Oxysterols exert proinflammatory effects in placental trophoblasts via TLR4-dependent, cholesterol-sensitive activation of NF-κB

Irving L.M.H. Aye1,*; Brendan J. Waddell2; Peter J. Mark2; and Jeffrey A. Keelan1

1School of Women’s and Infants’ Health, Faculty of Medicine, Dentistry and Health Sciences, King Edward Memorial Hospital, 374 Bagot Rd, Subiaco, The University of Western Australia, Perth, WA 6008, Australia 2School of Anatomy and Human Biology, Faculty of Life and Physical Sciences, The University of Western Australia, Perth, Australia

*Correspondence address. Tel: +61-8-9340-1881; Fax: +61-8-9381-3031; E-mail: iaye@meddent.uwa.edu.au

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ABSTRACT: Oxidized cholesterol metabolites (oxysterols) promote inflammation in a variety of cell types and are thought to be involved in a number of disease pathologies. Oxysterol concentrations are increased in pregnancy, together with systemic oxidative stress and inflammation. We tested the hypothesis that oxysterols 25-hydroxycholesterol (25-OHC) and 7-ketocholesterol (7-ketoC) promote placental trophoblast inflammation, and determined the mechanisms involved. Treatment of primary trophoblasts in culture with 25-OHC and 7-ketoC increased the production of proinflammatory cytokines (interleukin-6, macrophage inflammatory protein-1β and tumour necrosis factor-α) in a concentration-dependent fashion. Inhibition of TLR4 activation using selective inhibitors of TLR4 complex formation (OxPAPC) or signalling transmission (CLI095) prevented lipopolysaccharide (LPS)- and oxysterol-induced inflammatory cytokine production. Pretreatment of trophoblasts with selective inhibitors of IκB kinase activity (parthenolide and TPCA-1) reduced oxysterol- and LPS-stimulated inflammatory responses, consistent with the involvement of the nuclear factor kappa B (NF-κB) pathway downstream of TLR4 signalling. Both oxysterols also increased the phosphorylation and nuclear localization of NF-κB subunit p65/RelA. Oxysterols are also known to activate liver X receptors (LXRs) which can inhibit inflammatory signalling, either directly or indirectly via membrane cholesterol reduction. Treatment with the LXR agonist, T0901317, exerted significant anti-inflammatory effects, reducing LPS- and oxysterol-driven cytokine production. Treatment with methyl-β-cyclodextrin to deplete membrane microdomain cholesterol and thereby disrupt TLR4 signalling, similarly abrogated their effects. Together, these findings indicate that although oxysterols likely activate both pro- and anti-inflammatory pathways in the placenta, the predominant effect is the promotion of placental inflammation via TLR4-dependent activation of NF-κB.

Key words: cholesterol / 25-hydroxycholesterol / 7-ketocholesterol / cytokines (IL-6, TNF-α, MIP-1β) / nuclear factor kappa B

Introduction

Activation of placental inflammatory pathways is a feature of normal pregnancy and is increased in various placental pathologies (Desoye and Hauguel-de Mouzon, 2007; Keelan and Mitchell, 2007; Denison et al., 2010). Elevated levels of inflammatory mediators such as interleukin-6 (IL-6), IL-8, tumour necrosis factor-α (TNF-α) and macrophage inflammatory protein-1β (MIP-1β) are also observed in gestational tissues associated with infection (Lee et al., 2001; Abrams et al., 2003; Desoye and Hauguel-de Mouzon, 2007; Keelan and Mitchell, 2007; Denison et al., 2010). The innate immune system is activated by a variety of trigger molecules liberated during microbial division and/or lysis, eliciting a cellular inflammatory response typically characterized by increased cytokine/chemokine expression and production. In conditions of oxidative stress, which can accompany common pregnancy complications such as pre-eclampsia and gestational diabetes, oxidized lipids, glycolipids and carbohydrates may also activate a similar inflammatory response, possibly through autologous activation of the cell surface recognition system which recognizes and responds to pathogenic stimuli. Key components of this system are the Toll-like receptors (TLRs), of which 10 functional members have been identified in humans (TLR1 to TLR10); all 10 members have been reported to be expressed in the placenta (Nishimura and Naito, 2005; Koga and Mor, 2008). TLR4, which is activated...
by lipopolysaccharide (LPS)/endotoxin from the cell walls of Gram negative bacteria, is arguably one of the most important and most thoroughly studied (Lappas and Rice, 2007; Koga and Mor, 2010).

LPS activation of TLR4 requires various accessory molecules including LPS-binding protein (LBP), CD14 and MD-2, all of which associate with TLR4 at the extracellular surface. The aggregation of these accessory molecules is facilitated by the recruitment of the receptor complex into membrane microdomains enriched in cholesterol (Tian-taflou et al., 2002). Aggregation of these proteins at the membrane surface leads to receptor oligomerization and recruitment of adaptor proteins to the intracellular Toll-IL-1 receptor (TIR) domain (Akira et al., 2003). Recruitment of adapter proteins MyD88, TIRAP and TRAM to the TIR domain triggers a cascade of signalling events, including activation of the I-kB kinase (IKK) complex and phosphorylation of mitogen-associated protein kinases (MAPKs). This eventuates in the activation of the nuclear factor kappa B (NF-κB) signalling pathway via phosphorylation and nuclear localization of the p65/RelA protein which drives pro-inflammatory gene expression. In human gestational tissues, the NF-κB pathway plays an important role not only in triggering innate immunity in response to pathogens, but also in the normal parturition process (Keelan, 2011; Lappas and Rice, 2007). Although investigated to a lesser degree, recent evidence suggests that MAPK pathways including p38 MAPK and c-Jun N-terminal kinases (JNKs) also initiate inflammatory reactions in placental tissues (Lappas et al., 2007).

In addition to LPS, accumulating evidence indicates that endogenous or dietary factors can act as TLR2 and TLR4 agonists (Beg, 2002; Erridge, 2010). Fatty acids, which circulate in increased levels in obesity or diabetes, have been shown to activate TLR2/4 signalling in several tissues (Shi et al., 2006; Milanski et al., 2009), although oxidized fatty acids can also exert anti-inflammatory actions via peroxisome proliferator-activated receptors (Tontonoz and Spiegelman, 2008). Oxidized cholesterols (oxysterols) which can be obtained either from the diet or through oxidative modification in vivo

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**Figure 1** Oxysterols promote proinflammatory cytokine production in trophoblast cells. Primary term trophoblasts were treated with varying concentrations of 25-OHC or 7-ketoC or vehicle. Cytokine levels were measured by ELISA from culture media collected after 12 h (TNF-α) and 24 h (IL-6 and MIP-1β) following treatment. Data represents fold change from vehicle control (mean ± SD, n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle control. 25-OHC, 25-hydroxycholesterol; 7-ketoC, 7-ketocholesterol; IL-6, interleukin-6; MIP, macrophage inflammatory protein-1β; TNF-α, tumour necrosis factor-α.
Xenosterols and placental inflammation

Types (Dugas et al., 2002; Endo et al., 2010, 2011) and higher concentrations are reported in women with pre-eclampsia (Uzun et al., 2005; Qiu et al., 2006; Kim et al., 2007) and in the cord blood of neonates with intrauterine growth restriction (Leduc et al., 2011). These studies are all consistent with the expectation that in conditions associated with oxidative stress and/or inflammation in pregnancy, the placenta is exposed to elevated xenosterols levels.

To date, the link between increased levels of xenosterols or oxLDLs in pregnancy and placental inflammation has not been investigated. We have previously shown that xenosterols 25-hydroxycholesterol (25-OHC) and 7-ketocholesterol (7-ketoC) at physiological concentrations disrupt trophoblast differentiation and syncytialization and, at higher concentrations, promote apoptosis (Aye et al., 2010, 2011). In this study, we tested the hypothesis that xenosterols might also promote inflammation in placental trophoblasts via TLR2/4 activation, thereby providing a mechanistic link between oxidative stress in pregnancy and placental inflammation.

Materials and Methods

The following reagents were purchased from commercial sources as indicated: Dulbecco’s phosphate buffered saline (D-PBS), dispase-II, insulin-trasferin-sodium selenite cocktail, recombinant human epidermal growth factor (EGF), antibiotic-antimycotic solution, 0.05% trypsin with EDTA, goat anti-rabbit Cy3-conjugated antibodies and Pure Link RNA mini kit, from Invitrogen (Carlsbad, CA, USA); Hoechst 33258, methyl-β-cyclodextrin (MCD), horse-radish peroxidase conjugated antimouse and anti-rabbit antibodies were from Sigma-Aldrich (St. Louis, MO, USA); human IL-6, TNF-α, MIP-1β ELISA development kits from Peprotech Inc. (NJ, USA); phospho-p65 NF-kB (Ser536) antibody was from Cell Signaling Technology Inc. (MA, USA); Percoll was from GE Healthcare (Uppsala, Sweden); Medium 199 with Earle’s salts and L-glutamine from Mediatech Inc. (Manassas, VA, USA); DNAsel and complete protease inhibitors from Roche Diagnostics (Mannheim, Germany); fetal bovine serum (FBS) from Bovogen (VIC, Australia); T0901317 (LXR agonist) from Cayman Chemicals (Ann Arbor, MI, USA), 25-hydroxycholesterol (25-OHC) and 7-ketoC from Steraloids (Newport, RI, USA); Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) and CLI095 [Ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate, also known as TAK-242] were from InvivoGen (San Diego, CA, USA); Parthenolide and TPCA-1 from Calbiochem/Merck (Darmstadt, Germany). All other chemicals were of cell culturing grade from Calbiochem/Merck (Darmstadt, Germany).

Isolation and purification of primary trophoblast cells from term placentas for culture

Placentas were obtained with informed consent from women after delivery by Caesarean section at term. Primary trophoblast cells were isolated from term placenta by dispa ge as previously described (Aye et al., 2010, 2011). Briefly, villosus tissue was dissected free of membranes and blood vessels removed, washed in D-PBS and digested in dispase II (0.25% w/v) for 1 h at 37°C, with DNAsel (800 µg) added 15 min prior to the end of digestion. Tissue digests were then filtered through 70 µm cell filters and erythrocytes removed by incubating cell pellets in red cell lysis buffer (50 mM NH₄Cl, 10 mM NaHCO₃ and 0.1 mM EDTA). Digests were then incubated in 0.05% trypsin-EDTA for 1 min and trophoblast cells purified by centrifugation at 1200g for 20 min on a discontinuous Percoll gradient. Cells which migrated between the 20...
and 40% Percoll bands were collected and layered on a new Percoll gradient and centrifuged as before. Collected cells were cultured at a density of $1 \times 10^6$ cells/ml in M199 media supplemented with EGF (10 ng/ml), insulin (5 ng/ml), transferrin (10 ng/ml), sodium selenite (0.2 nM), 10% FBS and antibiotic/antimycotic solution in a 5% CO$_2$ humidified atmosphere at 37°C. Primary trophoblasts were washed twice in D-PBS following an overnight incubation and media replaced every 48 h. Cell purity was established by immunofluorescence staining with anti-cytokeratin-7 antibodies detected with Cy3-labeled secondary antibody and nuclei stained with Hoechst 33258 (5 µg/ml). Cytokeratin-7 positive cells in 5 fields at 200× magnification were counted using an Eclipse Ti inverted fluorescent microscope (Nikon, Tokyo, Japan) to determine trophoblast cell purity (≥95%).

**Trophoblast cell culture treatments**

Approximately 72 h following isolation, trophoblast cells were treated with the following compounds to modulate cellular cholesterol levels: T0901317 (400 nM) for 16 h, or MCD (10 mM) in serum-free media for 30 min prior to treatment with 25-OHC or 7-ketoC (6.7 µg/ml). Cytokine levels were measured by ELISA from culture media collected after 12 (TNF-α) and 24 h (IL-6 and MIP-1β) following treatment. Data represents percentage of oxysterol-stimulated response (mean ± SD, n = 4). *p < 0.05, **p < 0.01, ***p < 0.001. 25-OHC, 25-hydroxycholesterol; 7-ketoC, 7-ketocholesterol; IL-6, interleukin-6; MIP, macrophage inflammatory protein-1β; TNF-α, tumour necrosis factor-α.

**Figure 3** Inhibition of TLR4 activation attenuates oxysterol-stimulated cytokine production. Primary trophoblast cells were pretreated with TLR4 inhibitors OxPAPC and CLI095 for 30 min prior to treatment with 25-OHC or 7-ketoC (6.7 µg/ml). Cytokine levels were measured by ELISA from culture media collected after 12 (TNF-α) and 24 h (IL-6 and MIP-1β) following treatment. Data represents percentage of oxysterol-stimulated response (mean ± SD, n = 4). *p < 0.05, **p < 0.01, ***p < 0.001. 25-OHC, 25-hydroxycholesterol; 7-ketoC, 7-ketocholesterol; IL-6, interleukin-6; MIP, macrophage inflammatory protein-1β; TNF-α, tumour necrosis factor-α.

**Cytokine ELISAs**

Culture media was collected after 12 h (TNF-α) or 24 h (IL-6 and MIP-1β) following treatment with LPS or oxysterols for cytokine ELISAs. Secretion of IL-6, MIP-1β and TNF-α into incubation media was measured by ELISA according to the manufacturer’s instructions with modifications and the
assay calibrated to bovine serum albumin (BSA). Approximately 30 min for 15 min. Protein concentration was quantified by the bicinchoninic acid assay according to the manufacturer’s instructions. Oxysterol preparations at concentrations ranging from 0.8 to 30 μg/ml (Dugas et al., 2010; Palozza et al., 2010). Due to this variability amongst different placental tissues at concentrations ranging from 0.8 to 30 μg/ml (Dugas et al., 2010; Palozza et al., 2010). To ensure that the proinflammatory effects of oxysterols were not the result of endotoxin contamination, the LAL assay was performed according to the manufacturer’s instructions. Oxysterol preparations at 20 μg/ml and LPS at 2 and 0.02 μg/ml were tested for LAL activity.

Detection of endotoxin contamination using the limulus amoebocyte lysates assay

The limulus amoebocyte lysates (LAL) assay is commonly used to detect and quantify bacterial endotoxins in the quality assurance of medical devices, injectable drugs and biological preparations (Ding and Ho, 2010). To ensure that the proinflammatory effects of oxysterols were not the result of endotoxin contamination, the LAL assay was performed according to the manufacturer’s instructions. Oxysterol preparations at 20 μg/ml and LPS at 2 and 0.02 μg/ml were tested for LAL activity.

Immunoblotting analyses

In order to determine changes in NF-κB p65/RelA (Ser536) protein phosphorylation, trophoblasts were treated as described earlier, and cells lysed in buffer containing (50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 0.5% Na.Deoxycholate, 1% Triton X100 and complete protease inhibitor cocktail), and sonicated on a Polytron homogenizer (Kinematica, Lucerne, Switzerland). Insoluble material was removed by centrifugation at 900g for 15 min. Protein concentration was quantified by the bicinchoninic acid assay calibrated to bovine serum albumin (BSA). Approximately 30 μg of proteins were then separated under reducing conditions on a 4–12% BisTris precast polyacrylamide gradient gel (RunBlue, Expedeon Inc., San Diego, CA, USA) and transferred to a nitrocellulose membrane in an XCELL transfer module (Invitrogen). Nitrocellulose membranes were blocked in 5% non-fat milk powder for 1 h, and incubated overnight at 4°C with anti-phospho-p65/RelA (Ser536). Membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:2000) secondary antibody, and visualized by enhanced chemiluminescence using SuperSignal West-Femto Substrate (Pierce Chemical, Rockford, IL, USA). Resultant images were quantified on an ImageQuant 350 digital imaging and quantification system (GE Healthcare, Uppsala, Sweden).

Figure 4 Measurement of endotoxin levels in oxysterol and LPS preparations using the LAL assay. The extent of endotoxin contamination shown as enzymatic activity (in enzyme units/milliliter). LPS, lipopolysaccharide; 25-OHC, 25-hydroxycholesterol; 7-ketoC, 7-ketocholesterol.

Absorbance detected at 450 nm (corrected for background at 605 nm) on a VersaMax plate reader (Molecular Devices, CA, USA). Curve fitting and data extrapolation were performed using SoftMax Pro40 software (Molecular Devices, CA, USA). Due to the variability in basal cytokine secretion between placentas, detected cytokine levels were normalized against protein concentrations and presented as a percentage of stimulated response.

Statistics

All studies were performed three to four times in cultures from different placentas; descriptive statistics were performed for each data set, and the data combined for collective analysis after normalization to control. Graphs were plotted using Sigma Plot and statistics performed using Sigma Stat software (Systat Software Inc., Richmond, CA, USA). One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was carried out to determine the statistical significance of various treatments against vehicle treated controls or oxysterol treatments. *P* < 0.05 was considered to be significant.

Results

25-OHC and 7-ketoC promote proinflammatory cytokine production from placental trophoblasts

The oxysterols 25-OHC and 7-ketoC have previously been shown to promote proinflammatory cytokine expression in non-gestational tissues at concentrations ranging from 0.8 to 30 μg/ml (Dugas et al., 2010; Palozza et al., 2010). Due to this variability amongst different cell types, we sought to test a variety of concentrations to assess their effects on inflammation in trophoblasts.

Trophoblast cells were treated with increasing concentrations of 25-OHC or 7-ketoC and incubation media collected after 12 h for analysis of TNF-α concentration, or after 24 h for measurement of IL-6 and MIP-1β levels (time points were chosen based on pilot studies). Treatment with either 25-OHC or 7-ketoC led to increased production of all three cytokines to varying degrees (Fig. 1). Both oxysterols elicited increased production of proinflammatory cytokines in a dose-dependent manner, with statistically significant changes occurring at concentrations of 6.7 μg/ml or above.

TLR4-inhibition diminishes LPS- and oxysterol-stimulated proinflammatory cytokine production

In order to determine the role of TLR4 in oxysterol-mediated inflammatory response, we employed inhibitors to antagonize the activation of the TLR4 complex. Several different antagonists targeting different components of the TLR4 complex were employed. The first antagonist was an oxidized phospholipid, OxPAPC, which preferentially targets TLR4 (and to a lesser extent TLR2) by preventing its interaction with TLR4.
association with LBP, CD14 and MD-2 (Bochkov et al., 2002; Erridge et al., 2008; Oskolkova et al., 2010). In parallel experiments, we employed CLI095 which prevents the intracytoplasmic TIR domains of TLR4 complex from associating with intracellular adaptor molecules TIRAP and TRAM (Ii et al., 2006; Kawamoto et al., 2008; Matsunaga et al., 2011).

Before determining the effects of these antagonists on oxysterol response, we first characterized their effects on cytokine production stimulated by the archetypal TLR4 ligand, LPS. Pretreatment with OxPAPC (3 μM) significantly decreased MIP-1β production following LPS treatment, whereas the inhibitory effects of CLI095 were statistically significant for all cytokines examined at both concentrations tested (Fig. 2). The inhibitory effects of CLI095 were notably greater than those of OxPAPC: while OxPAPC reduced cytokine secretion by 15–35%, CLI095 decreased cytokine secretion by at least 50% even at the lowest concentration tested.

After establishing that OxPAPC and CLI095 were able to antagonize TLR4 activation by LPS, we determined if these inhibitors prevented the proinflammatory activities of oxysterols. As shown in Fig. 3, pretreatment of trophoblast cells with either OxPAPC or CLI095 significantly decreased both 25-OHC and 7-ketoC mediated proinflammatory cytokine secretion. At the highest inhibitor concentrations, cytokine secretion was reduced by ~50–80% by OxPAPC, and between 55 and 90% following CLI095 treatment.
25-OHC and 7-ketoC do not trigger LAL enzyme activity

To rule out the possibility that the proinflammatory effects of oxysterols were a result of endotoxin contamination, an LAL assay was performed. As shown in Fig. 4, neither 25-OHC nor 7-ketoC displayed any detectable LAL activity, while the LPS preparations exhibited high enzymatic activity as expected.

25-OHC and 7-ketoC promote nuclear translocation of NF-κB p65/RelA

The NF-κB pathway represents one of the major inflammatory pathways involving in mediating the effects of TLR4 activation on proinflammatory cytokine expression (Akira et al., 2003; Takeda and Akira, 2004). In this study, we investigated the involvement of the NF-κB pathway in the LPS- and oxysterol-mediated inflammatory response. One of the key events in NF-κB activation involves phosphorylation of the p65/RelA subunit followed by sequestration of this complex into the nucleus (Sakurai et al., 1999). As shown in Fig. 5, treatment with LPS, 25-OHC or 7-ketoC resulted in sequestration of phospho-p65 into the nucleus 12 h after stimulation. Stimulation also appeared to increase the amount and intensity of staining, although it should be noted that only a minor proportion of the total cellular population was stained by the antibody. The reasons for this are unknown; however, lack of sensitivity of the immunohistochemical assay, high levels of endogenous phosphatase activity in some cells and/or inherent heterogeneity in the cell culture are all possibilities.

Quantification of phospho-p65 stained nuclei revealed a 4-fold increase in phospho-p65 nuclear translocation following LPS treatment compared with control. Treatment with 25-OHC also observed a similar magnitude of change compared with control while 7-ketoC treatment resulted in ~3-fold increase in phospho-p65 nuclear translocation.

NF-κB activity is required for oxysterol-mediated inflammatory response

To clarify the role of NF-κB in oxysterol-induced inflammation, phosphorylation of the NF-κB subunit p65/RelA was investigated in the presence of IKK inhibitors parthenolide and TPCA-1. Parthenolide is a sesquiterpene lactone which specifically targets the IKK complex and prevents its association without directly inhibiting IKKα or IKKβ (Hehner et al., 1999), while TPCA-1 is a selective small molecule inhibitor of IKKβ (Podolin et al., 2005). At the concentrations used in this study, both compounds have previously been shown to effectively reduce LPS-stimulated cytokine production in cells derived from human placental tissues without affecting cell viability (De Silva et al., 2010).

Phosphorylation of the NF-κB subunit p65/RelA (at Ser536) was significantly increased following treatment with 25-OHC, 7-ketoC and LPS (Fig. 6). Pretreatment of trophoblasts with parthenolide decreased p65 (Ser536) phosphorylation following oxysterol or LPS stimulation, consistent with its role in IKK inhibition. Unexpectedly, TPCA-1 treatment did not significantly reduce p65 phosphorylation following 25-OHC or 7-ketoC treatment, although TPCA-1 did inhibit LPS-induced p65 phosphorylation. IKK inhibition by parthenolide or TPCA-1 significantly reduced the production of all cytokines following oxysterol or LPS stimulation (Fig. 7). Both these inhibitors reduced LPS and oxysterol-stimulated cytokine secretion by more than 50%.

LXR activation and cellular membrane cholesterol depletion attenuates LPS- and oxysterol-stimulated proinflammatory cytokine production

We have previously shown that oxysterols are functional liver X receptor (LXR) ligands in the placenta, increasing active efflux of...
Inhibition of NF-κB activation attenuates oxysterol-mediated cytokine secretion. Primary trophoblast cells were pretreated with IKK inhibitors parthenolide or TPCA-1 (10 μM) for 30 min prior to treatment with 25-OHC, 7-ketoC (6.7 μg/ml) or LPS (1 μg/ml). Cytokine levels were measured by ELISA from culture media collected after 12 (TNF-α) and 24 h (IL-6 and MIP-1β) following treatment. Data represents percentage of treatment response (mean ± SD, n = 3). *P < 0.05, **P < 0.01, ***P < 0.001. 25-OHC, 25-hydroxycholesterol; 7-ketoC, 7-ketocholesterol; IL-6, interleukin-6; MIP, macrophage inflammatory protein-1β; TNF-α, tumour necrosis factor-α; Parth, parthenolide; LPS, lipopolysaccharide.
cholesterol to apoA-I and HDL (Aye et al., 2010). LXR agonism not only regulates levels of cellular cholesterol, which can modulate TLR4 activity by influencing microdomain environment (Triantafilou et al., 2004), but may also exert anti-inflammatory effects via a range of other unrelated mechanisms. Therefore, to assess the anti-inflammatory effects of LXR activation we first treated our cells with the synthetic LXR agonist T0901317 prior to stimulation with 25-OHC, 7-ketoC (6.7 μg/ml) or 1 μg/ml LPS. Cytokine levels were measured by ELISA from culture media collected after 12 (TNF-α) and 24 h (IL-6 and MIP-1β) following treatment. Data represents percentage of stimulated response (mean ± SD, n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 versus LPS control. LPS, lipopolysaccharide; 25-OHC, 25-hydroxycholesterol; 7-ketoC, 7-ketocholesterol; T0, T0901317; MCD, methyl-β-cyclodextrin; IL-6, interleukin-6; MIP, macrophage inflammatory protein-1β; TNF-α, tumour necrosis factor-α.

To verify if the proinflammatory effects of oxysterols were similarly modulated by LXR agonism or membrane cholesterol manipulation, trophoblast cells were pretreated as described above prior to oxysterol stimulation. Similar to LPS stimulation, the proinflammatory response to 7-ketoC was significantly attenuated by T0901317 and MCD (Fig. 7). Although there was a propensity for a reduced inflammatory response to 25-OHC following T0901317 pretreatment, not all of the effects were statistically significant. T0901317 decreased 25-OHC stimulated MIP-1β and TNF-α secretion by ~65%; there was also a non-significant 40% decrease in IL-6 secretion (P = 0.101). Pretreatment with MCD also attenuated the inflammatory response to 7-ketoC, although despite the consistent negative trends, only MIP-1β levels were significantly reduced.
Discussion

Oxidized lipoproteins and oxysterols are commonly detected in atherosclerotic plaques (Brown and Jessup, 1999; Vaya et al., 2005). The accumulation of oxidized sterols may contribute to disease pathogenesis by promoting cell apoptosis and inflammation (Bjorkhem and Diczfalusy, 2002; Vejux et al., 2008; Vejux and Lizard, 2009). Previous studies have indicated that exposure of oxysterols in the placenta impairs trophoblast invasion (Pavan et al., 2004; Fournier et al., 2008), syncytialization (Aye et al., 2011), and at higher concentrations, apoptosis (Aye et al., 2010). In this study, evidence is presented that oxysterols 25-OHC and 7-ketoC activate the innate immune system in placental trophoblast cells leading to increased cytokine production. This proinflammatory phenomenon represents the net result of the activation of two opposing pathways: the primary effect is mediated via activation of the TLR4 signalling complex and NF-κB signalling; the other involves LXR activation and reduction of membrane cholesterol content.

Our findings have potentially important implications for the understanding and management of a number of common and serious pregnancy disorders in which oxidative stress and/or inflammation play an important role in placental pathophysiology. Although information is limited, evidence to date suggests that circulating concentrations of oxysterols and oxLDLs are increased in pregnant women with diabetes, obesity, pre-eclampsia and IUGR (Zwirska-Korczala et al., 2007; Endo et al., 2005; Qiu et al., 2006; Kim et al., 2007; Endo et al., 2008; Leduc et al., 2011; Makedou et al., 2011), all conditions in which oxidative stress has been described. With respect to the actual levels documented, several clinical studies have reported normal plasma levels of oxysterols in the mid to high nanogram/milliliter range (Dzeletovic et al., 1995; Iuliano et al., 2003; Endo et al., 2008). However, levels of oxysterols generated de novo in sites of oxidative stress, such as atherosclerotic lesions, are known to increase by ~90–200-fold depending on the severity of the lesion (Garcia-Cruset et al., 2001). Hence, we may predict oxysterol concentrations in focal lesions—such as acute atherosclerosis often observed in preeclamptic placentas—to be much higher than systemic oxysterol concentrations (Harsem et al., 2007; Staff et al., 2010), and the low microgram/milliliter levels used in this study are readily achievable under pathological conditions. Since placental inflammation is often observed in pregnancies complicated by oxidative stress, we hypothesize that oxysterols might be involved in mediating this phenomenon and hence could be of clinical interest as diagnostic biomarkers or therapeutic targets. Exploring this hypothesis is a topic for future studies.

There is mounting evidence that treatment with 25-OHC and 7-ketoC up-regulates the production of a number of inflammatory mediators including IL-1α, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1) and MIP-1B in a wide variety of cell types (Prunet et al., 2006; Joffre et al., 2007; Morello et al., 2009; Dugas et al., 2010; Mascia et al., 2010). However, the mechanisms underlying the proinflammatory response to oxysterols remain unclear. We aimed to identify whether oxysterols exert proinflammatory effects on trophoblasts, and clarify the mechanism of action of oxysterol-driven inflammatory activation by investigating the interactions between oxysterols, TLR4 and cholesterol as well as the involvement of the NF-κB pathway.

Treatment with 25-OHC and 7-ketoC led to a dose-dependent increase in the secretion of proinflammatory cytokines IL-6, MIP-1B and TNF-α from cultured placental trophoblast cells. Using specific antagonists, we determined that the TLR4 signalling complex is involved in these proinflammatory effects. Treatment with OxPAPC or CLI095 decreased cytokine production in cells treated with the classic TLR4 ligand LPS, and also decreased cytokine production in response to both 25-OHC and 7-ketoC to a similar extent. OxPAPC has been shown to inhibit both TLR2 as well as TLR4 signalling (Erridge et al., 2008), while CLI095 selectively inhibits TLR4 and has no effect on TLRs 1, 2, 3, 5, 7 or 9 (Li et al., 2006; Kawamoto et al., 2008; Matsunaga et al., 2011). As there is always a concern that pharmacological agents can exert multiple actions, the use of pharmacological inhibitors to address mechanistic studies should be treated with caution. Nevertheless, despite this caveat, in this study we used several pharmacological antagonists targeting different components of TLR4 signalling which resulted in similar results. Thus, taken together, these findings provide strong evidence that TLR4 plays a crucial role in the placental inflammatory response to oxysterols, although an involvement of TLR2 cannot be ruled out at this stage.

It has been proposed that several endogenous compounds may be incorrectly identified as TLR4 ligands due to contamination of these preparations with LPS (Erridge, 2010). Because of this concern, a LAL assay was performed on 25-OHC and 7-ketoC to determine if the oxysterols were contaminated with endotoxins. Neither of these compounds had detectable LAL activity at the highest dose used in this study, so it is highly unlikely that the inflammatory effects of these oxysterols can be attributable to endotoxin contamination.

Following activation of the TLR signalling complex at the plasma membrane interface, intracytoplasmic adaptor molecules are recruited to the TIR domain leading to the activation of a cascade of intracellular proteins eventuating in up-regulation of proinflammatory gene expression by transcription factors. NF-κB is the prototypical transcription factor, which plays a central role in innate immune response (Hayden et al., 2006). One of the key events leading to the activation of NF-κB is the phosphorylation of IκBα chaperone proteins by IKKβ. In addition to IκBα, IKKβ also phosphorylates the p65 NF-κB subunit on serine 536 (Ser536) in a time-dependent manner similar to IκBα phosphorylation (Sakurai et al., 1999). IKKβ mediated phosphorylation of serine 536 on p65 is necessary to increase the transcriptional activity of p65 (Yang et al., 2003). We observed increased translocation of the Ser536 phosphorylated p65 NF-κB subunit from the cytoplasm to the nucleus following treatment with LPS, 25-OHC and 7-ketoC. This is in agreement with a previous study showing that the same oxysterols promote NF-κB binding activity (Palozza et al., 2010), and is consistent with a TLR4-mediated inflammatory response. Furthermore, an increase in phospho-p65 staining was also observed following oxysterol and LPS treatments.

Using two IKK inhibitors (parthenolide and TPCA-1), proinflammatory cytokine production was attenuated in trophoblasts following 25-OHC, 7-ketoC or LPS treatments. Surprisingly, while inhibition of IKK complex formation with parthenolide decreased both 25-OHC and 7-ketoC-induced p65 (Ser536) phosphorylation, IKKβ inhibition using TPCA-1 did not reduce p65 (Ser536) phosphorylation, despite the decrease in cytokine production. Both IKK inhibitors, on the other hand, effectively reduced p65 phosphorylation at Ser536.
in LPS-treated cells. Therefore, it is possible that oxysterols may phosphorylate other NF-κB p65/RelA residues in addition to Ser536 to produce their inflammatory effects. In addition to NF-κB, activation of JNK and p38 MAPK pathways was also investigated. Although there was evidence of JNK and p38 MAPK phosphorylation, the extent of change was modest and inconsistent (data not shown). Overall, these findings indicate that NF-κB-dependent up-regulation of cytokine gene transcription plays a major role in the oxysterol-mediated inflammatory response.

While our data suggest that the effects of oxysterols observed are mediated by direct activation of TLR4, oxysterols are known to exert a wide range of cellular effects that are independent of TLR activation, some of which could indirectly result in changes in inflammatory responses and signalling. In particular, oxysterols are ligands for LXR-α and -β, transcription factors which regulate an array of genes involved in cholesterol disposition (Zelcer and Tontonoz, 2006). LXR agonists have been shown to reduce NF-κB activity, most likely through trans-repression of coactivators (Ghisletti et al., 2007; Zhang-Gandhi and Drew, 2007). Activation of LXR causes targeting at the promoters of TLR target genes and prevents the removal of nuclear receptor co-repressor (NCoR) complexes. TLR-responsive genes require NCoRs to be actively removed from the promoters of inflammatory genes to relieve basal repression. Therefore, LXR activation may prevent an inflammatory response by indirectly supporting the repression of TLR target genes activation which may modulate inflammatory signalling via several routes. We investigated the effects of LXR agonism on the oxysterol-driven inflammatory response using T0901317, a synthetic LXR agonist. Pretreating trophoblast cells with the LXR agonist led to a significant reduction in cytokine secretion in response to either LPS or oxysterols 25-OHC and 7-ketoC; confirming for the first time that in placenta LXR agonism is anti-inflammatory. Hence, the proinflammatory actions of oxysterols occur in spite of LXR agonism, consistent with findings previously reported in endothelial cells (Morello et al., 2009).

LXR agonism by oxysterols may also indirectly influence inflammatory signalling via their effects on cellular cholesterol levels. Oxysterols influence cholesterol homeostasis by inhibiting cholesterol synthesis, modulating intracellular trafficking of cholesterol or promoting cholesterol efflux (Otaegui-Arrazola et al., 2010). In the placenta, oxysterols up-regulate the expression of cholesterol efflux proteins ABCA1 and ABCG1 on the plasma membrane (Stefuli et al., 2009; Aye et al., 2010). As such, increased expression of these proteins through LXR activation may result in the disruption of lipid microdomains and its constituents by promoting membrane cholesterol efflux, since cholesterol forms a major component of these microdomains (Pike, 2006; Zhu et al., 2010). TLR4 and its accessory proteins CD14 and MD-2 are recruited into lipid microdomains along with MyD88; hence modulation of membrane cholesterol levels can diminish the inflammatory response by reducing TLR4 trafficking into lipid microdomains (Trian- tafkou et al., 2002, 2004; Wong et al., 2009). To clarify the role of membrane cholesterol in the inflammatory response of the placenta, we employed MCDs to disrupt lipid microdomains (Pitha et al., 1998). MCD is a water-soluble derivative which forms complexes with cholesterol and has been shown to disrupt formation of lipid microdomains in the plasma membrane (Pitha et al., 1998; Zhu et al., 2010). As expected, LPS-induced cytokine secretion was significantly reduced when trophoblast cells were pretreated with MCD. Similarly, in cells treated with oxysterols, the inflammatory response was reduced following MCD treatment. These findings indicate that inflammatory activity initiated by oxysterols is sensitive to membrane cholesterol content. Therefore, it appears that depletion of membrane cholesterol has a negative impact on the inflammatory effects of oxysterols, most likely through abrogation of TLR4 aggregation and activation. The fact that oxysterols promote inflammation, despite the expectation that they would also activate LXR and promote inhibition of inflammation, suggests that TLR4 activation at the concentrations employed is the predominant response. It is possible that the effects on membrane cholesterol levels mediated via LXR activation are too minor to have significant effects on TLR4 activity, or may be blocked in some way via NF-κB activation.

In conclusion, we have shown that the oxysterols 25-OHC and 7-ketoC promote proinflammatory cytokine secretion in cultured placental trophoblast cells via TLR4 activation, an effect that predominates over its anti-inflammatory effects mediated via LXR activation and/or cholesterol modulation. Taken together, these findings suggest that elevated circulating or local concentrations of oxysterols in conditions associated with oxidative stress may contribute towards placental inflammation. It remains to be determined whether steps taken to reduce cholesterol oxidation or to prevent excessive cholesterol accretion during pregnancy may be beneficial in pregnancy through the minimization of placental inflammation.

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Authors’ roles

I.L.M.H.A.: Study design, data acquisition, analysis, interpretation, wrote manuscript, approved final version. B.J.W., P.J.M.: Data interpretation, revised manuscript, approved final version. J.A.K.: Funding, study design, data interpretation, revised manuscript, approved final version.

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Conflict of interest

The authors declare no conflict of interest.

References


Aye IL, Waddell BJ, Mark PJ, Keelan JA. Placental ABCA1 and ABCG1 transporters efflux cholesterol and protect trophoblasts from oxysterol induced toxicity. *Biochim Biophys Acta* 2010;1801:1031–1024.


Mascia C, Maina M, Chiariotto E, Leonarduzzi G, Poli G, Biasi F. Proinflammatory effect of cholesterol and its oxidation products on...

Matsunaga N, Tsuchimori N, Matsumoto T, Li M. TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR4) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. Mol Pharmacol 2011;79:34–41.


