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27-Hydroxycholesterol induces production of tumor necrosis factor-alpha from macrophages

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ABSTRACT

Enhanced production of TNF-α from macrophages promotes development and instability of atherosclerotic plaques, but involvement of lipid component in TNF-α production has not been clarified in atherosclerosis. We attempted to determine whether cholesterol oxidation products (oxysterols) could modify TNF-α production. Treatment of THP-1 cells with 27-hydroxycholesterol (27OHChol) or 7α-hydroxycholesterol (7αOHChol) resulted in a profound increase in TNF-α transcription, while treatment with an identical concentration of cholesterol and 7-ketochoelsterol did not lead to any change in TNF-α expression. Treatment with 27OHChol resulted in increased synthesis, as well as secretion, of TNF-α, while 7αOHChol led to increased synthesis of TNF-α without affecting secretion of the cytokine. Co-treatment with 7αOHChol or 27OHChol and LPS resulted in synergistically enhanced or augmented secretion of TNF-α. Treatment with TO-901317, pertussis toxin, PP2, and LY294002 resulted not only in attenuated transcription of TNF-α induced by 27OHChol and 7αOHChol, but also secretion of TNF-α enhanced by 27OHChol. This is the first report demonstrating enhanced production of TNF-α in macrophages by treatment with oxysterols which are detected in abundance in atheromatous lesions; in addition, results of the current study provide evidence indicating that certain types of oxysterols contribute to development of atherosclerosis by promoting production of proinflammatory cytokines.

1. Introduction

Tumor necrosis factor-alpha (TNF-α), which is produced primarily by macrophages, is detected as granular and diffuse extracellular deposits in the connective tissue matrix within atherosclerotic plaques [1,2]. The cytokine plays critical roles in development and destabilization of atherosclerotic plaques. TNF-α induces expression of cell adhesion molecules, such as E-selectin, vascular cell adhesion molecule 1 and intercellular cell adhesion molecule 1 on endothelial cells to which blood leukocytes bind in the process of transmigration from the blood stream into the vascular wall [3,4]. In addition, TNF-α triggers migration, proliferation, and apoptosis of vascular smooth muscle cells [5–7] and facilitates rupture of atherosclerotic plaques and thrombus formation [8]. Therefore, understanding the mechanisms of TNF-α production is important for preventive therapeutics of plaque development as well as complication of atherosclerosis. Elevation of TNF-α expression in atheromatous plaques [1,2] suggests possible involvement of lipids in dysregulated TNF-α expression. However, lipids that enhance production of TNF-α have not been identified in atherosclerosis.

Atherosclerosis is characterized by accumulation of fatty deposit, including free cholesterol and free fatty acids, as well as calcium and cellular debris in the arterial wall [9,10]. The deposited cholesterol undergoes oxidative modification. 27-Hydroxycholesterol (27OHChol) is the major oxidized cholesterol (oxysterol) found in advanced atherosclerotic lesions; its level is approximately proportional to cholesterol levels and increases with increasing severity of atherosclerosis [11,12]. After 27OH, 7-ketocholesterol (7K) is the next most abundant oxysterol in advanced atherosclerotic lesions, followed by 7β-hydroxycholesterol (7βOHChol) and 7α-hydroxycholesterol (7αOHChol). These oxysterols comprized 75–85% of oxysterols detected in plaques from different sites [13,14]. The effects of oxysterols on vascular cells differ from those of cholesterol [15], and many of the atherogenic characteristics of oxidized low density lipoproteins are attributed to the oxysterol content of the lipoprotein [16]. Yet, roles of the above mentioned oxysterols in TNF-α production have not been determined.

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Because elevated production of TNF-α promotes atherosclerosis and plaque instability, identification of lipids responsible for upregulation of TNF-α and characterization of molecular mechanisms leading to TNF-α expression will provide new perspective on pathogenesis of as well as therapeutic strategy for treatment of atherosclerosis. We investigated the question of whether oxysterols could induce expression of TNF-α. To answer the question, we investigated the effects of subcytotoxic levels of 27OHChol, 7K, and 7αOHChol on TNF-α production. We demonstrated for the first time that particular types of oxysterols induce elevated expression of TNF-α in macrophages, and attempted to identify cellular molecules involved in upregulation of TNF-α in response to oxysterols.

2. Materials and methods

2.1. Cell culture and reagents

THP-1 cells purchased from American Type Culture Collection (ATCC, Manassas, VA) were maintained as suggested by the ATCC: THP-1 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO_2 in the presence of penicillin (50 units/mL) and streptomycin (50 μg/mL). TO-901317, pertussis toxin (PTX), and PP2 were purchased from Sigma–Aldrich Co. (St. Louis, MO). 7αOHChol, 27OHChol, cholesterol and 7-ketocholesterol (7K) were purchased from Research Plus, Inc. (Bayonne, NJ).

2.2. Analysis of the TNF-α gene transcript by reverse transcription coupled polymerase chain reaction (RT-PCR) or real-time PCR

RT-PCR and real-time PCR were performed as previously described [17]. In brief, total RNA isolated from THP-1 cells were reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase, followed by 25 cycles of PCR for amplification of TNF-α cDNA. Quantitative real-time PCR was performed in triplicate in 384-well plates containing SYBR Green PCR Master Mix and 10 pM forward primer and reverse primer for TNF-α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequence of TNF-α primers was 5-ATGAGCACTGAAAGCATGATCC-3 (forward) and 5-GAGGGCTGATTAG AGAGAGGTC-3 (reverse). Primers for GAPDH were 5-ATGGGGAAGGTGAAGGTCG-3 (forward) and 5-GGGGTCAT TGATGGCAACAATA-3 (reverse).

2.3. Enzyme linked immunosorbent assay (ELISA) of TNF-α

Levels of TNF-α protein released into the medium were measured using the ELISA kits purchased from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

2.4. Analysis of intracellular TNF-α protein

Phycoerythrin (PE)-conjugated anti-human TNF-α mAb and PE-conjugate isotype IgG1 were purchased from BD Biosciences (San Diego, CA), and intracellular TNF-α protein was stained according to the manufacturer's instructions. After stimulation for 48 h with oxysterols, THP-1 cells were incubated for 2.5 h with brefeldin A (10 μg/mL). Cells were collected by centrifugation, fixed using BD Cytofix/Cytoperm solution, permeablized using BD Perm/Wash buffer, and incubated for 40 min at 4 °C with the PE-conjugated TNF-α mAbs or PE-conjugated isotype-IgG1 diluted 1:50 in phosphate buffered saline (PBS). After two washes with PBS, cells were resuspended in 1% paraformaldehyde in PBS and analyzed using a FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA).

2.5. Statistics

Statistical analyses were performed using one-way ANOVA, followed by Tukey's multiple comparison test, using GraphPad PRISM (version 5.0) (GraphPad Software Inc., San Diego, CA).

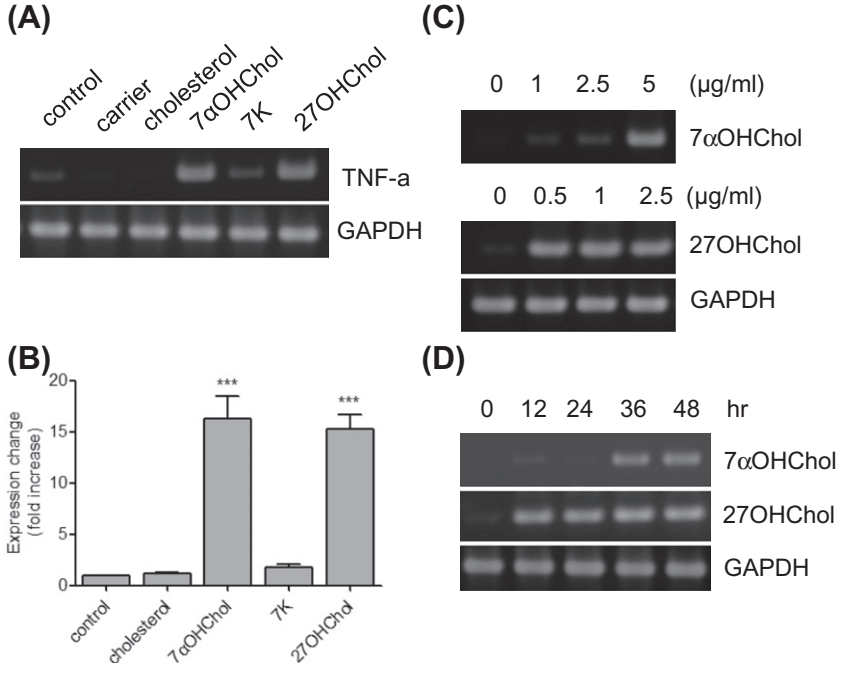

Fig. 1. The effects of cholesterol and oxysterols on TNF-α expression in THP-1 cells. (A) THP-1 cells (2.5×10^6 cells/a 100 mm culture dish) were serum starved for 24 h in 0.1% BSA (endotoxin free) in RPMI 1640 and stimulated for 48 h with cholesterol, 7αOHChol, 7K (5 μg/mL each) and 27OHChol (2.5 μg/mL) or an equal volume of ethanol, the carrier for oxysterols in the presence of 10% FBS. TNF-α transcripts were amplified by RT-PCR. (B) The relative expression ratio of TNF-α transcripts was determined by real-time PCR, and levels of TNF-α transcripts were plotted as averages of fold changes in each treatment group in comparison with control cells. Data are expressed as mean ± SD ($n = 3$ replicates/group). $^{***}P < 0.001$ vs. control. (C) THP-1 cells were treated for 48 h with the indicated amount of 7αOHChol and 27OHChol and TNF-α transcripts were amplified. (D) THP-1 cells were treated for the indicated time periods with 7αOHChol and 27OHChol and TNF-α transcripts were amplified.