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Research paper

# Betulinic acid downregulates expression of oxidative stress-induced lipoprotein lipase via the PKC/ERK/c-Fos pathway in RAW264.7 macrophages

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ABSTRACT

*Background:* Atherosclerosis is a major cause of coronary artery disease, which is characterized by cellular lipid accumulation. Lipoprotein lipase (LPL) is a key enzyme in lipid metabolism. Studies have shown that macrophage-derived LPL exhibits proatherogenic properties, and plays a major role in lipid accumulation in macrophages. Evidence suggests that oxidative stress can effectively enhance macrophage LPL production. Betulinic acid (BA) is a pentacyclic lupane triterpene with a potent antioxidant activity. In this study, we investigated whether BA affects the expression of macrophage LPL and how it regulates cellular lipid accumulation.

*Methods and results:* We revealed that BA downregulated $H_2O_2$-simulated macrophage LPL protein, mRNA levels and its activity in both concentration- and time-dependent manners. Furthermore, BA decreased LPL-involved total cholesterol and triglyceride levels in macrophages. In addition, cellular lipid staining by Oil Red O showed that BA decreased cellular lipid droplet deposition. Next, we confirmed that pretreatment with BA decreased $H_2O_2$-induced production of intracellular reactive oxygen species in a concentration-dependent manner. Further studies demonstrated that BA inhibited $H_2O_2$-induced membrane translocation of PKC, phosphorylation of ERK1/2 and c-Fos. Finally, the induction of LPL production and activity by $H_2O_2$ was abolished by BA, inhibition of PKC or ERK or depletion c-Fos, respectively.

*Conclusions:* BA, through its role of antioxidant activity, attenuated macrophage-derived LPL expression and activity induced by oxidative stress, and effectively reduced cellular lipid accumulation, likely through inhibition of the pathways involving PKC, ERK and c-Fos. These effects of BA may contribute to its mitigation of atherosclerosis and help develop BA as a therapeutic compound in treatment of atherosclerosis.



## 1. Introduction

Atherosclerosis is a major cause of coronary artery disease. Macrophage-derived cholesteryl ester-rich foam cells are formed within the arterial wall as a result of excessive internalization of lipoproteins, and subsequently promote early atherosclerotic plaque formation [1]. Lipoprotein lipase (LPL) is a key enzyme in the

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metabolism of lipoproteins, which hydrolyzes chylomicron and very-low-density lipoprotein (VLDL)-associated triglyceride [2,3]. Accumulated evidence has revealed that LPL secreted by macrophages in arterial intima is proatherogenic [4–6]. Indeed, LPL may cause nonenzymatic bridging of atherogenic lipoproteins to the extracellular matrix and subsequent retention and possible modification of the lipoproteins, which makes lipoproteins more atherogenic. Moreover, LPL lipolysis products have been reported to evoke inflammatory reactions and induce endothelial cell apoptosis in the blood vessel. In atherosclerotic lesions, macrophage-derived LPL also stimulates the production of the proinflammatory cytokines, and increases monocyte adhesion to endothelial cells [7,8], contributing to cellular lipid accumulation and macrophage foam cell formation. In addition, LPL has been reported to play an important role in VLDL-mediated induction of foam cell formation and production of inflammatory factors in macrophages [9,10].

Recently, increasing evidence has suggested that oxidative stress plays a crucial role in the pathogenesis and progression of atherosclerotic disease, and atherosclerosis represents a state of enhanced oxidative stress. Indeed, reactive oxygen species (ROS) may affect the atherosclerotic process through several different approaches, including endothelial dysfunction, arterial lipid metabolism and macrophage activation [11–13]. Several lines of evidence indicate that oxidative stress can also enhance LPL production by macrophages, and potentially promote the progression of atherosclerosis [14–17]. Therefore, it is possible to use antioxidants to prevent the pathogenesis of atherosclerosis.

Recent studies have revealed that many molecules are potentially important for the regulation of LPL expression and activity. LPL expression can be regulated at transcriptional, post-transcriptional, translational, and post-translational levels [18]. A variety of proteins that regulate LPL expression have been identified, including apolipoprotein (apo)-CII, apo-CIII, apo-AV, angiopoietin-like protein 3(ANGPTL3), and ANGPTL4 [19]. In addition, our previous research revealed miR-467b protects apolipoprotein E deficient ($apoE^{-/-}$) mice from atherosclerosis by reducing lipid accumulation and inflammatory cytokine secretion via down-regulation of LPL expression [20]. However, the effects of dietary and medicinal plants on the regulation of LPL expression are still poorly understood.

Betulinic acid (BA, 3β-hydroxy-lup-20(29)-en-28-oicacid) is a pentacyclic lupane triterpene present in betula plants and many fruits and vegetables, especially in birch bark, widespread throughout the tropics. It is a known natural compound shown to possess a wide variety of pharmacological and biochemical effects including anti-neoplastic activity in several human cancer cells, anti-human immunodeficiency virus(HIV), anti-malarial, anti-bacterial, anti-inflammatory and immunomodulatory properties [21,22]. A major advantage for therapy is that BA could activate metalloprotease and result in cell apoptosis without any cytotoxicity itself. It was also found that BA at the concentration of 500 mg/kg did not exhibit detectable toxic side effects [23]. Accumulated experimental evidence has revealed that BA also displayed strong free radical scavenging and antioxidant activities [24–26]. Given oxidative stress is effective enhancer of macrophage LPL production and modulates macrophage response to LPL, it was of interest to us to investigate the effects of BA on production of oxidant stress-induced macrophage-derived LPL and the possible molecular mechanism involved.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Betulinic acid, N-Acetyl-L-cysteine (NAC), calphostin C and PD98059 were purchased from Sigma Aldrich (Sigma Chemical Co, USA). LPL activity assay kit was purchased from Progen (Heidelberg, Germany). Tetrahydrolipstatin (THL) was purchased from Roche Diagnostics. Primary antibodies for PKC, phospho-ERK1/2, ERK1/2, c-Fos, phospho-c-Fos, β-actin and the secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tetrahydrolipstatin (THL) was provided by Roche Diagnostics. Lamin A antibody was purchased from Abcam (USA).

### 2.2. Cell culture

RAW264.7 macrophages were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin in 10 $cm^2$ dishes at 37 °C in an atmosphere containing 5% $CO_2$. Thereafter, cells were treated different factors.

### 2.3. Measurement of intracellular reactive oxygen species generation

The fluorescent probe, the cell-permeable fluorogenic dichlorofluorescein diacetate (DCFH-DA), which acts as a $H_2O_2$-sensitive fluorophore, was used to detect the ROS. Macrophages were pretreated with or without BA, and then cells were incubated with $H_2O_2$. The cells were washed prior to the addition of DCFH-DA (10 μM) probe at 37 °C for 30 min. At the end of incubation period, cells were washed with cold PBS, and then fixed with 2% paraformaldehyde. Then dichlorofluorescein fluorescence distribution of 20,000 cells was detected by fluorospectrophotometer analysis at an excitation wavelength of 488 nm and at an emission wavelength of 535 nm.

### 2.4. Determination of triglycerides (TG) and total cholesterol (TC) contents

RAW264.7 macrophages were starved overnight and treated with 50 μg/ml VLDL with or without LPL inhibitor (tetrahydrolipstatin, THL) for 24 h in FBS-free medium; or preincubated with and without BA (1 μg/ml) for 24 h. Cells were then, incubated with 50 μg/mL VLDL for 24 h with or without pretreatment with $H_2O_2$ (200 μM) for 18 h in FBS-free medium, followed by harvesting, treatment and detection as described previously [27]. Cellular TG and total TC were measured using the kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

### 2.5. Lipid accumulation evaluated by Oil Red O staining

RAW264.7 macrophages were cultured with serum-free medium containing 50 μg/ml VLDL with or without $H_2O_2$ for 24 h after pretreatment with or without BA. Cellular lipid accumulation was verified by fixing cells with 4% paraformaldehyde and then staining them with 0.5% Oil Red O. Hematoxylin was used as counterstaining, and cells were photographed at ×400 magnification.

### 2.6. Determination of extracellular LPL activity

Activity levels of LPL secreted by macrophages in the supernatants were determined using the LPL kit, the Confluolip kit (Progen, Heidelberg, Germany). When the media were assayed for LPL activity, 0.5 U/ml heparin was added to the medium 1 h before the end of incubation period. Levels of macrophage LPL activity were normalized to the levels of total cell proteins. To produce 1 μM free fatty acid per mg protein per hour in the reaction system is expressed as one active unit.