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Unsaturated fatty acids protect trophoblast cells from saturated fatty acid-induced autophagy defects

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ABSTRACT

Dysregulated serum fatty acids are associated with a lipotoxic placental environment, which contributes to increased pregnancy complications via altered trophoblast invasion. However, the role of saturated and unsaturated fatty acids in trophoblastic autophagy has yet to be explored. Here, we demonstrated that prolonged exposure of saturated fatty acids interferes with the invasiveness of human extravillous trophoblasts. Saturated fatty acids (but not unsaturated fatty acids) inhibited the fusion of autophagosomes and lysosomes, resulting in the formation of intracellular protein aggregates. Furthermore, when the trophoblast cells were exposed to saturated fatty acids, unsaturated fatty acids counteracted the effects of saturated fatty acids by increasing degradation of autophagic vacuoles. Saturated fatty acids reduced the levels of the matrix metalloproteinases (MMP)-2 and MMP-9, while unsaturated fatty acids maintained their levels. In conclusion, saturated fatty acids induced decreased trophoblast invasion, of which autophagy dysfunction plays a major role.

1. Introduction

Maternal obesity during pregnancy is associated with an abnormal intrauterine metabolic milieu and various obstetrical complications, such as premature labor, intrauterine growth restriction (IUGR), gestational hypertension, gestational diabetes, miscarriage, and pre-eclampsia (Andreasen et al., 2004; Cedergren 2004). Metabolic conditions such as obesity and metabolic syndromes, are correlated with high levels of free fatty acids in the serum (Zhang et al., 2014) and altered composition of serum free fatty acids, which comprise saturated, monounsaturated, and polyunsaturated fatty acids (Klein-Platat et al., 2005). Excessive free fatty acids in the serum have been reported to cause lipotoxic placental environment that contribute to various cellular stress (Jarvie et al., 2010; Saben et al., 2013; Saben et al., 2014).

Macroautophagy (hereafter referred to as autophagy) is a conserved intracellular clearance system by which cytoplasmic target components are degraded within the lysosome. During autophagy, cytoplasmic target components are sequestered into the double-membrane vesicles called autophagosomes, and subsequently combined with lysosomes to form autolysosomes (Yen and Klionsky 2008; Ravikumar et al., 2010). Autophagy is necessary for preserving protein homeostasis and protein quality control through selective degradation of aggregated proteins, damaged mitochondria, and pathogens (Kirkin et al., 2009; Youle and Narendra 2011). Failure of autophagy is associated with a number of pathological conditions, including obesity, inflammation, and cancer (Mizushima et al., 2008; Codogno and Meijer 2010; Park et al., 2014b). A recent study has suggested that inhibition of autophagy by soluble endoglin causes a disruption of extravillous trophoblast invasion, which contributes to the immune- pathology of preeclampsia (Nakashima et al., 2013).

In this study, we investigated the effects of saturated (SFA) and unsaturated fatty acids (UFA) on autophagic pathway and protein homeostasis in human extravillous trophoblasts. We found that SFA (but not UFA) interfere with trophoblast invasion and suppress autophagic degradation of protein aggregates. The accumulation of protein aggregates is mediated by the inhibition of autophagosomes–lysosomes fusion during SFA exposure. Moreover, UFA attenuate SFA-induced protein aggregate formation by restoring autophagosome–lysosome fusion, which in turn, rescues the impaired invasion of trophoblast cells from SFA conditions.

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2. Materials and methods

2.1. Cell culture and treatments

The human first trimester trophoblast cell line, Sw.71 (gift from Dr. Gil Mor, Yale University School of Medicine), was derived from 7-week first trimester placenta isolated from normal pregnancy and immortalized by telomerase-mediated transformation. Sw.71 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone) containing 10% fetal bovine serum (Corning), 100 U/mL penicillin-streptomycin (Gibco), 1 mM HEPES, 0.1 mM non-essential amino acids and 1 mM sodium pyruvates. All cultures were maintained in a 37 °C incubator with 5% CO_2. For free fatty acid treatment, cells were incubated in the presence of different free fatty acids/BSA, as described previously (Cousin et al., 2001). Briefly, 100 mM stock solutions of palmitate and oleate were prepared in 0.1 M NaOH by heating at 70 °C, added to 10% BSA solution (the 5 mM working solution) at 55 °C in a shaking water bath, and vortex mixed for 10 s followed by a further 10 min incubation. Same volume of 10% fatty acid free-BSA solution was used as the vehicle control. For the assessment of autophagic flux, cells were treated with bafilomycin A1 for the last 3 h before harvest.

2.2. Invasion assay

The invasiveness of cells was assayed in a Boyden chamber (Neuro Probe) according to the manufacturer's information. Briefly, polycarbonate membranes with 8 μm pores (Neuro Probe) were coated with 10 μl/ml matrigel (Corning). The bottom wells of a Boyden chamber were filled with DMEM medium containing 10% FBS. The cells in serum-free medium were trypsinized and seeded into the upper chamber at 4×10^6 cells per well. Fourteen hours later, the cells were fixed and stained with Diff-Quik stain solution (Sysmex). Non-invading cells on the upper side of the membrane were removed using a wiper tissue swab. The membranes were mounted onto microscope slides. The number of stained cells at the lower surface of the membrane was counted in at least five random microscope fields (at ×20 magnification). Data from experiments measuring invasion were expressed as the percentage of invaded cell number compared with the corresponding control.

2.3. Solubility fractionation

Sw.71 trophoblast cells were treated with 500 μM palmitate and/or 500 μM oleate for indicated time periods, washed in cold phosphate-buffered saline (PBS), and lysed in a lysis buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM NaPPi, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1% Triton X-100 and protease inhibitor cocktail). Cell lysates were centrifuged at 18,000*g* for 15 min at 4 °C to separate pellets from supernatants. Pellets were resuspended in a lysis buffer containing 2% SDS and then subjected to centrifugation at 15,000 r.p.m. for 15 min at 4 °C. Supernatants (Triton X-100-soluble fraction) or resuspended pellets (Triton X-100-insoluble fraction) were boiled in SDS sample buffer and subjected to SDS-PAGE and immunoblotting.

2.4. Immunoblotting

Immunoblotting was carried out as previously described (Park et al., 2014b). Cell lysates were obtained from whole cell lysate or fractionation and the protein concentration was measured by using the BCA Protein Assay (Pierce). The lysates were boiled in 1X SDS sample buffer for 5 min, separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes (Millipore) and probed with anti-ubiquitin antibody (1:1000, Santa Cruz Biotechnology), anti-LAMP1 antibody (1:1000, Santa Cruz Biotechnology), anti-SQSTM1 antibody (1:2000, Cell Signaling Technology), anti-LC3 antibody (1:2000, Cell Signaling Technology) or β-actin antibody (1:500, DSHB) as an internal control. After incubation with secondary antibodies conjugated with horseradish peroxidase (HRP), chemiluminescence signals were detected using Fusion Solo (Vilber Lourmat) systems. Densitometric analysis of the blots was performed using ImageJ (National Institutes of Health, USA).

2.5. Immunocytochemistry

Sw.71 cells grown on coverslip were treated with 500 μM palmitate and/or 500 μM oleate for indicated time periods at 37 °C. The cells were rinsed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were blocked in blocking solution for 1 h at room temperature and incubated with anti-ubiquitin antibody (1:500), anti-LC3 antibody (1:1000), or anti-SQSTM1 antibody (1:500) overnight at 4 °C in a humidified chamber. After washing, cells were incubated with Alexa Fluor 488- and 568-conjugated secondary antibodies (1:500, Invitrogen) for 1.5 h and counterstained with DAPI (Invitrogen). Confocal images were obtained with a laser confocal microscope (LSM 700, Carl Zeiss) and processed with ImageJ software (NIH). Ubiquitin aggregate size was quantified using ImageJ software. Co-localization of LC3 and LAMP1 in SW.71 cells was quantified by Pearson's coefficient using ImageJ software with JACoP plugin on images collected from triplicate experiments.

2.6. Gelatin zymography

Proteolytic activity of MMP-2 and MMP-9 were analyzed by substrate gel electrophoresis (zymography) using 10% polyacrylamide gels containing 1.5% gelatin (Sigma-Aldrich). Briefly, Sw.71 cells were plated in 6-well plates (35 mm diameter) and treated with 500 μM palmitate and/or 500 μM oleate for 16 h at 37 °C. The harvested culture media were diluted in 5 × sample buffer (5% SDS, 20% glycerol, 0.5% bromophenol blue, 0.4 M Tris pH 6.8), and equal amounts of protein were subjected to substrate gel electrophoresis. The gel was washed three times in 2.5% Triton X-100 for 10 min at room temperature and then incubated in development solution (50 mM $CaCl_2$, 0.5 M Tris pH 7.6) for 18 h at 37 °C. The gel was stained with Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid and destained in 40% methanol and 10% acetic acid until clear bands appeared. MMP-2 and MMP-9 activity were quantified by densitometric scanning of the zymographic bands using ImageJ.

2.7. Quantitative real-Time PCR

Total RNA was extracted from trophoblast cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and complementary DNA was made using Moloney-Murine leukemia virus reverse transcriptase (MMLV-RT, Promega) and random hexamers (Promega). Quantitative reverse transcription-PCR was performed on samples in triplicate with SYBR green real-time PCR master mix reagent (Toyobo) using the CFX96 Real-Time PCR Detection System (Bio-Rad). Relative mRNA expression was calculated from the comparative threshold cycle (C_t) values relative to human cyclophilin A. The following primers were used: *Cyclophilin A*; forward (fwd) 5′-GCAAAGT GAAAGAAGGCATGAA-3′, reverse (rev) 5′- CCATTCCTGGACCCAA AGC-3′ (Meur et al., 2010); *MMP-2*; fwd 5′-GTCTGTGTTGTCCAGAG GCA-3′, rev 5′-CTAGGCCAGCTGGTTGGTTC-3′; *MMP-9*; fwd 5′-GTACT CGACCTGTACCAGCG-3′, rev 5′-AGAAGCCCCACTTCTTGTCG-3′.

2.8. Statistical analysis

Statistical analysis was performed by the Microsoft Office Excel statistical package. Unless otherwise described above, results are presented as mean ± standard error of mean (s.e.m.), and are representative of at least three independent experiments. Statistical