 % CMC) and drug were administered via the intragastrical route (i.g) using oral gavage needle.

Determination of serum lipid profiles and cytokine levels

At the end of the experiment, 12-h fasting blood samples were collected via retroorbital puncture, and sera were obtained after centrifugation of the samples at 3000 rpm for 5 min at 4 °C. The serum samples were kept refrigerated at −80 °C until used. Serum levels of TC, TG, LDL-C, and HDL-C were determined using kits obtained from Nanjing Jiancheng Bioengineering Institute of P.R. China. Serum TNF-α, IL-1β and IL-6 were determined using ELISA kits (430902 and 431302, ELISA Max™ Sets; BioLegend Inc) in line with the manufacturer's instructions.

Quantitative RT- PCR analysis

Gene expression analysis was performed to assess mRNA levels, using standard RT-PCR method. Total RNA was extracted from liver tissue using RNA isolator reagent (Vazyme Biotech Co. Ltd.), and the RNA was reverse-transcribed to cDNA using HiScript® II 1st Strand cDNA Synthesis kit (Vazyme Biotech Co. Ltd., Nanjing, Jiangsu, P.R. China). The PCR amplification was performed as per protocol described previously [14]. The specific oligonucleotide primers corresponding to lipid metabolism genes (Table 1) were obtained commercially from vendors, with 36b4 mRNA as loading control. Total levels of respective mRNA was determined densitometrically.

Histological analysis

Mouse aorta specimens were fixed with formalin and embedded in paraffin. Then, 4-µm-thick aorta sections were obtained serially using a microtome. The slices were stained with hematoxylin/eosin (H & E) for routine histological analysis. For lipid levels, Oil Red O staining was performed on aorta sections mounted on glass slides. On completion of staining, high-resolution images were captured using LEICA DM500 microscope (Wetzlar, Germany) or Meta confocal Zeiss LSM5 microscope (Zeiss, Jena, Germany). Based on H & E staining images, necrotic cores were calculated and presented using planimetry (Diskus software). Lipid content of aorta proportional to plaque area was assessed with an image analysis system (Clemex Technologies Inc).

Western blotting assay for protein expression levels

Antibodies for PPAR-γ and HMG-CoA were purchased from Santa Cruz Biotechnology Inc., while NF-κBp65, TLR4, and β-actin antibodies with respective anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies, were provided by Cell Signaling Technology (Danvers, MA, USA). Protein concentration was determined using a BCA protein assay kit (Beyotime). Sodium dodecyl sulphate-PAGE gel (10 %) was carried out on protein extracts (40 µg/lane), followed by electro-blotting onto poly-vinylidene difluoride (PVDF) membranes (Millipore). Tris-buffered saline with 1% Tween 20 (TBST) solution containing 5% skimmed milk solution was used to block the membranes. Thereafter, the membranes were incubated overnight at 4 °C with primary antibodies for NF-κBp65, TLR4, PPAR-γ, HMG-CoA reductase and β-actin (1:800 or 1:500 dilution). Finally, the membranes were incubated with horse radish peroxidase-linked secondary antibody at room temperature for 60 min. Then, band absorbance was quantified using DAB, while band intensity was measured using ImageJ software.

Statistical analysis

Data analysis was performed using GraphPad Prism (GraphPad, San Diego, CA, USA). Results are expressed as mean ± SEM. Statistical significance was calculated using Student's t-test. Values of $p \leq 0.05$ were considered as significant.

RESULTS

Chrysophanol treatment mitigated abnormalities in serum lipid parameters and inflammatory markers in LDLR-/- mice

After 12 weeks of atherogenic diet feeding and CHR treatment, serum levels of TC and TGs were significantly restored in the CHR and simvastatin groups, when compared with corresponding levels in diet-alone group (Table 2). In particular, diet-induced abnormality in the levels of TC, TGs and LDL cholesterol were significantly reversed to near-normalcy in the CHR treatment group, whereas HDL cholesterol level was increased, relative to values in mice fed atherogenic diet alone (Table 2). These results indicate that CHR may improve lipid metabolism in atherosclerotic LDLR-/- mice.

CHR downregulated inflammatory mediators and positively regulated lipid metabolism genes in LDLR-/- mice

In order to evaluate the anti-inflammatory effect of CHR, the levels of inflammatory cytokines were measured using qRT-PCR. Results in Figure 1 show that CHR treatment markedly

suppressed the production of inflammatory molecules, and significantly relieved inflammatory response induced by atherogenic diet ($p \leq 0.05$). It upregulated the expressions of AdipoR1, PPAR-Y and HMCo-A reductase genes ($p \leq 0.05$). Thus, CHR mitigated impaired energy and lipid metabolism in mice induced by lipid storage in arterial walls due to prolonged intake of atherogenic diet (Figure 2). Furthermore, to substantiate the anti-inflammatory potential of CHR, inflammatory mediator molecules such as NF-κB and TLR4 were also measured. As shown in Figure 2, simvastatin produced significant effects on the expressions of genes related to lipid and glucose metabolism. Moreover, in mice fed atherogenic diet, CHR treatment also suppressed the overexpression of inflammatory mediators ($p \leq 0.05$). These data indicate that CHR improved the energy metabolism and attenuated the atherogenic diet-induced inflammatory events.

CHR reduced inflammatory mediators via prevention of lipid storage in the arterial wall of LDLR-/- mice

The expression of proteins regulating lipid metabolism were markedly down-regulated in mice fed atherogenic diet ($p \leq 0.05$). However, CHR administration up-regulated the expressions of these proteins to near-normalcy (Figure 3 and Figure 4). The same pattern of effect was seen in mice in positive control group given simvastatin. However, simvastatin failed to produce significant effect on inflammatory mediators, when compared to CHR.

CHR administration reduced aortic lipid deposition in LDLR-/- mice

Stained aortic sections showed that atherosclerotic plaque areas were significantly reduced in mice treated with CHR (25 mg/kg), when compared to sections from mice fed atherogenic diet alone ($p < 0.05$).

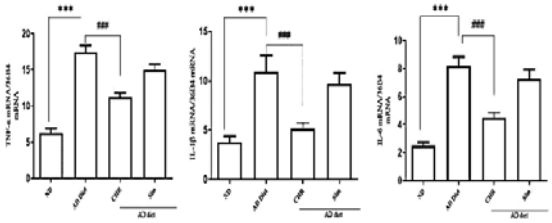

Figure 2: Effect of CHR on serum proinflammatory cytokines in LDLR-/- mice. Group I: NC (CMC 25 mg/kg); Group II: atherogenic diet; Group III: CHR (25 mg/kg); Group IV: Simvastatin (10 mg/kg). Values are expressed as mean ± SEM. ###Significant variation from Group II fed AD diet ($p < 0.05$). ***Significant variation from normal diet group ($p < 0.05$)

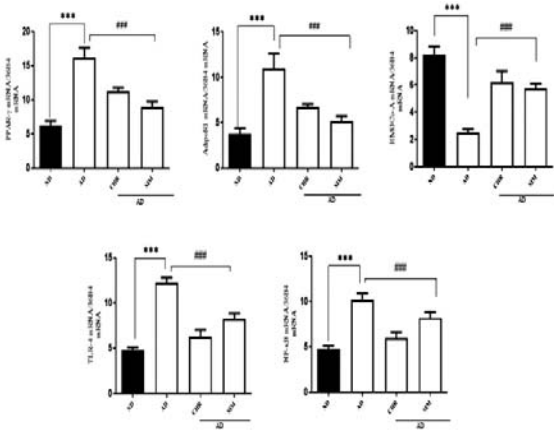

Figure 3: Effect of CHR on lipid biosynthesis and inflammatory mediator genes in LDLR-/- mice. CHR administration suppressed the expressions of inflammation-related factors and stimulated lipid biosynthesis molecules

Table 1: Oligonucleotide mouse gene primers used in this study

Gene	Forward (5'-3')	Reverse (5'-3')
36b4	CCCTGAAGTGCTCGACATCA	TGCGGACACCCTCCAGAA
AdipoR1	ACTACCGTCTCTACGGGTC	ATAGCGACTCCCGAAACAGTTC
PPAR-Y	GAAAGACAACGGACAAATCACCAT	CGGCTTCTACGGATCGAAACTG
HMGCR	TGTGGCCAGGAGTTTGGTGACTGA	TAAGATTCAACAACTCTGCTGACC
TLR4	GTTGCAGAAAATGCCAGGATG	CAGGGATTCAAGCTTCCTGGT
NF-κB	ACGACATTGAGGTTCGGTTC	ATCTTGTGATAGGGCGGTGT

Table 2: Effect of CHR administration on serum lipid profiles in LDLR-/- mice fed atherogenic diet

Group	TC (mg/dL)	LDL-c (mg/dL)	TG (mg/dL)	HDL (mg/dL)
NC	67.69 ± 2.11	50.31 ± 3.22	81.30 ± 1.42	12.38 ± 4.07
AD	141.22 ± 6.10 ***	112.28± 2.32 ***	152.64 ± 1.13 ***	25.21±1.15***
CHR, 25 mg/kg	87.49 ± 2.97 ###	78.13 ± 6.28 ###	101.43 ± 3.64 ###	15.36±2.33###
Simvastatin, 10 mg/kg	89.22 ± 3.84 ###	75.69 ± 7.54 ###	108.25 ± 4.13 ###	10.06±1.52###

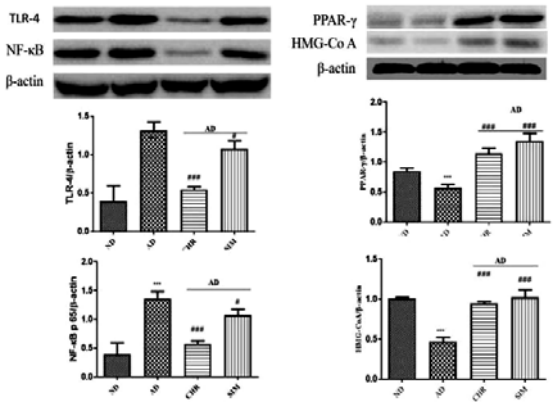

Figure 4: Effect of CHR on lipid biosynthesis and inflammatory mediator genes in LDLR-/- mice. Group I: NC (CMC 25 mg/kg); Group II: atherogenic diet; Group III: CHR (25 mg/kg); Group IV: simvastatin (10 mg/kg). Values are expressed mean ± SEM. ###Significant variation, compared to Group II (p < 0.05). ***Significant variation, compared to Group I (normal diet group; p < 0.05)

The protective effect of CHR against lipid deposition linked to atherosclerotic plaque was better than that of simvastatin, relative to mice fed atherogenic diet alone (p < 0.05). These histological observations in aorta demonstrate that CHR treatment produced protective effects against atherosclerosis in LDLR-/- mice (Figure 5).

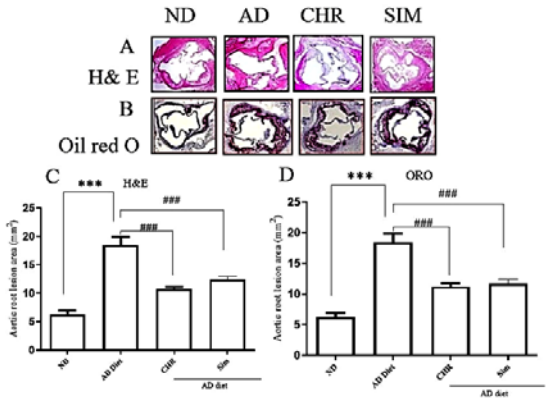

Figure 5: CHR prevented atherosclerotic lesion formation on in LDLR$^{-/-}$ mice. (A) Photomicrographs of aortic root sections of mice from various diet and treatment groups (n = 5). (B and D) Representative images and quantification data of aortic root sections stained with H & E (A and C) and ORO

DISCUSSION

This study demonstrates, for the first time, the protective effect of CHR against atherosclerosis-associated inflammation in LDLR-/- mice. In the LDLR-/- mice model, dietary cholesterol is essential for atherosclerosis development, with abnormal increase in the levels of circulating total cholesterol including LDL-C and very low-density lipoprotein [15]. The level of LDL is an index of the level of cholesterol present (LDL-C), and it is a major driving force in the development of atherosclerosis [16]. In this study, data on serum lipid parameter analysis suggest that CHR produced significant effect on reversal of AD diet-induced increases in lipid levels in LDLR-/- mice. Furthermore, it seems that simvastatin produced effects on serum lipid levels similar to those of CHR treated mice. These results are in agreement with results of previous studies on lipid profile normalization after treatment with oleanolic acid [1]. In addition, the mRNA levels of PPAR-γ, AdipoR1, AdipoR2, HMG-CoA reductase, TLR-4 and NF-κB (intra nuclear p65) were measured.

It is known that PPAR-γ is a nuclear receptor that acts as ligand activated transcription factor involved in glucose and lipid homeostasis in cells [17]. It has direct influence on vessel wall through its metabolic effect on fatty acid oxidation and uptake, thereby reducing progression of atherosclerosis. Hence, PPAR-γ can also suppress the expressions of pro-inflammatory genes during chronic inflammatory response [18,19]. Therefore, PPAR-Ƴ may be a well-established target for anti-atherosclerosis therapy. Transcription factor nuclear factor-κB (NF-κB) is a primary mediator of inflammation. Activation of NF-κB pathway is initiated by a number of extracellular events such as secretion of TNF and IL-1 via binding to their respective receptor molecules i.e., TNF and IL-1 receptor (TNFR1 & IL-1R) and interactaction with various Toll-like receptors (TLRs) [20]. Hydroxy methyl glutaryl-CoA reductase (HMG-CoA) is the enzyme involved in synthesis of LDL receptor (LDL-r), and cholesterol biosynthesis. In the liver, LDL-r acts as LDL-C lipo-protein receptor, and it is effective in the regulation of lipid metabolism and liver cholesterol [21]. Moreover, adiponectin molecules play crucial role in cardiovascular diseases via regulation of the adiponectin receptors (AdipoR1 & AdipoR2) [22].

In LDLR−/− mice, aorta mRNA expression analysis showed that CHR significantly restored the expressions of PPAR-γ, AdipoR1 and HMG-CoA reductase, and controlled the overexpressions of NF-κB and TLR-4. Results from ELISA analysis of serum pro-inflammatory cytokines also support the findings on mRNA expressions of inflammatory markers. Moreover, differentiation of monocytes to lipid-laden macrophages or foam cells is potentially initiated by inflammatory cytokines. This local

inflammatory response is subsequently amplified in the lesions, and it greatly enhances the chances of plaque rupture [4]. In blotting analysis, the over-expressions of lipid metabolism-associated proteins were alleviated by CHR. However, the inflammatory mediator protein molecule expressions were controlled more by CHR than simvastatin.

Histological examination of cross-sectional aorta showed that formation of atherosclerotic plaque was significantly reduced in mice treated with CHR and simvastatin, when compared to mice fed atherogenic diet alone. In line with this finding, it has been reported that curcumin reduced lipid deposits and plaque formation, thereby alleviating plaque inflammatory responses in an atherosclerosis animal model [23]. Therefore, CHR could prevent diet-induced atherosclerosis in LDLR−/− mice through downregulation of inflammatory mediators, ensure normal lipid metabolism, and prevent lipid deposition in atherosclerotic plaques.

CONCLUSION

The findings of this study indicate that atherosclerosis due to abnormalities in lipid storage and inflammation are effectively alleviated by chrysophanol. Therefore, chrysophanol is a potential agent for treating atherosclerotic inflammation, but clinical trials are required to ascertain this.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yuping Li, and Jun Zhang contributed to this work equally. The study was conceived and designed by Huawei Tian. Yuping Li and Jun Zhang perform experiments and analyse the data and both are contributed equally to this work. All the authors involved in drafting of manuscript and approve the present form of manuscript for publication.

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