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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).

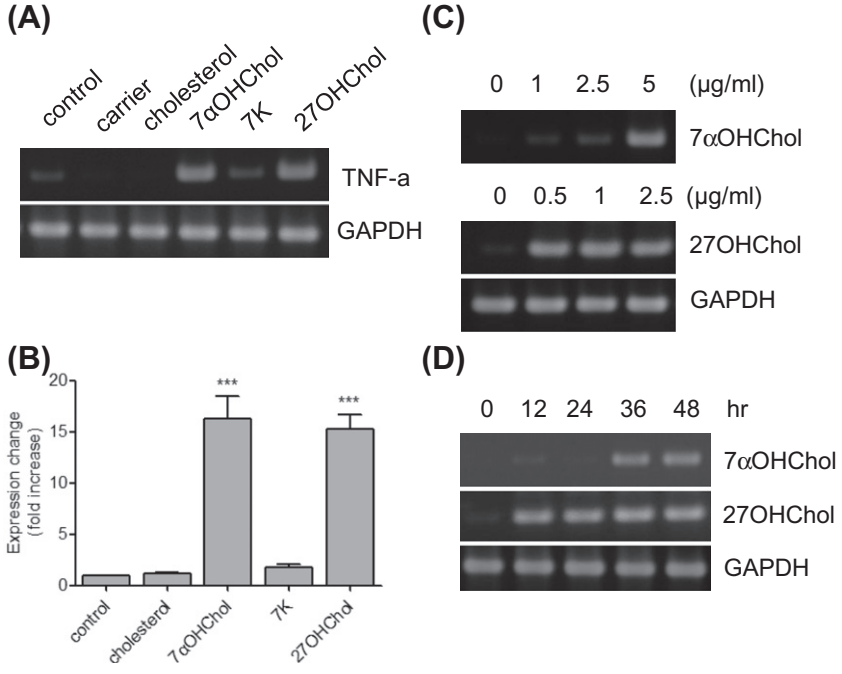

Fig. 1. The effects of cholesterol and oxysterols on TNF-α expression in THP-1 cells. (A) THP-1 cells (2.5×10^6 cells/a 100 mm culture dish) were serum starved for 24 h in 0.1% BSA (endotoxin free) in RPMI 1640 and stimulated for 48 h with cholesterol, 7αOHChol, 7K (5 μg/mL each) and 27OHChol (2.5 μg/mL) 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.

3. Results

3.1. 7αOHChol and 27OHChol induced expression of TNF-α in THP-1 cells

In order to investigate the effects of cholesterol and oxysterols on expression of TNF-α in macrophages, serum-starved THP-1 cells were treated for 48 h with cholesterol, 7αOHChol, 7K, and 27OHChol, prior to examination of TNF-α expression. Transcripts of TNF-α were rarely detected from THP-1 cells, while transcription of TNF-α showed a remarkable increase in the presence of 7αOHChol and 27OHChol, as determined by RT-PCR. However, cholesterol and 7K did not influence transcription of the cytokine (Fig. 1A). Quantitative real-time PCR was performed for analysis of changes in the levels of TNF-α transcripts. 7αOHChol and 27OHChol elevated levels of TNF-α transcripts by 16.3- and 15.2-fold, respectively, in comparison with control, while treatment with cholesterol and 7K did not result in increase in the levels of TNF-α transcripts (Fig. 1B). We performed concentration and time course experiments using 7αOHChol and 27OHChol. Transcription of TNF-α was induced at different concentrations of the oxysterols. Of the two oxysterols, 27OHChol induced expression of TNF-α at lower concentrations. Treatment with 5 μg/mL of 7αOHChol and with 0.5 μg/mL or more of 27OHChol led to expression of TNF-α (Fig. 1C). Transcription of TNF-α was induced at different time points after treatment with the two oxysterols. 7αOHChol and 27OHChol induced transcription of TNF-α at 36 h and at 12 h post-treatment, respectively, and levels of TNF-α transcripts induced by the oxysterols were sustained up to 48 h post-treatment (Fig. 1D).

3.2. 27OHChol, but not 7αOHChol, enhanced secretion of TNF-α protein

Because 7αOHChol and 27OHChol induced transcription of TNF-α, we investigated the question of whether treatment with 7αOHChol or 27OHChol resulted in production of TNF-α protein. Treatment with 27OHChol, but not with cholesterol and 7K, resulted in increased secretion of TNF-α from THP-1 cells, in agreement with real-time PCR results. However, treatment with 7αOHChol did not lead to secretion of TNF-α (Fig. 2A). We investigated the question of whether TNF-α protein was synthesized in response to 7αOHChol. We examined the effects of 7αOHChol on intracellular expression of TNF-α protein in parallel with 27OHChol. Because the primary pool of TNF-α in activated macrophages is held in the Golgi complex [18], THP-1 cells were treated with the oxysterols followed by incubation with brefeldin A, a potent transport inhibitor, in order to block Golgi function and TNF-α secretion.

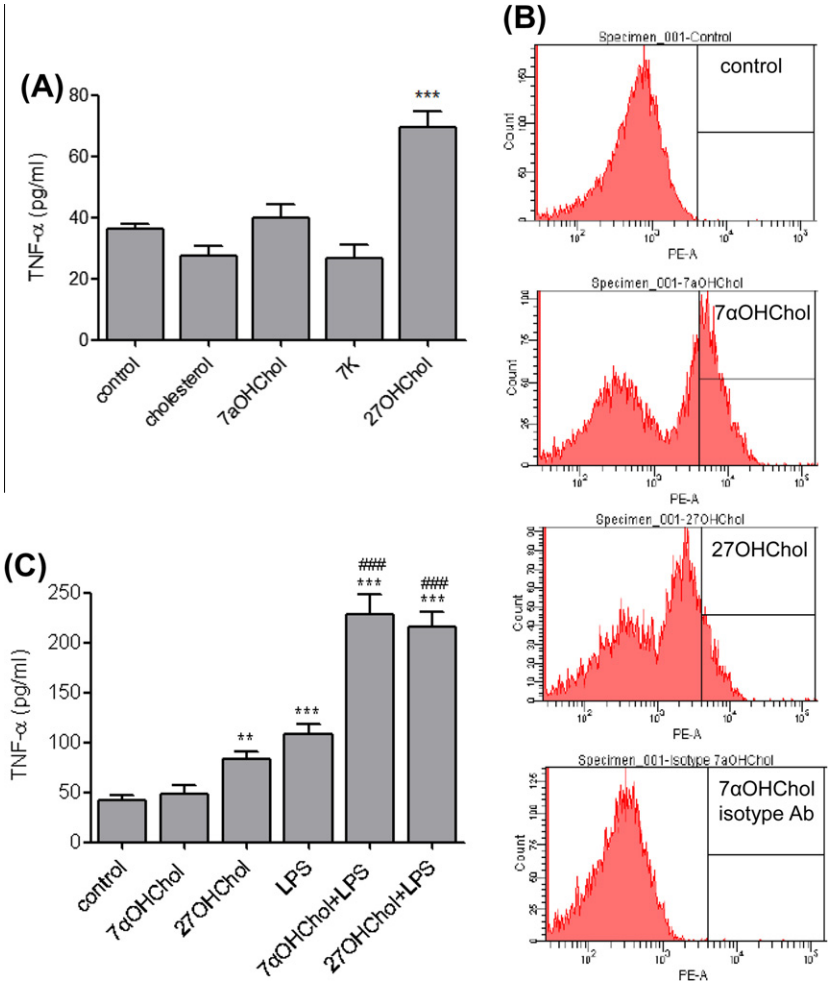

Fig. 2. The effects of oxysterols and LPS on TNF-α production. (A) THP-1 cells were incubated for 48 h with cholesterol or indicated oxysterols and the amount of TNF-α secreted into the culture media was determined by ELISA. $^{***}P < 0.001$ vs. control. (B) THP-1 cells were incubated for 48 h with 7αOHChol and 27OHChol, followed by treatment of cells for 2.5 h with brefeldin A (10 μg/mL). Cells were harvested and stained with PE-conjugated anti-human TNF-α antibody. Fluorescence was analyzed using flow cytometry. Each plot represents 10,000 events. (C) THP-1 cells were incubated for 48 h with 7αOHChol and 27OHChol in the absence and presence of LPS and the amount of TNF-α secreted into the culture media was determined. $^{**}P < 0.01$ vs. control, $^{***}P < 0.001$ vs. control, $^{\#\#\#}P < 0.001$ vs. 7αOHChol or 27OHChol or LPS.

Flow cytometry was performed for analysis of cells after staining with PE-conjugated anti-TNF-α antibodies. Compared with control, treatment with 7αOHChol and 27OHChol resulted in distinct shifts in the distribution of fluorescence by TNF-α. The percentage of TNF-α positive cells (i.e., in the lined region) was 0.1% in the absence of oxysterols, and the percentage of TNF-α positive cells increased to 39.1% and 30.5% in the presence of 7αOHChol and 27OHChol, respectively (Fig. 2B), indicating an increase in the level of intracellular TNF-α. In 7αOHChol-stimulated THP-1 cells stained with PE-conjugate non-immunized IgG, fluorescence remained unchanged. Next, we investigated the question of whether the presence of an additional stimulus, in addition to oxysterols, promoted secretion of TNF-α. THP-1 cells were incubated with 7αOHChol or 27OHChol in the absence and presence of lipopolysaccharide (LPS), followed by measurement of the amount of TNF-α secreted into culture medium (Fig. 2C). Treatment with 27OHChol or LPS, but not 7αOHChol, resulted in increased secretion of TNF-α, and treatment with oxysterols in combination with LPS further enhanced secretion of TNF-α. In addition, because the amount of TNF-α secreted in response to co-treatment was much higher than the summation of the amount of TNF-α secreted by each treatment, co-treatment with 7αOHChol and LPS seemed to result in synergistically enhanced production of TNF-α.

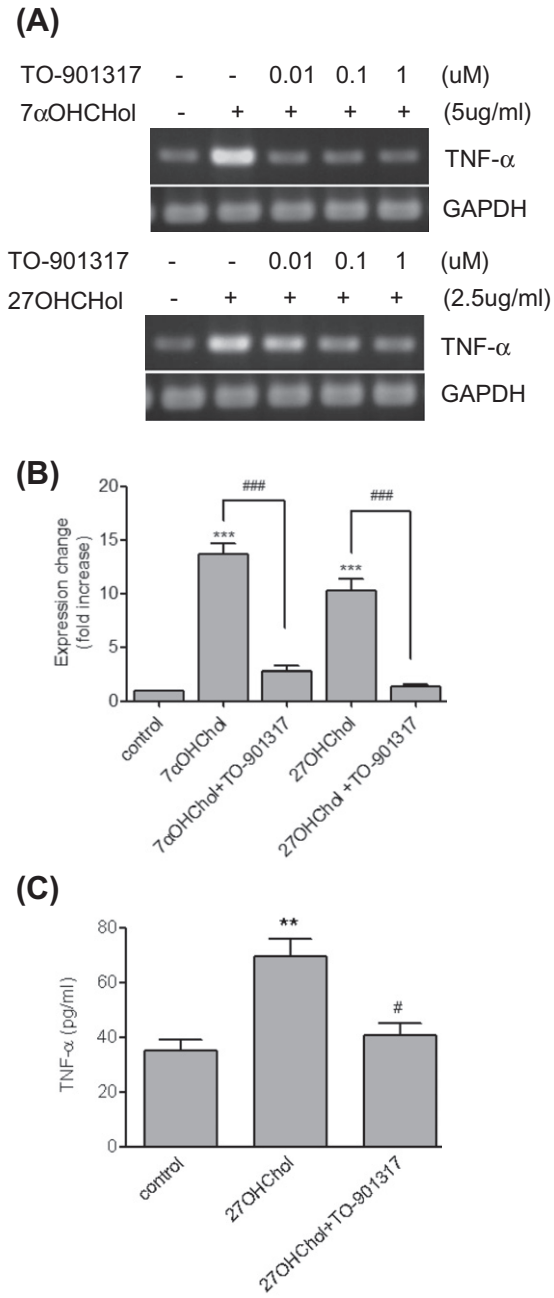

Fig. 3. The effects of TO-901317 on oxysterol-induced expression of TNF-α. (A) THP-1 cells were pre-incubated for 2 h with the indicated concentrations of TO-901317, prior to incubation for 48 h with 7αOHChol and 27OHChol. TNF-α transcripts were amplified. (B) The relative expression ratio of TNF-α transcripts 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. $^{###}P < 0.001$ vs. 7αOHChol or 27OHChol. (C) THP-1 cells were incubated for 48 h with 27OHChol in the absence and presence of TO-901317 (1 μM). The amount of TNF-α secreted into the culture media was determined. $^{**}P < 0.01$ vs. control, $^{#}P < 0.05$ vs. 27OHChol.

3.3. The liver X receptor (LXR) agonist TO-901317 inhibited oxysterol-induced expression of TNF-α

Because inhibited progression of atherosclerosis by treatment with T-0901317, a synthetic LXR agonist, has been demonstrated in mice fed high cholesterol diet [19,20], the effects of LXR agonist on oxysterol-induced expression of TNF-α were investigated. THP-1 cells were treated with 7αOHChol or 27OHChol in the presence of different concentrations of TO-901317, prior to examination of TNF-α expression. TNF-α transcription induced by 7αOHChol and 27OHChol was attenuated in the presence of TO-901317 (Fig. 3A and B). In comparison with control, treatment with 7αOHChol resulted in an increase in levels of TNF-α transcripts by 13.7-fold, which was reduced to 2.7-fold in the presence of TO-901317; treatment with 27OHChol resulted in an increase in the level of TNF-α transcripts by 10.4-fold, which was almost abrogated in the presence of TO-901317. Treatment with the LXR agonist also affected TNF-α production (Fig. 3C). Treatment with 27OHChol resulted in increased secretion of TNF-α, which was significantly reduced in the presence of TO-901317.

3.4. PTX, PP2 and LY294002 inhibited oxysterol-induced expression of TNF-α

To the best of our knowledge, transcription of TNF-α in the presence of 7αOHChol and 27OHChol has not been previously reported. We sought to identify the intracellular signaling molecules involved in 7αOHChol- and 27OHChol-induced expression of TNF-α in order to understand how both oxysterols exert their effects. Prior to assessment of TNF-α expression, THP-1 cells were treated with 7αOHChol or 27OHChol in the absence or presence of non-cytotoxic concentrations of diverse pharmacological inhibitors. Treatment with 7αOHChol or 27OHChol resulted in increased levels of TNF-α transcripts, which was significantly inhibited in the presence of PXT, PP2, and LY294002, as determined by real-time PCR (Fig. 4A–C) and RT-PCR (Supplement Fig. 1). In addition, secretion of TNF-α enhanced by 27OHChol was significantly attenuated in the presence of PXT, PP2, and LY294002 (Fig. 4D).

4. Discussion

7αOHChol, 7K, and 27OHChol, as well as free cholesterol, are present abundantly in the atherosclerotic lesion [9,12]. We demonstrated that 7αOHChol and 27OHChol induced significant elevation of the level of TNF-α mRNA, while cholesterol and 7K did not affect its level in THP-1 human macrophages. In observing transcription of TNF-α in response to treatment with 7αOHChol and 27OHChol, we questioned whether contaminating endotoxin, if any,

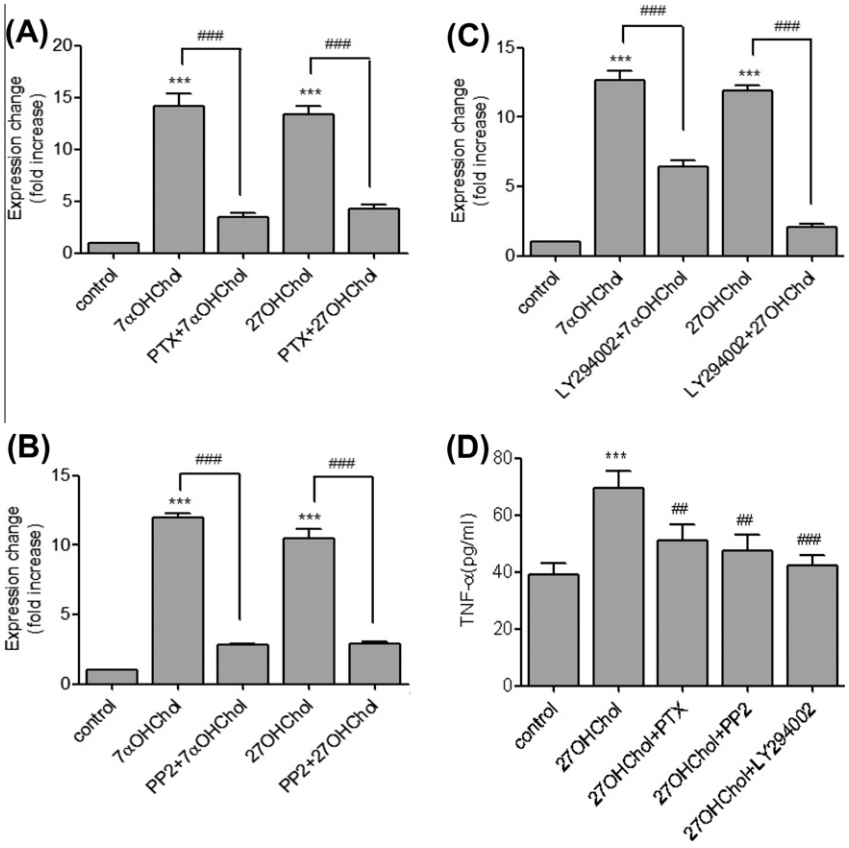

Fig. 4. The effects of PTX, PP2, and LY294002 on oxysterol-induced expression of TNF-α. (A–C) THP-1 cells were pre-incubated for 2 h with PTX (100 ng/mL) (A), PP2 (25 μM) (B), and LY294002 (C), prior to incubation for 48 h with 7αOHChol and 27-OHChol. TNF-α transcripts were amplified and the relative expression ratio of TNF-α transcripts was determined. Levels of TNF-α transcripts were plotted as averages of fold changes in each treatment group in comparison with control cells. $^{***}P < 0.001$ vs. control. $^{\#\#\#}P < 0.001$ vs. 7αOHChol or 27OHChol. (D) THP-1 cells were incubated for 48 h with 27OHChol in the absence and presence of the indicated inhibitors. The amount of TNF-α secreted into the culture media was determined. $^{***}P < 0.001$ vs. control, $^{\#}P < 0.05$ vs. 27OHChol, and $^{\#\#}P < 0.01$ vs. 27OHChol.

contributed to or might be responsible for expression of TNF-α through activation of Toll-like receptor (TLR)-4 [21]. To rule out that possibility, we used polymyxin B and OxPAPC. Polymyxin B binds to LPS in order to prevent its biological effects [22]. OxPAPC inhibits Toll-like receptors (TLRs)-2/4, thereby preventing expression of chemokines and cytokines in response to pathogen-associated molecular patterns (PAMPs) [23,24]. We found that treatment with polymyxin B and OxPAPC did not result in attenuated expression of TNF-α induced by the two oxysterols (Supplemental data). These results indicate that exposure of macrophages to 7αOHChol or 27OHChol alone is sufficient to induce expression of TNF-α via (TLRs)-2/4 independent mechanisms. These results are in agreement with those of a previous study reported by Erridge et al., who reported that some oxysterols might contribute to increased expression of certain inflammatory genes by mechanisms independent of TLR signaling [25].

The effects of oxysterols on production of TNF-α protein were investigated in parallel with LPS that enhances release of TNF-α from THP-1 cells with activation of secretory pathways [26,27]. Treatment with 27OHChol and LPS resulted in significantly enhanced secretion of TNF-α, while treatment with cholesterol, 7K, and 7αOHChol did not. The observation that 7αOHChol did not affect TNF-α secretion was interesting since marked elevation of the levels of TNF-α transcripts observed by treatment with 7αOHChol was as efficient as that observed by treatment with 27OHChol. We assumed that it may be due to lack of synthesis of TNF-α protein and/or failure in secretion. When intracellular TNF-α was assessed by flow cytometry, both 7αOHChol and 27OHChol induced an increase in immunoreactivity of TNF-α. Increase in immunoreactivity of TNF-α in the presence of 7αOHChol was comparable to that attained by treatment with 27OHChol. These data indicated that 7αOHChol was as effective as 27OHChol in terms of increasing intracellular expression of TNF-α protein. Because LPS activates multiple signaling molecules necessary for TNF-α secretion [26], we investigated the question of whether exposure to LPS could enhance TNF-α secretion. Exposure of THP-1 cells whose intracellular level of TNF-α protein was elevated after treatment with 7αOHChol resulted in synergistically enhanced production of TNF-α. The secretion was comparable to that released by THP-1 cells treated with 27OHChol plus LPS. Taken together, these data indicate that 7αOHChol, as well as 27OHChol, elevate intracellular TNF-α and that TNF-α secretion is further enhanced when cells were exposed to the oxysterols in combination with an additional stimulus, such as LPS.

Because treatment with TO-901317 resulted in reduction of atherosclerotic lesions in mice models, LXR receptors have been proposed as targets for atherosclerosis therapy. Treatment with TO-901317 resulted in suppression of vascular expression of the inflammatory transcription factor NF-κB, adhesion molecules (E-selectin, ICAM-1, and CD44), monocyte adhesion, and lesional macrophage content without increases in the levels of plasma triglyceride and total cholesterol, in addition to enhancing expression of genes promoting cholesterol efflux, like ABCA1 and ABCG1 [19,20]. We found that treatment with the synthetic LXR agonist, T-0901317, resulted in significantly inhibited oxysterol-mediated expression of TNF-α. Since, TNF-α plays a major role in expression of adhesion molecules via NF-κB and adhesion of monocytes onto endothelial cells [28,29], attenuated production of TNF-α will lead

to a reduction of the inflammatory process. The results of the current study imply that regulatory effects of TO-901317 on expression of TNF-α may contribute to anti-inflammatory and eventually atheroprotective effects of the LXR agonist.

Since the mechanisms through which 7αOHChol or 27OHChol induces expression of TNF-α are unknown, we attempted to elucidate signaling pathways involved in oxysterol-induced expression of TNF-α. We found that transcription of TNF-α induced by 7αOHChol and 27OHChol and secretion of TNF-α in response to 27OHChol were significantly inhibited in the presence of PTX, PP2, and LY294002. These results indicate that PTX-sensitive Gi-proteins, Src tyrosine kinase, and phosphoinositide 3-kinase (PI3K) are essential components for TNF-α expression in THP-1 cells. The mitogen-activated protein kinase (MAPK) pathway has been suggested as a key point in oxysterol-induced signal transduction, as inhibition of upstream MAPK kinase using PD98059 abolishes both increase of NF-κB nuclear binding by the oxysterol mixture and upregulation of monocyte chemoattractant protein-1 (MCP-1, CCL2) synthesis by either oxysterol mixture or 7αOHChol in human promonocytic U937 cells [30]. Therefore, we investigated the question of whether PD98059 had any effects on TNF-α expression. We were unable to obtain data demonstrating attenuation of expression of TNF-α induced by 7αOHChol or 27OHChol in the presence of the inhibitor. However, the inability of PD98059 to attenuate TNF-α expression appeared not to be due to lack of its inhibitory activity, since co-treatment with PD98059 significantly inhibited expression of CCL2 induced by 7αOHChol and 27OHChol (Supplemental data). Currently, we are working on elucidation of molecular mechanisms through which oxysterols induce expression of TNF-α and types of connections, if they exist, among PXT sensitive Gi-proteins, Src tyrosine kinase, and PI3K.

In conclusion, as demonstrated by our results, treatment of macrophages with 7αOHChol or 27OHChol resulted in a significant increase in synthesis as well as secretion of TNF-α, primarily 27OHChol, from macrophages. In addition, exposure of macrophages to LPS in the presence of 7αOHChol or 27OHChol resulted in augmented secretion of TNF-α. Taken together, our data suggest that certain types of oxysterols can modulate inflammatory responses and the effects of infectious burden via macrophages by enhancing production of proinflammatory cytokine, including TNF-α, thereby promoting atherosclerosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.021.

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