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Bornyl acetate suppresses ox-LDL-induced attachment of THP-1 monocytes to endothelial cells

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ABSTRACT

Leukocyte recruitment to the surface of the endothelium plays a pivotal role in the development of cardiovascular diseases. Bornyl acetate is the main volatile constituent present in numerous conifer oils, which has displayed its anti-oxidant and anti-inflammatory properties in different types of tissues and cells. However, little information regarding the effects of bornyl acetate on vascular endothelial inflammation has been reported before. In the current study, we aimed to investigate the pharmacological roles of bornyl acetate against ox-LDL-induced leukocyte adhesion to the endothelium. Our findings indicate that bornyl acetate ameliorated ox-LDL-induced reduction in cell viability of HUVECs. Additionally, bornyl acetate inhibited the attachment of THP-1 monocytes to HUVECs induced by treatment with ox-LDL through ameliorating the expression of ICAM-1, VCAM-1, and E-selectin. Mechanistically, we found that bornyl acetate could suppress activation of the IκBα/NF-κB signaling pathway. Lastly, our results indicate that bornyl acetate mitigated expression of the pro-inflammatory cytokines TNF-α and IL-1β. Our results suggest the therapeutic potential of bornyl acetate in patients with atherosclerosis.

1. Introduction

As a chronic inflammatory pathological process occurring on the artery wall, atherosclerosis leads to several cardiovascular diseases such as coronary heart disease, myocardial infarction, and peripheral arterial disease [1]. Pro-atherosclerotic stimuli, such as the oxidized form of LDL-cholesterol (ox-LDL) and adhesion of leukocytes to the endothelium induced by pro-inflammatory cytokines, play an important role in all stages of atherosclerosis [2,3]. Oxidative stress plays a causative role in ageing and the pathogenesis of cardiovascular diseases [4]. LDLs act as the major carriers of blood cholesterol and contain a large amount of polyunsaturated fatty acids, which are the main substrate for lipid peroxidation. The oxidation of LDL is a complicated biological process during which both proteins and the lipids undergo oxidative changes and form complex products due to metabolic imbalances including hyperlipidemia, hyperglycemia, and insulin resistance. Indeed, the oxidative modification hypothesis of atherosclerosis recognizes the crucial role of ox-LDL as a byproduct of LDL exposure to ROS [5]. Ox-LDL has been considered as an important oxidative stress biomarker and a non-traditional, pro-atherogenic emerging risk factor for coronary heart disease.

Multiple lines of evidence have shown that ox-LDL is more important in the genesis and progression of atherosclerosis than native unmodified LDL cholesterol [6]. Excessive production of circulating ox-LDL is associated with increased levels of endothelial adhesion molecules in the immunoglobulin superfamily, such as vascular cell adhesion molecule (VCAM-1), E-selectin, and intercellular cell adhesion molecule (ICAM-1), which mediate leukocyte adhesion to the endothelium [7].

Notably, overproduction of circulating ox-LDL leads to activation of inflammatory signaling by stimulating expression of the transcriptional regulator nuclear factor-kappa B (NF-κB), which governs the expression of many inflammatory genes that encode mediators of atherogenesis, including inflammatory chemokines and adhesion molecules [8]. Preventing the attachment of leukocytes to the endothelium induced by ox-LDL has been considered as a potential therapeutic strategy for atherosclerosis treatment.

Leukocytes have been closely associated with ox-LDL. Expressed by leukocytes in atherosclerotic plaques but not in normal vessels, 12/15-lipoxygenase catalyzed the oxidation of LDL [9]. Inhibition of 12/15-lipoxygenase using several different inhibitors can decrease oxidation of LDLs [10]. The heme enzyme myeloperoxidase, which can be found in human atherosclerotic plaques, is another agent secreted by neutrophils and leukocytes that modifies LDLs [11]. Additionally, generation of ROS such as superoxide anions has previously been reported to play a major role in the oxidation of LDL to a cytotoxin by activated

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leukocytes [12]. Native LDLs are internalized by macrophages at a pace too low to account for the formation of foam cells owing to LDL receptor downregulation. However, oxidative modification of LDLs allows them to be easily internalized and avidly accumulated by leukocyte scavenger receptors, leading to foam cell formation.

Fixed oil and essential oil are the most important chemical compositions of extracts from plants, including trees, shrubs, and vines. Multiple biological activities have been attributed to fixed oil derived from leaves, seeds, stems, and roots. Importantly, fixed oil has been reported to play essential pharmacological roles in several chronic diseases. For example, repeated administration of fixed oil extracted from Caryocar coriaceum Wittm. (Pequi) displayed hypolipemic effects by reducing total serum cholesterol and triglycerides while increasing HDL-C in mice [13]. Fixed oil of thymoquinone, the active constituent of Nigella sativa, has been shown to possess preventive potential against ethanol toxicity in liver and kidney tissues in rats through reducing lipid peroxidation and inflammation, as well as interrupting apoptosis [14]. Nigella sativa fixed oil exhibited an anti-aging effect in a D-galactosein-induced mice model of chronic aging by attenuating oxidative stress and apoptosis [15]. The fixed oil component α-linolenic acid extracted from Ocimum sanctum L. leaves provides renal protection against diabetes by exerting anti-hyperglycemic, anti-hyperlipidemic, and free radical scavenging effects in rats [16].

Bornyl acetate is the main volatile constituent present in numerous conifer oils and several other oils including red pine (Pinus densiflora) and valerian (valeriana officinalis) [17,18]. As a functional bicyclic monoterpene, bornyl acetate has been intensively used as the chemical and active composition in skin creams, wound care, and natural antiseptic agents [19]. In addition, bornyl acetate has displayed its antioxidant and anti-inflammatory properties in different types of cells and tissues [20]. For example, it has been recently reported that bornyl acetate obviously lowered the production of LPS-induced pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in vivo and in vitro. Bornyl acetate has been suggested as a preventive agent for lung inflammatory diseases [21]. However, little information regarding the effects of bornyl acetate in endothelial inflammation has been reported before. In the current study, we aimed to investigate the pharmacological roles of bornyl acetate against ox-LDL-induced leukocyte adhesion to the endothelium.

2. Materials and methods

2.1. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were commercially obtained (ATCC, USA). Cells were cultured in endothelial cell growth medium (Lonza, USA) with supplemental growth factors in accordance with the manufacturer's instructions (Cell Systems Corp., USA). Human monocytic leukemia cell line THP-1 cells were obtained from ATCC, USA. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), antibiotic/antimycotic solution (1%), and L-glutamine (Life Technologies, USA). HUVECs were seeded at 2.5×10^5 cells/mL in a 6-well plate for 12 h before any treatment. Ox-LDLs (Biosynthesis Biotechnology Company, Beijing, China) were dissolved in PBS at 1 g/L and added into cell culture medium at the final concentration of 100 mg/L. Bornyl acetate was dissolved in dimethyl sulfoxide (DMSO) at 1 g/L and added into the cell culture medium at the final concentration of 50, 100 μg/ml. Cells were treated with ox-LDL (100 mg/L) in the presence or absence of bornyl acetate (50, 100 μg/ml) for 24 h, pH7.4.

2.2. Assessment of cell viability

Cell viability of HUVECs was determined using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) kit (Thermo Fisher Scientific, USA). After washing 3 times with PBS, cells were loaded with MTT at a final concentration of 1 mg/ml and incubated for 4 h in a CO_2 incubator at 37 °C. The resultant insoluble formazan crystals were dissolved by dimethyl sulfoxide (DMSO). Absorbance of the formazan dye was recorded at 570 nm using a microtiter plate reader. The intensity of the OD value was used to index cell viability.

2.3. RNA isolation and real-time PCR

Total RNA was isolated from HUVECs using a commercial RNA isolation kit (Roche, USA). Two μg total RNA was used for reverse transcription using the iScript cDNA Synthesis kit (Bio-Rad, USA). Real-time PCR analyses of target genes (IL-1β, TNF- α, ICAM-1, VCAM-1, and E-selectin) were performed using the Roche universal probe library detection system. Relative quantification of gene expression was performed using the comparative threshold (CT) method. Changes in mRNA expression levels of target genes were calculated following normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta CT}$ method. The following primers were used in this study: human VCAM-1, (forward) 5′-CTTAAAATGCCTGGGAAGATGGT-3′; (reverse) 5′-GTCAATGAGACGGAGTCACCAAT-3′; human ICAM-1, (forward) 5′-GGCTGGAGCTGTTTGAGAAC-3′; (reverse) 5′-CTGACAAGTTGTGGGGGAGT-3′); E-selectin, (forward) 5′-GCC TGC AAT GTG GTT GAG TG-3′; (reverse) 5′-ACG AAC CCA TTG GCT GGA TT-3′; human TNF-α, (forward) 5′-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3′; (reverse) 5′-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3′), human IL-1β, (forward) 5′-GCCCATCCTCTGTGACTCAT-3′; (reverse) 5′-AGGCCACAGGTATTTTGTCG-3′), human GAPDH, (forward), 5′-CCACATCGCTCAGACACCAT-3′; (reverse) 5′-CCAGGCGCCCAATACG-3′.

2.4. Protein isolation and western blot analysis

Total intracellular protein was isolated from human umbilical vein endothelial cells (HUVECs) using RIPA lysis buffer (Thermo Fisher Scientific, USA) with protease inhibitor and phosphatase inhibitor cocktails following the instructions from the manufacture. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Sigma-Aldrich, USA). Equal amounts (20 μg) of total protein were resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA). The membrane was blocked with 5% none-fat milk diluted in TBST for 2 h at room temperature and incubated with the corresponding antibodies ICAM-1, VCAM-1, E-selectin and IκBα (all diluted 1:1000 in primary antibody dilution buffer) overnight at 4 ℃ with β-actin (as control diluted 1:5000 in primary antibody dilution buffer). Membranes were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:2000 in secondary antibody dilution buffer) for 2 h at room temperature. Immunodetection was carried out using an enhanced chemiluminescence reagent according to the manufacturer's instructions (BeyoECL Plus, USA). Equivalent protein loading and transfer efficiency were verified by staining for β-actin.

2.5. ELISA analysis

Protein levels of intracellular IL-1β and TNF-α were determined using ELISA kits (R&D Systems, USA) in accordance with the manufacturer's protocols. Briefly, ELISA plates were coated with mouse anti-IL-1β or anti-TNF-α antibody and stored overnight at 4 °C. The plates were then blocked with PBS containing 1% BSA and 5% sucrose for 1 h at room temperature and then washed with a wash buffer (0.05% Tween 20 in PBS) for twice (10 min/time). One-hundred μl culture supernatant was collected and incubated in the ELISA plate overnight at 4 °C. The plates were rinsed in wash buffer and then incubated sequentially with appropriate second antibody for 2 h at room temperature. After washing, 100 μl tetramethylbenzidine substrate (Sigma-