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Oregonin reduces lipid accumulation and proinflammatory responses in primary human macrophages

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ABSTRACT

Inflammation in the vascular wall is important for the development of atherosclerosis. We have previously shown that inflammatory macrophages are more abundant in human atherosclerotic lesions than in healthy arteries. Activated macrophages produce reactive oxygen species (ROS) that promote local inflammation in atherosclerotic lesions. Here, we investigated the role of oregonin, a diarylheptanoid, on proinflammatory responses in primary human macrophages and found that oregonin decreased cellular lipid accumulation and proinflammatory cytokine secretion. We also found that oregonin decreased ROS production in macrophages. Additionally, we observed that treatment of lipopolysaccharide-exposed macrophages with oregonin significantly induced the expression of antioxidant-related genes, including *Heme oxygenase-1* and *NADPH dehydrogenase quinone 1*. In summary, we have shown that oregonin reduces lipid accumulation, inflammation and ROS production in primary human macrophages, indicating that oregonin has anti-inflammatory bioactivities.

1. Introduction

Macrophages that are derived from foam cells play integral roles in atherosclerosis and are key regulators of lipid-driven proinflammatory responses that promote atherosclerosis [1]. We have previously shown that ischemia induces lipid accumulation in primary human macrophages [2]. An inflammatory subset of macrophages accumulates in atherosclerotic lesions and produces proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, and IL-6 [1,3]. Direct evidence for the critical role of inflammation in regulating early atherogenic events in atherosclerosis was provided by the finding that sub-endothelial monocytes could develop into foam cells after trans-endothelial migration across a TNF-α activated endothelium [4,5]. The numbers of proinflammatory macrophages have been shown to be increased in symptomatic compared with asymptomatic lesions [6]. Additionally, proinflammatory markers are expressed at higher levels in macrophages from human carotid lesions, suggesting a role for these cells in the atherosclerotic process. High levels of reactive oxygen species (ROS) in the artery wall are widely considered to promote atherosclerosis progression and possibly even initiation [7]. ROS are highly reactive molecules that are continuously produced by the mitochondrial electron transport chain and by ROS producing enzymes, such as NADPH-oxidases, xanthine oxidases, and lipoxygenases. At physiological levels, ROS perform important regulatory functions within cells and in tissues. At excessive levels, ROS irreversibly damage proteins, lipids, carbohydrates, and DNA. ROS levels have been reported to be high in stable atherosclerotic arteries, and such levels are even higher in unstable lesions [8].

Oregonin, an open-chain diarylheptanoid glycoside containing 3-carbonyl and 5-xylosyloxy groups, is found in the *Alnus* plants. Evidence indicates that this compound has anti-inflammatory and anti-oxidant properties. For example, *Alnus japonica* bark has been

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used as a health food to enhance immunity against influenza [9]. Previous studies have shown that oregonin from *A. japonica* suppresses inflammation in rabbit macrophages by regulating NF-κB signaling [10]. Furthermore, other studies have shown that oregonin reduces the expression of adhesion molecules and IL-1β in TNF-α-stimulated human endothelial cells [11]. Moreover, studies have shown that *Alnus acuminata* bark extracts possess strong anti-inflammatory bioactivities [12]. However, little is known about the effects of oregonin from *Alnus incana* on lipid accumulation and anti-inflammatory responses in primary human macrophages.

Here, we investigated the effect of oregonin from *A. incana* on foam cell formation, inflammation, and ROS production in human monocyte-derived macrophages and found that oregonin decreases cellular lipid accumulation, ROS production, and the secretion of proinflammatory cytokines.

2. Materials and methods

2.1. Oregonin isolation

Bark from the grey alder *A. incana* was collected in southwestern Latvia. Freeze-dried and powdered (fine enough to pass through a 420-μm sieve) bark was sequentially extracted using a Soxhlet apparatus with solvents of increasing polarity (first hexane, then ethyl acetate). The open-chain diarylheptanoid, 1,7-bis-(3,4-dihydroxyphenyl)-heptan-3-one-5-O-β-D-xylopyronoside, known as oregonin (Fig. 1A), was isolated from the bark ethyl acetate extract to 95% purity using the Biotage (Charlottsville, VA, USA) SP1 preparative chromatography system with a reverse-phase column (KPC18-HS, 35–70 mm, 90A, Biotage) using aqueous ethanol [13].

2.2. Primary human macrophages

Buffy coats were obtained from volunteer healthy adult blood donors at Kungälv Hospital, Sweden, and samples were made anonymous before handling. Human mononuclear cells were isolated by centrifugation over a discontinuous gradient of Ficoll-Paque (GE Healthcare, Little Chalfont, UK). To obtain proinflammatory macrophages, isolated cells were seeded in sterile 6-well plates (TPP, Switzerland) at a cell density of 2×10^6 cells/mL in RPMI medium and incubated for 2 h at 37 °C in a 5% CO_2 incubator to allow adherence of monocytes. After washing off non-adherent cells, monocytes were cultured in Macrophage-SFM media (Gibco, Carlsbad, CA, USA) containing granulocyte macrophage colony stimulating factor (GM-CSF) to allow differentiation. After 3 days, the media was changed to RPMI media without GM-CSF and cells were cultured for 4 days before use in experiments.

2.3. Quantification of Oil Red O-stained lipid droplets

Primary human macrophages were seeded in 8-well chamber slides at a cell density of 400 000 cells/well (Lab-Tek Systems) as described above. The cells were incubated under ischemic conditions (1% oxygen) for 24 h, in the absence or presence of 50 μmol/L oregonin. This oregonin concentration is shown to lower cholesterol levels in rats with induced hypercholesterolemia [14]. Cells were stained with Oil Red O and hematoxylin, and viewed with a Zeiss Axioplan 2 microscope. We analysed20 randomly selected images, containing approximately 20 cells/image, for each condition and blinded quantification of the total area (μm^2) of Oil Red O-stained lipid droplets per cell was performed using BioPix iQ 2.2.1 (Gothenburg, Sweden; see www.biopix.se for further information) [15].

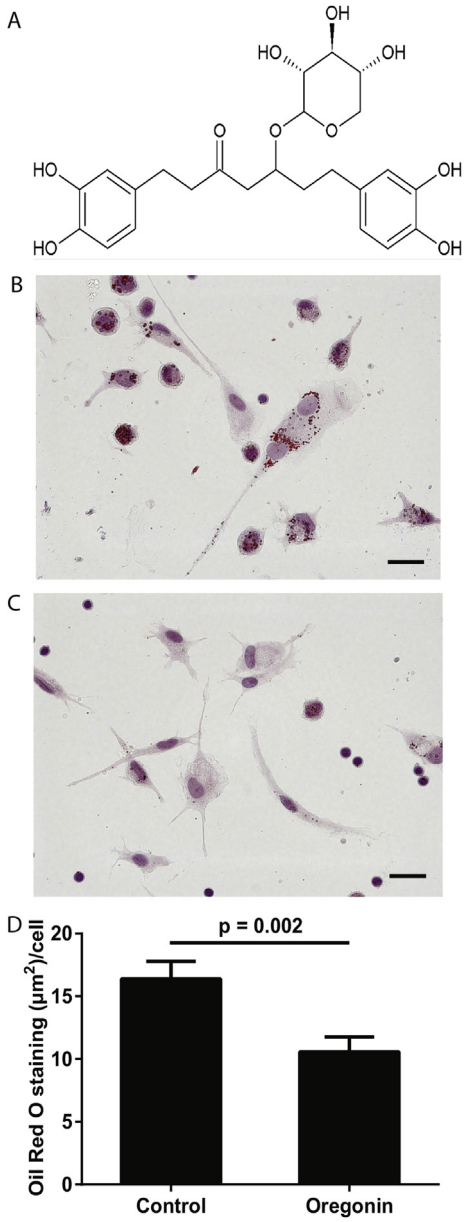

Fig. 1. Decreased cytosolic lipid droplets in oregonin-treated primary human macrophages. **A)** Oregonin, the open-chain diarylheptanoid, 1,7-bis-(3,4-dihydroxyphenyl)-heptan-3-one-5-O-β-D-xylopyronoside, was found to be the major bioactive compound in bark extracts from the grey alder (*Alnus incana*). **B–D)** Primary human macrophages were grown on chamber slides and incubated under ischemic conditions (1% oxygen) for 24 h, in the absence or presence of 50 μmol/L oregonin. Lipid accumulation was analyzed using Oil Red O staining. Representative images showing Oil Red O staining of **B)** control and **C)** oregonin-treated macrophages. **D)** Quantification of the total area (μm^2) of Oil Red O-stained lipid droplets per cell. Each bar indicates the mean ± SEM of all cells present in 20 randomly selected micrographs from four different donors. Bar 20 μm.

2.4. Cell viability

The cell viability was measured by using the Trypan blue exclusion test. Cells incubated with LPS or with oregonin in 6-well plate were cultured under normoxic conditions or hypoxic conditions for 24 h. Then the cells were washed with PBS and 0.5 mL of 0.2% trypan blue solution in PBS was added onto the cells and incubated for 5 min. Both the blue dead cells and living cells were counted.

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