25-Hydroxycholesterol-3-sulfate attenuates inflammatory response via PPARγ signaling in human THP-1 macrophages

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Xu L, Shen S, Ma Y, Kim JK, Rodriguez-Aguado D, Heuman DM, Hylemon PB, Pandak WM, Ren S. 25-Hydroxycholesterol-3-sulfate attenuates inflammatory response via PPARγ signaling in human THP-1 macrophages. Am J Physiol Endocrinol Metab 302: E788–E799, 2012.—The nuclear receptor peroxisome proliferator-activated receptors (PPARs) are important in regulating lipid metabolism and inflammatory responses in macrophages. Activation of PPARγ represses key inflammatory response gene expressions. Recently, we identified a new cholesterol metabolite, 25-hydroxycholesterol-3-sulfate (25HC3S), as a potent regulatory molecule of lipid metabolism. In this paper, we report the effect of 25HC3S and its precursor 25-hydroxycholesterol (25HC) on PPARγ activity and on inflammatory responses. Addition of 25HC3S to human macrophages markedly increased nuclear PPARγ and cytosol IkB and decreased nuclear NF-κB protein levels. PPARγ response element reporter gene assays showed that 25HC3S significantly increased luciferase activities. PPARγ competitor assay showed that the K_i for 25HC3S was ~1 μM, similar to those of other known natural ligands. NF-κB-dependent promoter reporter gene assays showed that 25HC3S suppressed TNFα-induced luciferase activities only when cotransfected with pcDNA1-PPARγ plasmid. In addition, 25HC3S decreased LPS-induced expression and release of IL-1β. In the PPARγ-specific siRNA transfected macrophages or in the presence of PPARγ-specific antagonist, 25HC3S failed to increase IkB and to suppress TNFα and IL-1β expression. In contrast to 25HC3S, its precursor 25HC, a known liver X receptor ligand, decreased nuclear PPARγ and cytosol IkB and increased nuclear NF-κB protein levels. We conclude that 25HC3S acts in macrophages as a PPARγ ligand and suppresses inflammatory responses via the PPARγ/IkB/NF-κB signaling pathway.

Peroxisome proliferator-activated receptor-γ; oxysterols; oxysterol sulfation; cholesterol metabolites; inflammatory response; macrophages; nuclear factor-κB signaling pathway

MACROPHAGES ARE THE KEY CELLULAR PLAYERS IN THE PATHOGENESIS OF Atherosclerosis. In the early stage of atherosclerosis, macrophages in arterial walls accumulate lipids. These lipid-laden macrophages, termed foam cells, are characteristic of a reversible early cellular phase of atherosclerotic lesions. Progressive lipid accumulation leads to further escalation of inflammatory responses and infiltration of inflammatory cells (26). Through this process, early cellular lesions are transformed to late, fibrous, atherosclerotic plaques. Physiological or pharmacological maneuvers that reduce macrophage lipids and inflammatory responses may be effective in preventing or reversing atherosclerosis.

Nuclear receptors are ligand-activated transcription factors that regulate the expression of target genes to affect processes as diverse as reproduction, inflammation, development, and metabolism (17). Nuclear receptor peroxisome proliferator-activated receptors (PPARs) play major roles in the regulation of lipid metabolism, glucose homeostasis, and inflammatory processes and may be ideal targets for therapeutic management strategies for cardiovascular diseases (4, 6, 11, 16, 23, 42). PPARγ appears particularly important in regulating genes involved in lipid metabolism and inflammation (1, 8, 20, 21). The PPARγ:RXR heterodimer exists in both an active and an inactive state as a corepressor exchange mechanism (5). When inactive, it is bound to corepressors such as the nuclear receptor corepressor (NCOR) or the silencing mediator for retinoid and thyroid hormone receptor (SMRT). In the presence of ligand for either PPAR or RXR, the corepressor dissociates so that the ligand can bind and activate coactivators, such as steroid receptor coactivator (SRC1) and PPARγ coactivators 1 and 2 (5). Activation of PPARγ inhibits inflammatory responses by preventing the activation of nuclear transcription factors such as NF-κB, activating protein-1, and signal transducer and activator of transcription 1. Since inflammation plays an important role in atherosclerosis, this anti-inflammatory effect of PPARγ helps to reduce the risk of atherosclerosis (14, 15, 45). IkBα is one of the key regulatory elements and plays a central role in the inflammatory responses; its intracellular levels regulate inflammatory responses, and its expression is highly regulated (25, 54, 59). When IkBα is phosphorylated and degraded, NF-κB will be translocated to nuclei and up-regulate IkBα expression. PPAR response element (PPRE) has been identified in the IkBα promoter region and has shown the ability to bind to PPARγ and PPARδ with a similar activity (7). Thus, IkBα expression can be upregulated not only by NF-κB but also by nuclear receptor ligands such as glucocorticone, dexamethasone (12, 40), and PPAR ligands (7).

Oxysterols play an important role in the maintenance of lipid homeostasis (27). Recently, we identified a novel oxysterol, 5-cholenstein 3,25-diol 3-sulfate (or 25-hydroxycholesterol-3-sulfate, 25HC3S), which accumulates in hepatocyte nuclei following overexpression of the mitochondrial cholesterol delivery protein StarD1 (44, 50, 51). Macrophages are able to synthesize this oxysterol (38). This oxysterol appears to be synthesized from 25-hydroxycholesterol (25HC) by sterol sulfotransferase-2B1b (SULT2B1b) (36). Overexpression of SULT2B1 impairs the response of oxysterol receptor liver X receptor (LXR) to multiple oxysterol ligands. The reaction catalyzed by SULT2B1 is hypothesized to be an inactivation process (18). However, addition of 25HC3S to primary hepatocytes downregulates the expression of key enzymes involved in lipid metabolism and decreases lipid biosynthesis by inactivating the LXR/SREBP-1c signaling pathway in hepatocytes.
and macrophages (38, 52, 58). Furthermore, overexpression of SULT2B1b decreases intracellular lipid levels in human aortic cells (3).

Several studies show that inflammation is closely associated with dysregulation of lipid metabolism (24, 29). Infection and inflammation induce the acute-phase response (APR), leading to multiple alterations in lipid and lipoprotein metabolism. APR increases plasma triglyceride levels, de novo hepatic fatty acid synthesis, and suppression of fatty acid oxidation (34). The molecular mechanism during the APR involves coordinated changes in several orphan nuclear receptors, including PPARs, LXRs, and RXRs (34, 57). PPARγ as well as LXRs reciprocally regulate inflammation and lipid metabolism (4, 10). The processes by which these events occur are not fully understood. The cholesterol metabolites 25HC and 25HC3S have been shown to be the coordinated regulators of lipid metabolism via the LXR/SREBP signaling pathway (38, 52). It is possible that, in addition to and in conjunction with their regulation of lipid metabolism, 25HC and 25HC3S are able to regulate inflammatory responses through the PPAR signaling pathway.

In the present study, we report that 25HC3S increases nuclear PPARγ levels and represses inflammatory responses. In contrast, 25HC elicits opposite effects on these responses. We hypothesize that the effects of the oxysterols on inflammatory responses are mediated via activation of the PPAR/Îºb/NF-κB signaling pathway in THP-1-derived macrophages.

**MATERIALS AND METHODS**

**Materials.** Cell culture reagents and supplies were purchased from GIBCO-BRL (Grand Island, NY); the reagents for real-time RT-PCR were obtained from AB Applied Biosystems (Foster City, CA). Antibodies against human PPARγ (Cat. no. sc-7273), ÎºbX (Cat. no. sc-371), NF-κb (Cat. no. sc-372), and lamin B1 (Cat. no. sc-56145) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against human phosphorylated ÎºbX (p-ÎºbX; Cat. no. 9246) was purchased from Cell Signaling (Danvers, MA); FuGENE HD transfection reagent was obtained from Roche Applied Science (Indianapolis, IN). Single Analyte ELISArray kits were purchased from Superarray (Frederick, MD). The Dual-Glo Luciferase Assay System and pGL3-NF-κb-luc were purchased from Promega (Madison, WI). IKK inhibitor X was from Calbiochem (Gibbstown, NJ); PPARγ agonist rosiglitazone (Rosi) and antagonist T0070907 were from New Cayman Chemical (Ann Arbor, MI). Arachidonic acid (AA), docosahexaenoic acid (DHA), and 15-deoxy-d12,14-PGJ2 (PGJ2) were from Sigma (St. Louis, MO). pGL3-PPARγ response element (PPRE)-luciferase reporter containing three copies of PPRE from the promoter of rat acyl-CoA oxidase and the receptor expression plasmids pcDNA-PPARγ1 were kindly provided by Dr. Gabor Tigiyi (University of Tennessee Health Science Center) (39).

**Cell culture.** Human THP-1 monocytes and H441 (human lung adenocarcinoma cell line) cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained according to the supplier’s protocols. THP-1 monocytes were differentiated to macrophages by adding 100 nM phorbol 12-myristate 13-acetate (PMA). When cells reached ~90% confluence, oxysterols in DMSO or in ethanol (final concentration in media was <0.1%) were added as indicated. The cells were harvested at the times indicated. Nuclear and cytosolic fractions were isolated using NE-PER, Nuclear, and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL).

**Western blot analysis of nuclear PPARγ, NF-κb, intracellular ÎºbXa, and p-ÎºbXa levels.** Fifty micrograms of total cell lysates or nuclear protein extracts, otherwise as indicated, was separated on 10% SDS-PAGE gels and transferred onto a polyvinyldene difluoride membrane as described previously (49). Membranes were blocked in TBS containing 5% nonfat dried milk for 1 h. The specific proteins were determined by incubation with specific antibodies against human PPARγ, NF-κbX, ÎºbXa, or p-ÎºbXa at 4°C with shaking overnight. After washing, the membrane was incubated in a 1:3,000 dilution of a secondary antibody (goat anti-rabbit or anti-mouse IgG-HP conjugate; Bio-Rad, Hercules, CA) at room temperature in the washing buffer (Tris-buffered solution containing 0.5% Tween 20) for 1 h. The protein bands were visualized using Western Lightening Chemiluminescence Reagent (PerkinElmer, Waltham, MA). The protein expression levels were normalized to β-actin, nuclear protein to lamin B1.

**Detection of intracellular distribution of PPARγ in THP-1-derived macrophages.** THP-1-derived macrophages were cultured on coverslips in six-well plates and treated with different concentrations of 25HC and 25HC3S for 4 h. The cells on coverslips were washed with PBS, fixed with 3.7% formaldehyde for 10 min at 4°C, and rinsed three times with PBS at room temperature. They were permeabilized with PBS containing 0.1% Triton X-100 for 3 min and washed with PBS before blocking by incubation with 5% normal goat serum in PBS overnight at 4°C. For interaction with primary antibodies, cells were incubated with 2.5% normal goat serum in PBS containing PPARγ antibody for 1 h in an incubator. Cells were washed twice with PBS containing 0.05% Tween 20 (3 × 10 min). The bound primary antibodies were visualized with Alexa fluor 488 goat anti-mouse IgG. The minor groove of double-stranded DNA as a nuclear marker was stained with DAPI. After washing, coverslips were mounted on slides and viewed with a Zeiss LSM 510 Meta confocal microscope.

**PolarScreen PPARγ competitor assay.** PPARγ binding affinities were measured using a PolarScreen PPARγ competitor assay kit based on the competition principle according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Twenty microliters of a test compound and 20 µl of 2× PPAR-LBD/Fluoromon PPAR Green complex were dispensed and mixed in the microwell plate. The assay was incubated at room temperature for 2 h, avoiding light with a cover, and the fluorescence polarization value of each well was determined by a fluorescence polarization plate reader. Competition data were generated in 384-well black plates, and polarization values were plotted against different concentrations of competitors 25HC3S, 25HC, Rosi, PGJ2, AA, DHA, cholesterol, and cholesterol 3-sulfate. The IC50 for each competitor is the concentrations at a half-maximal shift in polarization, which represents the relative affinity of each competitor with PPARγ ligand binding domain. The binding affinity of the inhibitor 9 was calculated from the IC50 using the Chen-Prusnook equation as: $K_i = IC_{50}/(1+[L]/K_d)$, where $[L]$ = 5 nM and $K_d$ = 9 nM (19). The curve was plotted using the following equation: $Y = mp_{100\%} \times (mp_{100\%} - mp_{0\%})/(1 + [L]/K_d - X)$ Hill slope, where $Y = mP, X = log [inhibitor], mp_{100\%} = 100\%$ inhibition, and $mp_{0\%} = 0\%$ inhibition. Curve fitting was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com).

**PPARγ response element binding assay.** PPARγ response element (PPRE) binding activities were measured using an enzyme-linked immunosorbent assay (ELISA; PPARγ transcription factor assay kit; Cayman Chemical, Ann Arbor, MI). The 96-well plate was preimmobilized with deoxyxylonicucleotides containing PPRE. THP-1-derived macrophages were treated with 25HC3S at indicated concentrations or Rosi for 4 h. The cells were then rinsed, and nuclear proteins were extracted according to the manufacturer’s instructions. Total nuclear extract protein, 10 µg from each sample, was added to the plate. The kit provided two negatives as zero controls, one positive as maximal binding activity of each competitor with PPARγ ligand binding domain. The binding affinity of the inhibitor $K_i$ was calculated from the IC50 using the Chen-Prusnook equation as: $K_i = IC_{50}/(1+[L]/K_d)$, where $[L]$ = 5 nM and $K_d$ = 9 nM (19). The curve was plotted using the following equation: $Y = mp_{100\%} \times (mp_{100\%} - mp_{0\%})/(1 + [L]/K_d - X)$ Hill slope, where $Y = mP, X = log [inhibitor], mp_{100\%} = 100\%$ inhibition, and $mp_{0\%} = 0\%$ inhibition. Curve fitting was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com).
90–95%, the cells were transfected with an expression plasmid as indicated using a lipid-based FuGENE HD transfection reagent according to the manufacturer’s instructions (Roche). A synthetic Renilla luciferase reporter, phrG-TK (Promega), was used as a luciferase internal standard. For PPRE reporter gene assay, 50 ng of pGL3-PPRE-acyl-CoA oxidase luciferase reporter, 50 ng of expression plasmid pcDNAI-PPARγ1, and 50 ng of phrG-TK vector (internal standard) were cotransfected per the manufacturer’s instructions. At 24 h after the transfection, different concentrations of 25HC3S, Rosi, and/or T0070907 were added and incubated for another 24 h. Luciferase activity was determined using the Dual-Glo Luciferase Assay System according to the manufacturer’s protocol. The amount of luciferase activity was measured using a TopCount NXT Microplate Scintillation and Luminescence Counter (Packard, Meriden, CT) and normalized to the amount of phG-TK luciferase activity. Transfections were carried out in triplicate for each sample, and each experiment was repeated three times.

**Determination of mRNA levels by real-time RT-PCR.** Total RNA was isolated from THP-1–derived macrophages following treatments for 6 h or otherwise as indicated, using an SV Total RNA Isolation Kit (Promega), which includes DNase treatment. Two microgram of total RNA was used for first-strand cDNA synthesis as recommended by the manufacturer (Invitrogen). Real-time RT-PCR was performed using SYBR Green as indicator on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The final reaction mixture contained 10 ng of cDNA, 100 nM of each primer, 10 μl of SYBR Green PCR Master Mix (Applied Biosystems) and RNase-free water to complete the reaction mixture volume to 20 μl. All reactions were performed in triplicate. PCR was carried out for 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescence was read during the reaction, allowing a continuous monitoring of the amount of PCR product. The ΔΔC_{T} method was used to calculate relative mRNA expression levels, and each targeting mRNA expression was normalized by GAPDH mRNA expression levels. The sequences of primers used were as recommended by http://pga.mgh.harvard.edu/primerband/.

**ELISA analysis of cytokine releases.** A total of 1 × 10⁶ macrophages was treated with LPS (1 μg/ml) and/or different concentrations of 25HC or 25HC3S for 24 h. Supernatants were harvested, and cytokine IL-1β concentrations were measured by ELISA according to the manufacturer’s instructions (SuperArray Bioscience). siRNA-mediated macrophage RNA interference. pSilencer2.1-U6 neo siRNA expression vector and three negative scrambled controls for RNAi were purchased from Ambion (Austin, TX). Three human PPARγ oligonucleotide sequences, GACTCAGCTCTACAATAAG (siRNA1), GCCGATTCTTCAGTATC (siRNA2), and GCCATTCTAGCATGC (siRNA3), were selected as specific siRNAs to target human PPARγ. Synthetic sense and antisense oligonucleotides were annealed and incubated at 90°C for 3 min and then at 37°C for 1 h. The double-stranded oligonucleotides were cloned into the BamHI-HindIII sites of the pSilencer2.1-U6 neo vector according to the manufacturer’s protocol. The control RNA interference (RNAi) sequence was randomly scrambled and was not found in the mouse, human, or rat genome databases. All of the constructs were confirmed by sequencing. THP-1 macrophages were transfected with a PPARγ siRNA or control RNAi using FugeneHD reagent according to the manufacturer’s instructions (Roche Applied Science). After incubation for 4 h, the medium was changed with normal medium, and compounds were added at appropriate concentrations as indicated. Cells were harvested after 48 h following the addition, PPARγ protein levels were determined using Western blot, and mRNA levels of inflammatory response factors were measured by real-time RT-PCR analysis.

**Statistics.** Data are reported as means ± SD. Western blot results were repeated at least three times. Statistics were performed using Student’s t-test. *P < 0.05 was considered statistically significant.

**RESULTS**

25HC3S inhibits NF-κB nuclear translocation and inflammatory responses. NF-κB plays an important role in inflammatory responses. To study the effect of 25HC3S on inflammatory responses, NF-κB translocation from cytosol to nuclei following addition of oxysterols was determined. In the absence of LPS, the addition of 25HC3S to THP-1–derived macrophages significantly decreased nuclear NF-κB levels. In contrast, the addition of 25HC increased its levels, indicating that 25HC is expected, in macrophages pretreated with 12-EP3, and TRAF1, similar to previous reports (31, 32). As expected, in macrophages pretreated with 12 μM 25HC3S, LPS failed to induce NF-κB target gene expression. Furthermore,
25HC3S significantly inhibited LPS-induced IL-1\beta release. In contrast, addition of 25HC significantly increased the release of IL-1\beta. Both of the effects were concentration dependent, as shown in Fig. 1C. The results provide evidence for the suppression of inflammatory responses by 25HC3S through inhibition of NF-κB nuclear translocation.

25HC3S suppresses NF-κB nuclear translocation by inducing cytoplasmic IkBα expression. Activation of NF-κB is initiated by the LPS-activated IkB kinase (IKK) signal followed by phosphorylation and degradation of IkB. With the degradation of IkB, NF-κB in the NF-κB/IkB complex is free to translocate to the nucleus, where it can induce the expression of specific genes. To assess the regulation of this inflammatory response by 25HC3S/25HC through IkB/NF-κB signaling pathway, the effects of these two oxysterols on IkBα expressions were examined. Addition of 25HC3S to THP-1 macrophages led to significant time- and concentration-dependent increases in the expression of IkBα at mRNA level.

Table 1. Relative mRNA levels (fold)

<table>
<thead>
<tr>
<th>NCBI Gene ID</th>
<th>Gene</th>
<th>DMSO</th>
<th>DMSO + LPS</th>
<th>25HC3S + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3552</td>
<td>IL-1α</td>
<td>1</td>
<td>20.1 ± 1.0**</td>
<td>16.9 ± 1.6†</td>
</tr>
<tr>
<td>3553</td>
<td>IL-1β</td>
<td>1</td>
<td>12.7 ± 0.8**</td>
<td>7.6 ± 0.6††</td>
</tr>
<tr>
<td>3569</td>
<td>IL-6</td>
<td>1</td>
<td>263.7 ± 45.3**</td>
<td>48.1 ± 27.4‡‡</td>
</tr>
<tr>
<td>5743</td>
<td>COX-2</td>
<td>1</td>
<td>25.6 ± 2.4**</td>
<td>12.3 ± 2.2‡†</td>
</tr>
<tr>
<td>5970</td>
<td>RelA/p65</td>
<td>1</td>
<td>4.5 ± 0.5**</td>
<td>2.5 ± 0.2‡†</td>
</tr>
<tr>
<td>7124</td>
<td>TNFα</td>
<td>1</td>
<td>43.6 ± 3.5**</td>
<td>14.8 ± 2.1‡†</td>
</tr>
<tr>
<td>7185</td>
<td>TRAF1</td>
<td>1</td>
<td>11.2 ± 1.3**</td>
<td>6.7 ± 1.0††</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD (n = 3). After incubation with 12 μM 25HC3S or DMSO for 24 h, THP-1 macrophages were treated with 100 ng/ml LPS for 3 h. mRNA expression levels were determined by real-time RT-PCR analysis. IL, interleukin; COX-2, cyclooxygenase-2; RelA/p65, v-rel reticuloendotheliosis viral oncogene homolog A; TNFα, tumor necrosis factor-α; TRAF1, TNF receptor-associated factor 1. **P < 0.01 vs. DMSO treatment; †P < 0.05, ††P < 0.01 vs. DMSO + LPS treatment.

Fig. 2. Effects of 25HC3S on IkBα expression. Effects of 25HC3S on IkBα mRNA expression were determined by qPCR analysis in time-dependent manner at 25 μM (A) and concentration-dependent manner following the treatment for 6 h (B). Protein expression was analyzed by Western blot analysis in time-dependent manner at 25 μM (C) and concentration-dependent manner following treatment for 24 h (D). Real-time PCR values are expressed as means ± SD (n = 3). Western blot data represent a typical 1 of 3 experiments. Relative levels were analyzed by t-test from 3 independent experiments. *Statistical significant difference vs. DMSO treatment (P < 0.05).
It should be noted that the increases of IkBα mRNA expressions reached maximum at 6 h posttreatment of 25HC3S. Western blot analysis also showed that 25HC3S increased and 25HC decreased IkBα protein levels in time- (Figs. 2C) and concentration- (Fig. 2D) dependent manners. However, addition of 25HC had no significant effect on IkBα mRNA levels (P > 0.05; data not shown), but the protein levels were significantly concentration-dependently decreased (P < 0.05; Fig. 2D), indicating that 25HC induces IkB protein degradation.

To further examine the increase in IkBα levels by 25HC3S via upregulation of its expression, p-IκBα protein levels were determined. As shown in Fig. 3A, addition of LPS to THP-1 macrophages significantly decreased cytoplasmic IkBα protein levels during the 15- to 60-min incubation; and the IkBα protein levels rebounded by 2 h and further increased during incubation, which was consistent with previous reports (47, 48). Thus, 30-min incubation with LPS was chosen for the study of effects of 25HC3S on the IkB/NF-κB signaling pathway. Pretreatment of 25HC3S significantly decreased LPS-induced cytoplasmic IkBα degradation in THP-1 macrophages, as shown in Fig. 3B. To study whether 25HC3S decreases IkBα degradation via IKK, synthetic IKK inhibitor X was used (13, 30). Surprisingly, p-IκBα protein levels were not changed in the presence of 25HC3S, whereas IKK inhibitor X successfully inhibited IkBα protein degradation (Fig. 3B). As expected, pretreatment with 25HC3S or IKK inhibitor X significantly reduced LPS-induced NF-κB nuclear translocation (Fig. 3C). It was noticed that the nuclear NF-κB protein levels were further decreased by the cotreatment of 25HC3S and IKK inhibitor X as shown in Fig. 3C, providing the evidence that the inactivation of NF-κB by 25HC3S and IKK inhibitor X is mediated through different pathways. These results support that 25HC3S repressed NF-κB translocation by increasing IkBα expression.

25HC3S increases nuclear PPARγ levels. To examine the effect of 25HC3S and its precursor 25HC on PPARγ translocation, total nuclear proteins were extracted, and PPARγ protein levels were determined by Western blot analysis. Addition of 25HC3S to the macrophages led to significant concentration- (Fig. 4A, top) and time- (data not shown) dependent increases in nuclear PPARγ protein levels. To confirm that the 52-kDa band was PPARγ protein, a synthetic PPARγ ligand, Rosi, was used as a positive control and a specific antagonist,
showed that the 25HC3S IC50, 1.7 μM, performed. Rosi was used as positive control. The results suggested that 25HC3S functions as a potent PPARγ agonist. To further confirm that 25HC3S binds with the same molecule as the synthetic antagonist, a competitive assay was performed. In the presence of the antagonist T0070907, 25HC3S failed to increase the nuclear PPARγ levels to the maximum. However, the levels of inhibition could be partially reversed following increasing the concentration of 25HC3S as shown in Fig. 4B. In contrast, Rosi increased nuclear PPARγ levels, and the increased levels could be significantly inhibited by the presence of 25HC, as shown in Fig. 4B, middle. These results suggested that 25HC3S/T0070907 and 25HC/Rosi are competitive. Real-time RT-PCR analysis showed that 25HC3S was able to increase PPARγ mRNA levels slightly, whereas 25HC did not (data not shown). Confocal microscopy analyses confirmed the increases in PPARγ protein levels in the nuclei and decreases in cytosol following addition of 25HC3S in the macrophages, as shown in Fig. 5. The results indicate that 25HC3S induces PPARγ nuclear translocation.

25HC3S directly binds to PPARγ. To confirm that 25HC3S directly binds to PPARγ, a PPARγ competitor assay was performed. Rosi was used as positive control. The results showed that the 25HC3S IC50 was 1.7 μM, and Ki, 1.1 μM, are similar to those of other natural ligands AA, DHA, and PGJ2 but of lower affinity than Rosi (synthetic), (Fig. 6, A and B, and Table 2). However, 25HC, cholesterol, and cholesterol 3-sulfate did not significantly bind to PPARγ, IC50s >100 μM (Table 2). These results suggest that more than two different binding sites may exist in the PPARγ binding pocket. One possible reason is that the synthetic Fluormone Green ligand binds with different residues from the natural ligands in the pocket in this assay; thus, the values may not represent the actual binding affinity. Because of the fixed Green ligand, the assay is unable to determine whether 25HC3S competes with 25HC or other compounds. Further study may be needed to establish a new binding affinity assay using natural ligand as the reporter, such as using [3H]25HC3S to reevaluate their affinities with the receptors.

25HC3S increases PPARγ-response transcriptional activities. To study the transcriptional activities of the nuclear extracts from 25HC3S-treated THP-1 macrophages, PPRE-immobilized ELISA and PPRE reporter gene assays were carried out as shown in Fig. 6C. ELISA showed that addition of 25HC3S significantly increased the PPRE binding activities of the nuclear extracts, which is concentration dependent (P < 0.01; Fig. 6C). PPRE reporter gene assays were performed in H441 cells because these cells are an easily transfectable and have low levels of endogenous PPARs. At 25 μM 25HC3S, the activity reached maximum. In the presence of the antagonist T0070907, 25HC3S failed to increase PPARγ reporter gene activity, with cooverexpression of ACOX-PPRE reporter and PPARγ plasmids (Fig. 6D). Interestingly, in the presence of T0070907, the lower concentrations of Rosi failed to stimulate the reporter gene activity (P < 0.01), but the higher concentrations still could increase the activities, as shown in Fig. 6E. The results further confirm that 25HC3S specifically binds PPARγ and
increases its activity. To investigate whether the IκB/NF-κB signaling pathway is PPARγ dependent, H441 cells were transfected with p-­NC-κB-dependent reporter gene-Luc expression plasmid alone or cotransfected with PPARγ expression plasmid. In the absence of PPARγ expression plasmid, TNFα induced the NF-κB luciferase activities 10-fold and 25HC3S failed to suppress its induction, as shown in Fig. 6F. In the cotransfected cells, with the presence of PPARγ expression TNFα still induced the reporter gene expression 10-fold, but 25HC3S reduced its induction by 50% (Fig. 6G). Furthermore, in the presence of PPARγ antagonist, the suppression was blunted (Fig. 6G). It was noticed that addition of T0070907 alone had no significant effect on the NF-κB luciferase activities (P > 0.05; data not shown). Addition of PPARγ agonists showed that synthetic GW-1929 substantially and natural AA significantly increased IκBα expression (Fig. 6H) but not DHA and PGEJ2 (data not shown). The results demonstrate that the suppression of TNFα-induced NF-κB activation by 25HC3S requires the presence of PPARγ protein.

In the presence of PPARγ antagonist T0070907, IκBα mRNA expression levels induced by 25HC3S were blunted, as shown in Fig. 7A, indicating that IκBα upregulation by 25HC3S is PPARγ dependent. To confirm that the suppression of LPS-induced proinflammatory cytokines TNFα and IL-1β expression, is PPARγ dependent, specific siRNAs were used to knock down PPARγ. Following transfection of the recombinant plasmids encoding specific siRNAs or control RNAi for 48 h, ~75% of the cells were viable and harvested. We observed a 90% suppression of PPARγ protein levels, which were not changed in the presence of 12 μM 25HC3S, as shown in Fig. 7B. Consistent with earlier finding (Fig. 2, A and B), under control condition, 25HC3S significantly increased IκBα mRNA levels 2.5-fold, and in the presence of TNFα, 25HC3S increased the levels 6-fold, which could be abolished by PPARγ-specific siRNA (Fig. 7C). The results indicate that 25HC3S upregulates IκBα expression in a different site of the promoter region from NF-κB and that they coordinately rather than competitively regulate IκBα expression. As shown in Fig. 7, D–F, LPS stimulated TNFα expression 40-fold, IL-1β expression 12-fold, and NF-κB expression 4-fold. As expected, LPS-induced expressions of TNFα, IL-1β, and NF-κB were significantly suppressed by 12 μM 25HC3S treatment (P < 0.05). However, in the siRNA-expressed cells, 25HC3S failed to suppress these expressions (Figs. 7, D–F). These results indicate that the PPARγ/IκB/NF-κB signaling pathway is involved in 25HC3S/25HC-regulated inflammatory responses in THP-1 macrophages.

**DISCUSSION**

We previously reported that 25HC and 25HC3S act as ligands, an agonist and antagonist, respectively, of LXR nuclear receptors (38, 52). The intracellular 25HC3S levels are extremely low, so we hypothesize that this oxysterol is
an intracellular sterol hormone (low levels and high efficiency) (50). THP-1 macrophages express SULT2B1b and are able to synthesize 25HC3S (38). It has been reported by our laboratory that the expression is dramatically upregulated by insulin and downregulated by 25HC3S in both hepatocytes (36) and macrophages (38). In vitro, only 5% of our added 25HC3S entered the cells. It is predicted that the effective intracellular concentration is much lower than what we see in these in vitro experiments. In the present study, we show that 25HC3S increases nuclear PPARγ and

Table 2. Comparison of PPARγ binding affinities with natural or synthetic ligands

<table>
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<tr>
<th></th>
<th>Rosi</th>
<th>25HC3S</th>
<th>PGJ2</th>
<th>AA</th>
<th>DHA</th>
<th>25HC</th>
<th>C</th>
<th>C-3S</th>
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<tr>
<td>IC₅₀ (nM)</td>
<td>81.5</td>
<td>1,722</td>
<td>879.7</td>
<td>913.6</td>
<td>245.6</td>
<td>&gt; 100 mM</td>
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</tr>
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<td>Kᵢ (nM)</td>
<td>52.4</td>
<td>1,107</td>
<td>565.5</td>
<td>587.3</td>
<td>157.9</td>
<td>&gt; 64.2 mM</td>
<td>&gt; 64.2 mM</td>
<td>&gt; 64.2 mM</td>
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The concentration of the test compound that results in a half-maximal shift in polarization value equals the IC₅₀ of the test compound. The curve was plotted using the following equation as described in MATERIALS AND METHODS. IC₅₀, half maximal inhibitory concentration; Kᵢ, equilibration dissociation constant of ligand; Rosi, rosiglitazone; 25HC3S, 25-hydroxycholesterol-3-sulfate; PGJ2, 15-deoxy-Δ12,14-PGJ2; AA, arachidonic acid; DHA, docosahexaenoic acid; 25HC, 25-hydroxycholesterol; C, cholesterol; C-3S, cholesterol 3-sulfate.
its transcriptional activities, increases cytosol IκBα expression, decreases NF-κB nuclear translocation, and subsequently inhibits TNFα- and LPS-induced expression of inflammatory genes in human THP-1-derived macrophages. In contrast, its precursor, 25HC, a known LXR ligand, basically has the opposite effects. Thus, the present results provide evidence that the ratio of cellular 25HC to 25HC3S may not only coordinately regulate lipid metabolism by LXR/SREBP-1c (38, 52) but also inflammatory responses by the PPARγ/IκB/NF-κB signaling pathway.

IκBα plays a central role in inflammatory responses; its intracellular levels regulate inflammatory responses via regulating NF-κB translocation, and its expression is highly regulated by nuclear receptors and their ligands (12, 25, 40). When IκBα is phosphorylated and degraded, NF-κB will be translocated to nuclei and upregulate IκBα expression. The IκBα promoter region contains PPRE and has been shown to be a PPARα/γ target gene (7). A recent report shows that losartan inhibits LPS-induced inflammatory signaling through a PPARγ-dependent mechanism in human THP-1 macrophages (2). PPARs are ligand-inducible transcription factors and regulate gene expression by binding with RXR as a heterodimeric partner to PPRE (43). This complex then binds to cognate sequences in promoter regions of target genes involved in lipid metabolism and inflammatory responses (37). Addition of 25HC3S increased, whereas its precursor 25HC decreased, the nuclear PPARγ protein levels and IκBα expression; furthermore, 25HC3S increased IκBα much higher in the presence of TNFα (Fig. 7B), indicating that the mechanism of upregulation by 25HC3S is different from that of NF-κB. On the basis of the present results, nuclear PPARγ protein levels, promoter binding ability, and knockdown of PPARγ suppressing IκB expression, we propose that precursor 25HC and product 25HC3S of oxysterol sulfation regulate inflammatory responses via PPARγ regulating IκBα expression. However, the mechanism of suppression of this signaling by 25HC is unknown. Based on the finding that 25HC does not change PPARγ and IκBα mRNA levels but decreases their protein levels, it is reasonable to hypothesize that 25HC directly acts on protein degradation. One of the possibilities is that the suppression is through ubiquitination, like its effect on HMG-CoA reductase (35), and subsequent upregulation of inflammatory responses.

The role of oxysterols in inflammation is controversial. The nuclear receptors LXR and PPARγ regulate inflammation in different ways and respond to distinct signaling pathways (4). PPARs and LXRs both exert positive and negative control over the expression of a range of metabolic and inflammatory genes. Although LXRs as well as PPARγ can repress several inflammatory genes in a similar manner, comparative cDNA microarray studies have identified overlapping but distinct subsets of genes that are repressed by
ligand binding (22, 33, 41, 55). Why these nuclear receptors use parallel molecular mechanisms to negatively regulate similar but distinct gene subsets in the same cell type remains an open and intriguing question (56). In the present study, we have shown that 25HC3S functions as an agonist of PPARγ and 25HC as an LXR agonist. Thus, both oxysterols can regulate inflammatory responses but via two different pathways. 25HC can suppress inflammatory responses via the LXR signaling pathway but stimulate the response via the PPARγ pathway. Unlike oxysterol 25HC, which interferes with the inflammation responses through the LXR pathway, 25HC3S suppresses responses via the PPARγ pathway. Several laboratories have found that oxysterols, inclusive of 25HC, can activate LXRs and subsequently repress a set of inflammatory genes after LPS and cytokine stimulation (41, 53). However, many other studies found that oxysterols, including 25HC, induce inflammation and oxidation in different kinds of cells. For example, 25HC substantially increased the IL-1β mRNA expression and secretion induced by LPS in monocyte-derived macrophages. 25HC is also a potent inducer of MCP-1, MIP-1β, and IL-8 secretion in vitro (53, 46). 25HC treatment results in a significant increase in NF-κB transcriptional activity, not only by affecting IkB degradation and the translocation of p65/NF-κB to the nucleus, but also by regulating p65/NF-κB transactivation (9). Oxysterols also induce inflammation and oxidation by inducing slight mitochondrial dysfunctions and increasing reactive oxygen species (ROS) (9). Our previous studies have shown that 25HC3S decreases LXRs activity and its target gene expression in THP-1 macrophages. In contrast, the LXR ligand 25HC increases nuclear LXR levels following a short incubation time, subsequently increasing LXR target gene expression, inclusive of ABCA1/G1 and SREBP-1c mRNAs. Furthermore, 25HC3S blocked the stimulation of target gene expressions induced by 25HC or synthetic LXR agonist. On the basis of these observations, we conclude that 25HC3S activates PPARγ and modulates inflammatory responses through the PPARγ but not the LXR signaling pathway in THP-1 macrophages.

Previous studies have shown that PPARγ suppresses target gene expression of NF-κB, nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), and signal transducers and activator of transcription (STATs) in response to a variety of inflammatory stimuli, including cytokines and TLR ligands (28). The mechanisms involved in the repressive effects of PPARγ have yet to be elucidated. In the present study, we found that 25HC3S decreases NF-κB protein levels and increases PPARγ in nuclei (Figs. 1B, 3C, and 4), suppresses the expression of TNFα-induced NF-κB-dependent reporter gene, which is PPARγ-dependent (Fig. 6, B and C), induces IκBα expression (Figs. 2 and 3B), and inhibits a number of NF-κB target gene expressions including IL-1β, TNFα, etc. (Table 1). TNF binds to TNF receptor while LPS binds to TLR4, and both signaling pathways stimulate inflammatory responses by activation of IκB kinase (IKK). 25HC3S suppresses inflammatory responses via downstream elements, at IκBα levels rather than blocking the TLR activation by LPS, because 25HC3S suppresses both stimulation (Fig. 7, C–F), indicating that the suppression is not mediated through IκB phosphorylation but by its expression. We propose that 25HC3S increases nuclear PPARγ protein levels, which binds to PPRE, induces IκBα expression, and inhibits NF-κB translocation. IκBα protein inhibits inflammatory responses by binding and inactivating NF-κB. Meanwhile, the activated PPARγ inhibits TNFα expression, which directly decreases IκBα ubiquitination and degradation. Thus, 25HC3S attenuates inflammatory responses by increasing IκBα expression and decreasing IκBα ubiquitination and degradation through the PPARγ/IκBα/NF-κB signaling pathway.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.X., S.S., Y.M., J.K.K., and D.R.-A. performed experiments; L.X. and S.R. analyzed data; D.M.H. and S.R. interpreted results of experiments; S.R. conception and design of research; S.R. approved final version of manuscript.

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