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Impairment of trophoblast survival and differentiation by LXR ligands is prevented by cholesterol but not ABCA1 silencing

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ABSTRACT

Introduction: The Liver X Receptors (LXRs) drive the transcriptional response to excess intracellular cholesterol. Oxysterols, the products of cholesterol oxidation, are activating ligands for LXR that can accumulate under conditions of oxidative stress and disrupt cholesterol homeostasis. While activation of LXR inhibits trophoblast differentiation, the impact of LXR on trophoblast physiology or cholesterol homeostasis is incompletely understood. We sought to determine if the effects of LXR activation can be ameliorated through modification of cholesterol bioavailability or inhibition of LXR-driven cholesterol efflux in trophoblasts.

Methods: We measured the effect of oxysterol exposure on BeWo cells and primary human trophoblasts (PHT cells) cultured in lipoprotein-deficient medium. We also measured the effect of the synthetic, LXR-specific ligand T0901317 on PHT cell differentiation and survival. Finally, we silenced the ATP-binding cassette transporter A1 (ABCA1), a transcriptional target of LXR that drives cholesterol efflux, to determine if inhibition of cholesterol efflux could block the effects of T0901317.

Results: Oxysterols inhibited BeWo survival and PHT cell differentiation, and these effects were blocked by cholesterol supplementation. T0901317 also inhibited PHT cell differentiation, and this effect was similarly blocked by cholesterol. Unlike cholesterol however, ABCA1 silencing did not modify the effect of T0901317 on PHT cell differentiation.

Discussion: Oxysterols and LXR inhibit trophoblast survival and differentiation exclusively in conditions of cholesterol scarcity. These findings underscore the importance of cholesterol homeostasis in the maintenance of placental function and suggest that pathways regulating cholesterol homeostasis may represent therapeutic targets to mitigate harmful sequelae of placental injury.

1. Introduction

Placental dysfunction is associated with fetal growth restriction (FGR) and its harmful sequelae. Incomplete remodeling of the spiral arteries is a common feature of FGR, characterized by intermittent vasoconstriction and turbulent flow into the intervillous space [1–3]. The resulting irregular oxygen delivery promotes formation of reactive oxygen species (ROS) and oxidative stress, hallmarks of placental injury and FGR [4–6].

Cholesterol is a ubiquitous lipid molecule required for basic cellular functions and multifold synthesis and signaling pathways. Cholesterol excess and deficiency are cytotoxic, necessitating the exquisite regulation of cellular cholesterol homeostasis [7]. The Liver X Receptors (LXRs) and Sterol Response Element Binding Protein 2 (SREBP2) are the primary transcriptional regulators of this intricate homeostasis. Both isoforms of LXR (LXRα and LXRβ) promote cholesterol efflux in response to excess intracellular cholesterol, while SREBP2 stimulates cholesterol uptake and synthesis in response to cholesterol deficiency [8,9]. Oxysterols, the products of cholesterol oxidation, inhibit SREBP2 and are activating ligands for LXR. As such, they promote lipid droplet formation, cholesterol efflux, and inhibit cholesterol synthesis and uptake [10,11].

Placental cholesterol uptake is needed to sustain steroid synthesis and cholesterol delivery. However, circulating cholesterol is highly susceptible to oxidation [12,13], and levels of cholesterol oxidation products are increased in maternal serum, fetal serum and placental tissues of pregnancies affected with FGR [14–16]. Furthermore, oxysterols inhibit trophoblast survival and differentiation *in vitro* [17–19], and activation of LXR inhibits extravillous cytotrophoblast invasion [20,21].

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One transcriptional target of LXRs relevant to placental physiology is the ATP-binding cassette transporter A1 (ABCA1), a transmembrane protein that mediates efflux of cholesterol to lipid-poor apolipoprotein A1 (ApoA1) [22]. ABCA1-mediated cholesterol efflux has been posited as a crucial component of maternal-fetal cholesterol transport [23,24]. Nonetheless, the localization and function of ABCA1 in the placenta remains controversial. Prior studies indicate that ABCA1 effluxes cholesterol from the placenta to fetus [24] and is expressed in villous endothelium [23] and syncytiotrophoblast [25]. Yet contrasting reports indicate no expression of ABCA1 in syncytiotrophoblasts [26] or villous endothelium [27], and suggest that ABCA1 does not drive cholesterol efflux in choriocarcinoma-derived BeWo cells [28].

We sought to probe the effect of oxysterols and LXR on trophoblast differentiation and survival, testing two hypotheses: (1) the inhibitory effect of LXR on trophoblast survival and differentiation can be ameliorated through modification of cholesterol bioavailability; and (2) the effect of LXR activation in trophoblasts is attributable to the induction of ABCA1-mediated cholesterol efflux.

2. Methods

2.1. Cell culture and ligands

Primary human trophoblast (PHT) cells were purified as described by Kliman et al. with published modifications [17,29]. Placentas were collected following uncomplicated term delivery at the Magee-Womens Hospital under a protocol approved by the University of Pittsburgh IRB. All placentas were from pregnancies without a clinical indication for placental histopathology and would otherwise have been discarded. Cells were plated at 350,000 cells/cm^2 in DMEM (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and 1% Penicillin/Streptomycin/Fungizone in 5% CO_2. After 6 h, cells were washed with PBS and 10% FBS medium was replaced. After 24 h, cells were washed, placed in medium containing 5% lipoprotein-deficient serum (LPDS) (Alfa Aesar, Haverhill, MA), and exposed to the oxysterol 25-hydroxy cholesterol (25OHC) (Cayman Chemical, Ann Arbor, MI) (24–72 h after plating) or the LXR-specific ligand T0901317 (Sigma) (24–48 h after plating) [30]. All experimental exposures were continued with subsequent media changes. Control cells were exposed to equivalent volumes of vehicle (100% ethanol, maximum 0.2% v/v). 5% LPDS medium was supplemented with water-soluble cholesterol (Sigma). Medium was replaced every 24 h and all analyses were performed after 72 h.

BeWo cells were cultured in Ham's F12 media (Fisher, Walkersville, MD) supplemented with 5% LPDS, L-Glutamine (Fisher) and 1% Penicillin/Streptomycin (Fisher) incubated at 37 °C in 5% CO_2. Data from BeWo experiments were collected at multiple time points, with time 0 defined as initial exposure to 5% LPDS. 25OHC and cholesterol were added to BeWo cells at time 0, and culture medium was not changed between time 0 and collection.

2.2. Viral transduction

The lentiviral packaging plasmid pCD/NL-BH*DDD and envelope plasmid pLTR were obtained from Addgene. Three shRNA oligonucleotides (in MISSION TRC2 pLKO.5 backbone) targeting ABCA1 (Sigma) were tested for knockdown efficiency in 293T cells. We selected the most efficient construct (5′-ACCTATGTGAAACTCTATTAT-3′) for subsequent experiments with a sequence targeting GFP (5′—CCGG CGTGATCTTCACCGACAAGATCTC-3′) (Sigma) and cells unexposed to lentivirus as controls.

Lentivirus vectors were produced by transfection of 293T cells using polyethylenimine. 20 × 10^6 cells were plated in 15-cm dishes in Opti-MEM (Gibco, Gaithersburgh, MD) with 10% FBS in 5% CO_2. Cells were transfected with 18 μg of the transfer vector with the indicated shRNA insert, 9 μg pLTR, and 13 μg pCD/NL-BH*DDD. Medium was changed 18 h after transfection, and culture medium was collected 48 h and 72 h after plating. All media were combined and centrifuged at 300 × g to remove cellular debris. The supernatant was vacuum filtered (0.45 μm) (Millipore, Bedford, MA) and centrifuged at 25,000 rpm for 2 h. The lentivirus pellet was suspended in PBS. Viral concentration was determined using Lenti-X qRT-PCR titration kit (Takara, Mountain View, CA) with serially diluted virus (10^3–10^6) used to titer transduction efficiency in 293T cells. For PHT cell transduction, lentivirus was added 6 h after plating for 18 h.

2.3. Cell viability measurements

Viability measurements were made using the Cell Titer-Glo assay kit (Promega, Fitchburg, WI). This kit generates a luminescent signal proportional to the quantity of ATP, an indicator of metabolically active cells. All measurements were made in duplicate.

2.4. Detection of human chorionic gonadotropin (hCG)

ELISA kits were used to quantify hCG in cell medium (DRG International, Mountainside, NJ). Duplicate assays were performed after 72 h in culture.

Isolation of RNA and reverse transcriptase-quantitative PCR (RT-qPCR).

Cells were lysed using Tri-Reagent (Molecular Research Center, Cincinnati, OH) and genomic DNA removed using the DNA-Free kit (Invitrogen). Extracted RNA was quantified by 260/280 absorbance ratio using a NanoDrop-1000 spectrophotometer (Fisher-Thermo). 1 μg RNA was used for reverse transcription with the High Capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). Duplicate qPCR reactions were performed with 250 nM primer concentrations (Supplementary Table 1) using 384 well plates with 25 ng cDNA per 10 μL, SYBR Green PCR Master Mix (Applied Biosystems), analyzed with Applied Biosystems' ViiA 7 Sequence Detection System, and normalized to YWHAZ [31] using the ΔΔCt method [32].

2.5. Western immunoblotting

Cells were lysed in RIPA (Santa Cruz biotechnology, Santa Cruz, CA) and sonicated. Protein content was determined using the Pierce BCA assay (Pierce, Rockford, IL). 30 μg of protein was loaded in 2.5% β-mercaptoethanol in tris-glycine SDS sample buffer (BioRad, Hercules, CA) and incubated at room temperature (RT) for 15 min. Proteins were separated on a 4–15% SDS-polyacrylamide gel (BioRad) at 100 V for 1 h and transferred to polyvinylidene fluoride membranes (Immobilon; Millipore, Bedford, MA) using a wet transfer system (BioRad). Membranes were blocked with 5% nonfat dry milk in 10 mM Tris (pH 7.4), 150 mM NaCl (TBS)-0.05% Tween-20 (TBS-T) and incubated with anti-ABCA1 rabbit polyclonal antibody (Novus, Littleton, CO) at 1:500 overnight at 4 °C. Blots were washed with TBS-T and incubated with goat anti-rabbit IgG-HRP (Abcam, Cambridge, MA) at 1:30,000 for 1 h at RT. Protein bands were detected by enhanced chemiluminescence (Fisher). Membranes were stripped with Restore Buffer (Fisher), blocked for 1 h at RT, and incubated with a mouse monoclonal anti-GAPDH antibody (Abcam) at 1:2000 for 1 h at RT. Blots were washed and incubated with goat anti-mouse IgG-HRP at 1:30,000 for 1 h at RT.

2.6. Fluorescent immunohistochemistry

For *in vivo* analysis, placental biopsies were collected under the same IRB protocol used for collection and isolation of primary trophoblasts. Paraffin embedded placental sections (5 μm) were deparaffinized in xylene and ethanol before antigen retrieval in sodium citrate (10 mM, pH 7.5) for 20 min at 90 °C. Cultured cells were fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton X-100/PBS. Cells and tissue samples were blocked in immunobuffer (3% goat