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Oleate attenuates palmitate-induced endoplasmic reticulum stress and apoptosis in placental trophoblasts

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Abstract

Pre-pregnancy obesity is increasingly common and predisposes pregnant women and offspring to gestational diabetes, pre-eclampsia, fetal growth abnormalities and stillbirth. Obese women exhibit elevated levels of the two most common dietary fatty acids, palmitate and oleate, and the maternal blood containing these nutrients bathes the surface of trophoblasts of placental villi *in vivo*. We test the hypothesis that the composition and concentration of free fatty acids modulate viability and function of primary human villous trophoblasts in culture. We found that palmitate increases syncytiotrophoblast death, specifically by caspase-mediated apoptosis, whereas oleate does not cause enhanced cell death. Importantly, exposure to both fatty acids in equimolar amounts yielded no increase in death or apoptosis, suggesting that oleate can protect syncytiotrophoblasts from palmitate-induced death. We further found that palmitate, but not oleate or oleate with palmitate, increases endoplasmic reticulum (ER) stress, signaling through the unfolded protein response, and yielding CHOP-mediated induction of apoptosis. Finally, we show that oleate or oleate plus palmitate both lead to increased lipid droplets in syncytiotrophoblasts, whereas palmitate does not. The data show palmitate is toxic to human syncytiotrophoblasts, through the induction of ER stress and apoptosis mediated by CHOP, whereas oleate is not toxic, abrogates palmitate toxicity and induces fat accumulation. We speculate that our *in vitro* results offer pathways by which the metabolic milieu of the obese pregnant woman can yield villous trophoblast dysfunction and sub-optimal placental function.

Introduction

Pre-pregnancy body mass index determines pregnancy outcome (Nelson *et al*. 2010, Flegal *et al*. 2012), and more than half of pregnant women in the United States are overweight or obese. The latter are at risk for gestational diabetes, pre-eclampsia, Caesarean delivery and stillbirth (American College of Obstetricians and Gynecologists 2013, Marchi *et al*. 2015). Moreover, infants of obese women are at increased risk for congenital anomalies, macrosomia, birth injury and childhood obesity (American College of Obstetricians and Gynecologists 2013, Marchi *et al*. 2015). The mechanisms contributing to these adverse outcomes are poorly understood.

Pre-pregnant obese women exhibit abnormal lipid profiles compared to lean controls (Chen *et al*. 2010*b*). Saturated fatty acids circulating in obese women commonly have more lipotoxic potential than non-saturated fatty acids (Poitout *et al*. 2010, Leamy *et al*. 2013). Palmitate is a saturated fatty acid found in the highest concentration in most diets and oleate is a dominant monounsaturated fatty acid (van Dijk *et al*. 2009). Notably, obese pregnant women have higher circulating levels of free fatty acid compared to lean controls, commonly showing higher-than-control concentrations of palmitate and oleate (Chen *et al*. 2010*b*). Although total concentrations of serum fatty acids influence health (Pilz *et al*. 2006, Khaw *et al*. 2012), levels of specific fatty acids and ratios of saturated to unsaturated fatty acids, also influence health (Micha & Mozaffarian 2010). Although a limited number of studies have investigated serum free fatty acid levels in pregnant women (Villa *et al*. 2009, Chen *et al*. 2010*b*), no studies have yet directly addressed if the relative amounts of specific fatty acid types (e.g., saturated vs unsaturated) are altered in obese pregnant women compared to lean women, or if altered lipid ratios affect pregnancy outcomes.

Palmitate and oleate differentially affect the fate of cells in a way that depends on cell type, substrate

concentration and ratio of these fatty acids (Miller *et al*. 2005, Karaskov *et al*. 2006, Wei *et al*. 2006). Palmitate, for example, induces apoptotic cell death in multiple cell types, including pancreatic beta cells (Karaskov *et al*. 2006), cardiac myocytes (Miller *et al*. 2005) and hepatocytes (Wei *et al*. 2006). Additionally, palmitate can induce endoplasmic reticulum (ER) stress, and the resultant activation of the ER stress pathway can be the proximate cause leading to apoptosis and cell death (Karaskov *et al*. 2006, Cunha *et al*. 2008, Pfaffenbach *et al*. 2010, Peng *et al*. 2011, Lu *et al*. 2012). In contrast, oleate does not induce apoptosis, and, remarkably, can be protective against palmitate-induced apoptosis (Listenberger *et al*. 2003, Miller *et al*. 2005, Soumura *et al*. 2010, Sommerweiss *et al*. 2013). Several studies have suggested that oleate-mediated protection from palmitate-induced cell death is related to the formation of neutral lipids, such as triglycerides, which are then stored as lipid droplets (Listenberger *et al*. 2003, Soumura *et al*. 2010, Thorn & Bergsten 2010, Peng *et al*. 2011).

At the interface between two circulations, the placental villus mediates exchange of nutrients, oxygen and wastes between the maternal and fetal circulations. The human placental villus is thereby positioned to be a target for the metabolic effects from the maternal circulation in obese or lean women during pregnancy. Specifically, a multinucleated epithelial layer, called the syncytiotrophoblast, overlies the chorioallantoic villi and is bathed in the maternal blood that circulates through the intervillous space. This microanatomy allows maternal blood fatty acid levels, and the ratios of saturated and unsaturated fatty acids, to directly affect syncytiotrophoblast function and, ultimately, to modify placental function. We tested the hypothesis that the concentration and ratio of palmitate and oleate differentially modulate viability and function of primary cultures of human syncytiotrophoblasts.

Materials and methods

Isolation and culture of primary human trophoblasts

The Institutional Review Board of Washington University School of Medicine approved this study, and written informed consent was obtained to utilize the placentas. Primary human trophoblasts were isolated from placentas retrieved immediately after repeat C-section under epidural anesthesia at 39-week gestation, in non-labored, uncomplicated pregnancies, as previously described (Chen *et al*. 2010*a*, 2012).

Trophoblasts were cultured at 37°C in 5% CO_2/ambient air in phenol red-free DMEM (GE Life Sciences, Logan, UT, USA) with 20 mM HEPES (pH 7.4) (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin (obtained from the Washington University tissue culture support center). Trophoblasts were plated at a density of 300,000 cells/cm² in medium supplemented with 10% fetal bovine serum (FBS)

(Thermo Fisher Scientific), cultured for 4 h to allow attachment and rinsed thoroughly with medium to eliminate dead cells and villous fragments; this time was designated as time zero of culture. Medium supplemented with 10% charcoal-stripped FBS was changed each 24 h during culture. We assayed the effects on trophoblasts for levels of fatty acids that are typical of those present in human serum: 100 µM palmitate or oleate, which approximate those found in lean, healthy pregnant women, whereas 200–400 µM of these lipids are present in obese individuals (Villa *et al*. 2009, Chen *et al*. 2010*b*).

Fatty acid, tunicamycin and Z-VAD-FMK treatment

Oleic acid and palmitic acid were purchased from Sigma. Fatty acid micelles were prepared according to Listenberger and Brown (2007), stored at 4°C and used within one week of preparation. Cultures were supplemented with 100, 200 or 400 µM of oleate, palmitate or an equimolar mixture of these fatty acids (Sigma) for 24 h prior to harvest for the concentration-dependent studies. Syncytiotrophoblasts were treated with 200 µM oleate, 200 µM palmitate or with 200 µM of each lipid for 0, 3, 8 or 24 h for time-dependent studies. Where indicated, tunicamycin (5 µg/mL, Sigma) or dimethyl sulfoxide (DMSO) vehicle control was added for 24 h prior to harvest. Where indicated, the pan-caspase inhibitor Z-VAD-FMK (20 µM, BD Biosciences, Franklin Lakes, NJ, USA) or DMSO vehicle control was added one hour prior to and throughout lipid exposure.

Assessment of cell viability

Culture medium was harvested for lactate dehydrogenase (LDH) released from cells as a marker of global cell death and assessed by CytoTox 96 non-radioactive cytotoxicity assay (Promega) according to the manufacturer's instructions. Results are expressed as relative amount of LDH released into the culture medium in experimental vs control conditions. Cell viability was also assessed using the MTS colorimetric assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay; Promega) according to the manufacturer's instructions. Results are expressed as percent cell viability in experimental vs control conditions.

Western blotting

Trophoblasts cultured in 22 mm diameter tissue culture-treated plastic plates were rinsed with PBS and lysed with 120 µL of RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate and 0.1% SDS in PBS) containing protease and phosphatase inhibitors (Sigma). Samples were sonicated and centrifuged for 10 min at 4°C, and the supernatant was transferred to a fresh tube. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, blocked for >1 h at room temperature with 5% nonfat dry milk in PBST (PBS with 0.05% Tween 20) and incubated overnight at 4°C with primary antibody (Table 1) in either 5% NFDM or 5% BSA in PBST, as recommended by the manufacturer. Blots were washed with PBST for 30 min and then incubated for 4 h with appropriate horseradish peroxidase-conjugated donkey anti-

Table 1 Antibodies used for Western blotting.

Protein target	Name of antibody	Manufacturer, catalog #	Species raised in	Concentration	Dilution used
Actin	Actin (I-19)	Santa Cruz Biotechnologies, Dallas, TX, #sc-1616	Goat	200 µg/mL	1:2000
BiP	BiP (C50B12)	Cell Signaling Technology, Danvers, MA; #3177	Rabbit	65 µg/mL	1:1000
CHOP	CHOP	Proteintech, Chicago, IL; #15204-1-AP	Rabbit	373 µg/mL	1:300
Cleaved caspase 3	Cleaved Caspase-3 (Asp175) (5A1E)	Cell Signaling Technology #9664	Rabbit	80 µg/mL	1:1000
Cleaved PARP	Cleaved PARP (Asp214)	Cell Signaling Technology #9541	Rabbit	35 µg/mL	1:1000
Cleaved cytokeratin 18	M30 CytoDEATH	Roche, Indianapolis, IN; #12140322001	Mouse	16 µg/mL	1:1000
Goat IgG	Anti-goat IgG HRP conjugate	Thermo Scientific, Waltham, MA; #PA1-28664	Donkey	2 mg/mL	1:5000
Mouse IgG	Anti-mouse IgG HRP-linked antibody	Cell Signaling Technology #7076	Horse	170 µg/mL	1:5000
Rabbit IgG	Anti-rabbit IgG HRP-linked antibody	Cell Signaling Technology #7074	Goat	66 µg/mL	1:5000

BiP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; PARP, poly(ADP-ribose) polymerase.

goat, horse anti-mouse or goat anti-rabbit IgG antibodies at room temperature. Blots were washed with PBST, and targeted proteins were detected by chemiluminescence (Thermo Scientific). Protein expression levels were determined by densitometry, normalized to actin and quantified using ImageJ software (NIH).

Quantitative real-time PCR

RNA isolation, cDNA generation and quantitative real-time PCR (qRT-PCR) were done as described previously (Chen *et al.* 2015). Briefly, nucleic acid was isolated from primary trophoblasts with TRI Reagent (Life Technologies), DNA was removed by DNAse treatment (Turbo DNA-free, Life Technologies) and cDNA generated by reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a 50 µL reaction containing 0.5 µg RNA, 1× RT buffer, 50 mM deoxy-NTP, random hexamers at 1× as recommended by the manufacturer, 0.4 units/µL RNase inhibitor and 2 units/µL MultiScribe reverse transcriptase, at 25°C for 10 min, 37°C for 120 min and 85°C for 10 min. qRT-PCR reactions were carried out in a CFX96 real-time PCR machine (Bio-Rad Laboratories), using 1 µL of cDNA with 500 nM each of the forward and reverse gene-specific primers (Table 2) and Universal SYBR green supermix (Bio-Rad Laboratories) in a total reaction volume of 10 µL. Dissociation curves were evaluated for all reactions to verify amplification of a single product with the appropriate melting temperature. Agarose gel electrophoresis verified the generation of a single product of the expected size for all primer pairs used for qRT-PCR. RNA expression levels were normalized to parallel reactions with primers specific for *YWHAZ* (Meller *et al.* 2005, Murthi *et al.* 2008). The fold increase gene expression in experimental relative to control conditions was determined by utilizing the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak 2008).

Gene silencing by siRNA

At 24 h of culture, trophoblasts were transferred to OPTI-MEM with 10% csFBS and transfected with 50 nM siCTRL or si*CHOP* RNA (OriGene, Rockville, MD, USA, SR30004, SR301166B) prepared using DharmaFECT 1 (Dharmacon) according to the manufacturer's instructions. After 24-h culture

in the transfection medium, medium was replaced with DMEM with vehicle or DMEM with 200 µM palmitate and culture was continued for another 24 h prior to harvest.

Lipid droplet staining

Primary cultures were fixed with 3% paraformaldehyde in PBS for 30 min at room temperature, and then permeabilized and blocked with 0.01% saponin in 3% BSA for 45 min at room temperature. Lipid droplets were stained with BODIPY 493/503 (1:500; Molecular Probes) and nuclei were stained with TOPRO3 (1 µM; Life Technologies) for one hour at room temperature. Images were obtained using a Nikon E800 C1 confocal microscope at 1024×1024 px resolution using a final magnification of 600×. At least four representative areas of syncytiotrophoblasts for each condition from three separate placentas were outlined, and the area was measured using

Table 2 Primers for RT-PCR.

Gene	Acc. No.	Sequence (5′–3′)
BCL2	NM_000633.2	F: CGGGAGATGTCGCCCCTGGT
		R: CAAAGGCATCCCAGCCTCCGT
BiP	NM_005347.4	F: CGGGCAAAGATGTCAGGAAAG
		R: TTCTGGACGGGCTTCATAGTAGAC
CHOP	NM_001195053.1	F: ACCAAGGGAGAACCAGGAAACG
		R: TGACCATTCGGTCAATCAGAGC
PUMA	NM_014417	F: GACCTCAACGCACAGTACGAG
		R: AGGAGTCCCATGATGAGATTGT
XBP1 (T)	NM_005080.3 and NM_001079539.1	F: CTGGAACAGCAAGTGGTAGA
		R: CTGGGTCCTTCTGGGTAGAC
XBP1 (US)	NM_005080.3	F: CAGCACTCAGACTACGTGCA
		R: ATCCATGGGGAGATGTTCTGG
XBP1 (S)	NM_001079539.1	F: CTGAGTCCGAATCAGGTGCAG
		R: ATCCATGGGGAGATGTTCTGG
YWHAZ	NM_001135702.1	F: TTTGGTACATTGTGGCTTC
		R: CCCGCCAGGACAAACCAG

XBP(T) primers are used to amplify simultaneously both unspliced (NM_005080.3) and spliced (NM_001079539.1) forms of *XBP1* mRNA by non-quantitative PCR for analysis by gel electrophoresis. All other primer pairs were used for qRT-PCR.
Acc. No.: NCBI RefSeq nucleotide accession number; *BCL2*, B-cell lymphoma 2; *BiP*, binding immunoglobulin protein; *CHOP*, C/EBP homologous protein; FOR, forward; *PUMA*, p53 upregulated modulator of apoptosis; REV, reverse; *XBP1*, X-box binding protein 1; *YWHAZ*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide.

ImageJ software (NIH). Lipid droplets and nuclei within these areas were then quantified. Results are presented as number of lipid droplets per unit area.

Statistical analysis

The number (*n*) of placentas for each set of experiments is indicated in the figure legend or on the figure. Data are mean ± s.d. One-way ANOVA with Bonferroni *post hoc* test for ≥3 conditions or Student's *t*-test for two comparisons was performed using KaleidaGraph software (Synergy Software, Reading, PA, USA).

Results

Palmitate, but not oleate, decreases syncytiotrophoblast viability

Primary cytotrophoblasts fuse beyond 24 h in culture and >85% of nuclei are in syncytiotrophoblasts by 52 h, as we previously described (Wang *et al.* 2016). Primary cultures exposed to 200 or 400 µM palmitate for 24 h showed significantly increased levels of LDH in the culture medium, compared to vehicle control (Fig. 1A and B). This effect was time dependent, with LDH significantly increased above control after 24 h of exposure (Fig. 1B). In contrast, oleate did not result in increased LDH levels in the medium, even after 24 h of exposure to 400 µM (Fig. 1A and B). Notably, syncytiotrophoblasts exposed to an equimolar mixture of oleate and palmitate showed LDH release is indistinguishable from that of control or oleate-only exposed cells and significantly less than that in palmitate-only exposed cultures (Fig. 1A and B). Together, these data indicate that palmitate, but not oleate, is cytotoxic for syncytiotrophoblasts, and that oleate can protect syncytiotrophoblasts from palmitate-induced cell death.

Palmitate, but not oleate, induces syncytiotrophoblast apoptosis

We next sought to determine whether palmitate-induced cell death occurred via apoptosis. We found that syncytiotrophoblasts exposed to palmitate, but not to oleate, showed elevated levels of the cleaved, active form of the effector caspase, caspase 3, compared to control (Fig. 2). This activation depended on the palmitate concentration and length of exposure (Fig. 2A and B). We buttressed these results by assay of palmitate-exposed syncytiotrophoblasts for cleaved poly(ADP-ribose) polymerase (PARP) (cl-PARP), a nuclear-localized marker of caspase-mediated apoptosis, and for cleaved cytokeratin 18 (cl-CYT18), a caspase substrate in the cytoplasm. Compared to control, both markers of apoptosis showed higher levels of cleaved substrates in palmitate-treated cultures compared to control cells, in agreement with the increase of active caspase 3 (Fig. 2A and B). In contrast,
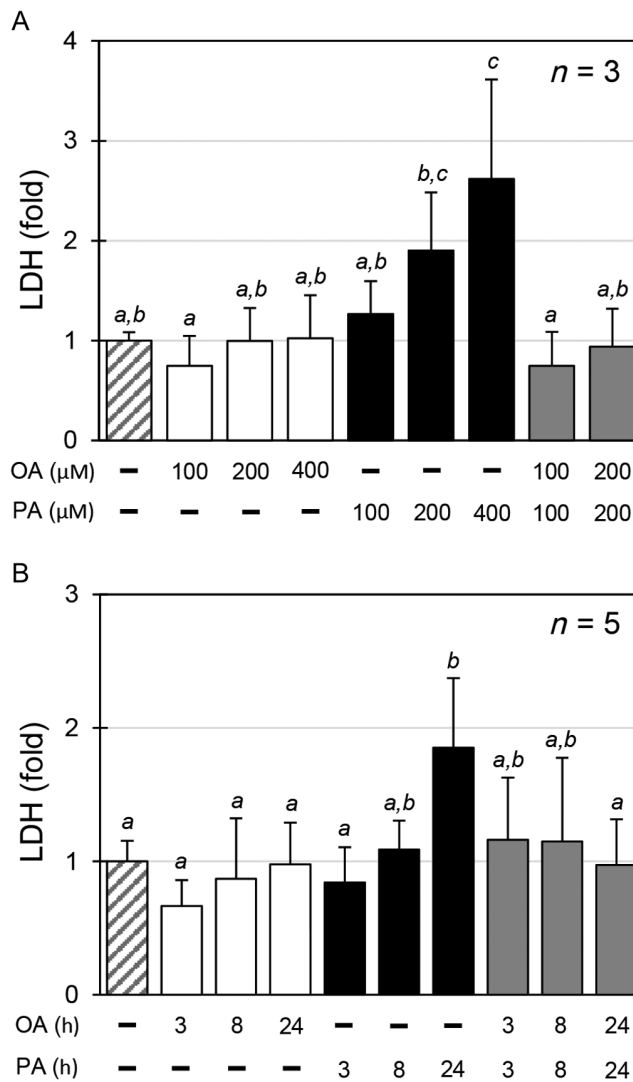

Figure 1 Palmitate, but not oleate or palmitate with oleate, increases syncytiotrophoblast LDH release. Media LDH levels were quantified as a marker of cell death and are expressed as fold change from control with no added lipid. (A) Syncytiotrophoblasts were treated with oleate or palmitate at increasing concentrations or with equimolar concentrations of both lipids, for 24 h. (B) Syncytiotrophoblasts were treated with 200 µM oleate, 200 µM palmitate or with 200 µM of each lipid, for the indicated times. Data are mean ± s.d. with the number of primary cultures indicated by *n*. Conditions with a common italicized letter do not differ as analyzed by one-way ANOVA with Bonferroni *post hoc* test (*P* < 0.05).

there was no effect of oleate compared to control for the activation of caspase 3 or on the levels of cl-PARP or cl-CYT18 (Fig. 2A and B). Moreover, co-incubation of syncytiotrophoblasts with oleate and palmitate at equimolar concentrations prevented both the activation of caspase 3 and caspase-mediated cleavage of PARP and cytokeratin 18 (Fig. 2A and B). Collectively, these data indicate that palmitate, but not oleate, induces caspase-mediated death of syncytiotrophoblasts and that oleate reduces palmitate-induced apoptosis.
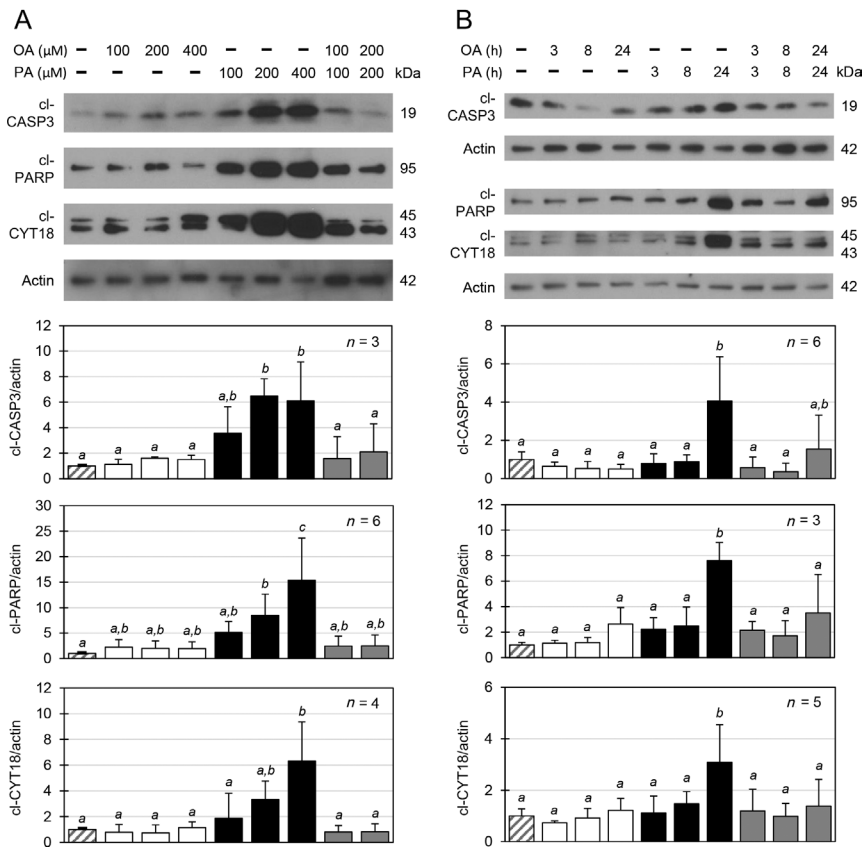
Figure 2 Palmitate, but not oleate or palmitate with oleate, increases caspase activation and apoptosis in syncytiotrophoblasts. (A and B) Top panels, representative Western blots of active (cleaved) caspase 3 (cl-CASP3), cleaved PARP (cl-PARP) and cleaved cytokeratin 18 intermediate filaments (cl-CYT18); bottom panels, quantified protein levels normalized to actin. (A) Syncytiotrophoblasts were treated with oleate or palmitate at increasing concentrations for 24 h. (B) Syncytiotrophoblasts were treated for the indicated times with 200 μM oleate, 200 μM palmitate or with 200 μM of each lipid. Data are mean ± s.d. with the number of primary cultures indicated by *n*. Conditions with a common italicized letter do not differ as analyzed by one-way ANOVA with Bonferroni *post hoc* test ($P < 0.05$).

Palmitate activates caspase-mediated apoptosis

Palmitate may induce cell death independent of caspase-mediated apoptosis (El-Assaad *et al.* 2003) or through alternative pathways (Schilling *et al.* 2013). We thus determined if caspase activity was required for palmitate-induced syncytiotrophoblast death. We found that the pan-caspase inhibitor Z-VAD-FMK prevented caspase activity in response to palmitate exposure, as indicated by reduced levels of cl-PARP (Fig. 3A) and cl-CYT18 (Fig. 3B). Z-VAD-FMK also reduced LDH release (Fig. 3C) and enhanced cell viability by MTS assay (Fig. 3D) in palmitate-exposed syncytiotrophoblasts, compared to cells exposed to palmitate in the absence of Z-VAD-FMK. Together, these data indicate that palmitate-enhanced apoptosis and cell death of syncytiotrophoblasts occurs through a caspase-mediated pathway.

Palmitate, but not oleate, increases endoplasmic reticulum stress in syncytiotrophoblasts

ER stress occurs when cellular alterations lead to accumulation of misfolded proteins in the ER. This activates a series of cellular pathways known as the unfolded protein response (UPR), which is mediated by three main stress sensors expressed at the ER membrane: PKR-like ER kinase (PERK), inositol-requiring transmembrane kinase/endonucleases

(IRE1α and β) and activating transcription factor 6 (ATF6) (Dufey *et al.* 2014). We tested the hypothesis that palmitate-induced apoptosis was mediated by ER stress in syncytiotrophoblasts. We found that palmitate-treated syncytiotrophoblasts showed higher than control mRNA and protein levels of binding immunoglobulin protein (*BiP/GRP78/HSPA5*), a chaperone for misfolded proteins in the ER (Fig. 4A and C). Notably, BiP mRNA and protein levels were not increased after exposure to either oleate or the combination of oleate and palmitate (Fig. 4A and C).

As a positive control, we exposed syncytiotrophoblasts for 24 h to tunicamycin, a well-characterized inducer of ER stress (Finnie 2001, Xu *et al.* 2005, Tabas & Ron 2011) and analyzed the expression of spliced and unspliced forms of X-box-binding protein 1 (*XBP1*). The spliced form of *XBP1* (*XBP1s*) encodes a transcription factor, and *XBP1* splicing is induced downstream of activation of IRE1α after the induction of ER stress (Dufey *et al.* 2014). As expected, and consistent with the work of others on tunicamycin-induced ER stress in trophoblasts (Yung *et al.* 2008, 2012a,b), we found that tunicamycin yielded an 9.5 ± 2.1-fold increase in *XBP1s* levels ($n = 6$, $P < 0.05$, Student's *t*-test) compared to vehicle control. Additionally, tunicamycin-exposed trophoblasts also exhibited a ~50% increase in LDH release compared to vehicle control ($n = 6$, $P < 0.05$, Student's *t*-test). Similar to tunicamycin, palmitate also
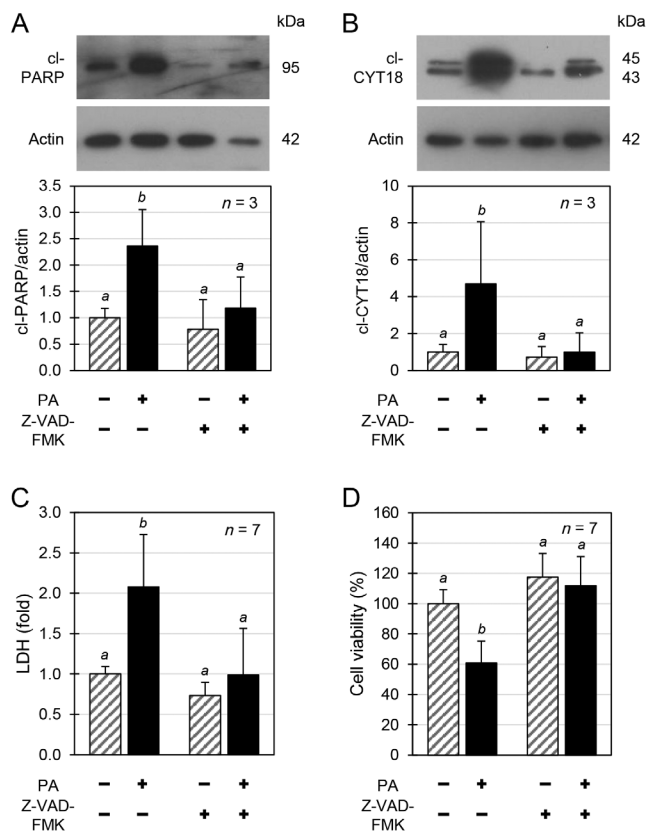
Figure 3 Palmitate-increased cell death requires caspase activity. (A, B, C and D) Syncytiotrophoblasts were treated without or with 200 μM palmitate for 24 h in the absence or presence of 20 μM Z-VAD-FMK. (A and B) Top panels, representative Western blots of cl-PARP and cl-CYT18; bottom panels, quantified protein levels, normalized to actin. (C) Media LDH levels were quantified as a marker of cell death and are expressed as fold change from vehicle control. (D) Syncytiotrophoblast viability was assessed by MTS assay and is expressed as percent viability compared to control with no added lipid and without Z-VAD FMK. Data are shown as mean ± S.D. with the number of primary cultures indicated by *n*. Conditions with a common italicized letter do not differ as analyzed by one-way ANOVA with Bonferroni *post hoc* test ($P < 0.05$).

resulted in increased *XBP1* splicing, as assayed by both qRT-PCR (Fig. 4A) and gel electrophoresis of products using primers that simultaneously amplify both *XBP1s* and *XBP1us* (Fig. 4B). In contrast, oleate did not induce *XBP1* splicing (Fig. 4A and B). Remarkably, co-exposure of cells to oleate and palmitate also did not yield increased *XBP1* splicing (Fig. 4A and B). Together, these data show that palmitate increases *XBP1* splicing by syncytiotrophoblasts (Fig. 4B), whereas oleate does not and that oleate can reduce or prevent palmitate-induced *XBP1* splicing.

We next pursued the effects of palmitate exposure on C/EBP homologous protein (*CHOP/GAD153/DDIT3*). Importantly, CHOP is a downstream target for both PERK and activating transcription factor 4 (ATF4) (Hockenbery *et al*. 1993, Dufey *et al*. 2014), and we

found CHOP mRNA and protein expression was higher than control in palmitate-treated cultures (Fig. 4A and C). CHOP links ER stress to the induction of apoptosis (Tabas & Ron 2011, Urra *et al*. 2013) via CHOP-mediated reduction in levels of the anti-apoptotic protein B-cell lymphoma 2 (BCL2) (Hockenbery *et al*. 1993) and CHOP-mediated increased mRNA expression of the pro-apoptotic p53-upregulated modulator of apoptosis (PUMA) (Urra *et al*. 2013). As would be predicted, palmitate decreased mRNA levels of *BCL2* and enhanced the levels of mRNA for *PUMA* in human syncytiotrophoblasts (Fig. 4A). Notably, we found an increased level of CHOP protein after 8 h of palmitate exposure (Fig. 4C), whereas clear induction of apoptosis required 24 h (Fig. 2B), indicating that CHOP expression precedes apoptosis induction in palmitate-exposed syncytiotrophoblasts. These results contrasted with results where syncytiotrophoblasts were exposed to oleate alone, to equimolar concentrations of oleate and palmitate, or to vehicle control, where there was no change in expression of mRNA for *XBP1s*, *XBP1us*, *CHOP*, *BCL2* or *PUMA* (Fig. 4A) or expression of protein for BiP or CHOP.

CHOP mediates palmitate-induced apoptosis

We next tested the hypothesis that CHOP mediates the induction of apoptosis by palmitate. Exposure of syncytiotrophoblasts to silencing RNA (siRNA) specific for *CHOP* (si*CHOP*) for 24 h prior to exposure to 200 μM palmitate for 24 h reduced the induction of *CHOP* mRNA level 86% compared to siCTRL (Fig. 5A). Moreover, si*CHOP* exposure under these conditions resulted in a ~65% reduction in CHOP protein level (Fig. 5B). Importantly, the reduction of CHOP reduced the expression of both markers of apoptosis, cl-PARP and cl-CYT18, by 35% and 60% respectively (Fig. 5B). Consistent with these findings, si*CHOP* also reduced LDH release in palmitate-exposed cells (Fig. 5C). Collectively, these data indicate that CHOP activation plays a significant role in palmitate-induced apoptotic cell death of placental syncytiotrophoblasts, although non-CHOP-mediated events may also be involved.

Oleate, but not palmitate, enhances lipid droplet formation in syncytiotrophoblasts

We sought to determine why oleate, unlike palmitate, did not trigger cell death in human syncytiotrophoblasts. As lipid droplet formation avoids cell toxicity (Cnop *et al*. 2001, Listenberger *et al*. 2003, Mantzaris *et al*. 2011), we investigated the induction of lipid droplet formation in response to each of the two free fatty acids. Notably, numbers (Fig. 6A and C) and size (data not shown) of lipid droplets increased in trophoblasts exposed to increasing
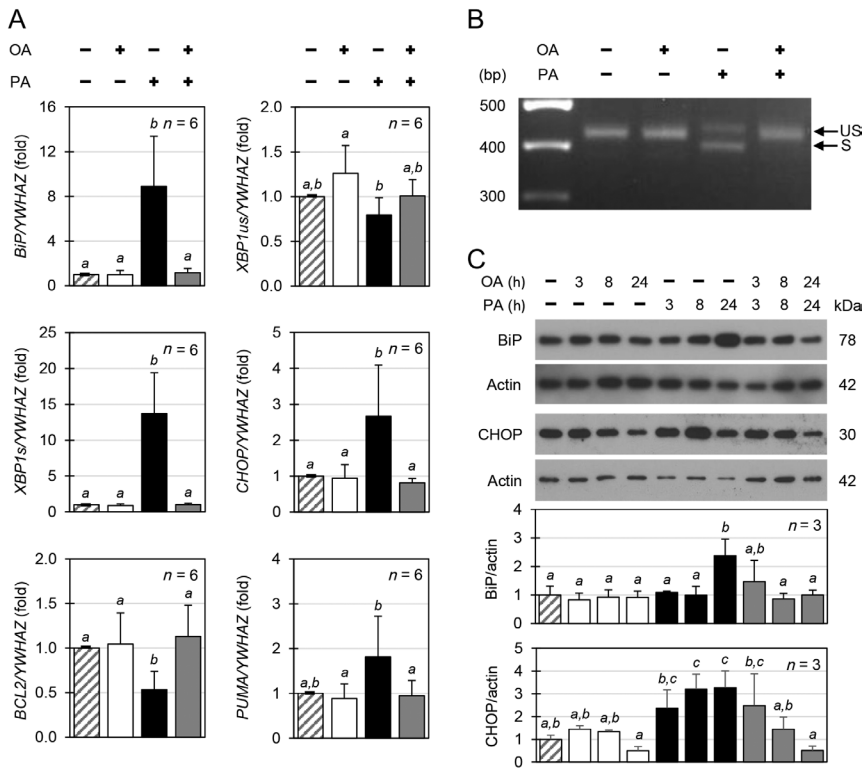
Figure 4 Palmitate, but not oleate or palmitate with oleate, induces ER stress in syncytiotrophoblasts. (A and B) Syncytiotrophoblasts were treated with 200 μM oleate, palmitate or 200 μM of each lipid for 24 h. (A) Quantitative RT-PCR was performed and expression was normalized to *YWHAZ* as a stable reference gene. (B) Agarose gel electrophoresis of quantitative real-time PCR (qRT-PCR) products of *XBP1* splice forms after treatment with vehicle control or the indicated lipids. (C) Syncytiotrophoblasts were treated with 200 μM oleate, palmitate or 200 μM each for the indicated times. Top panels, representative Western blots of binding immunoglobulin protein (BiP) and C/EBP homologous protein (CHOP); bottom panels, quantified protein levels normalized to actin. Data are mean ± s.d. with the number of primary cultures indicated by *n*. Conditions with a common italicized letter do not differ as analyzed by one-way ANOVA with Bonferroni *post hoc* test ($P < 0.05$).

concentrations of oleate, with a 17-fold increase in numbers after exposure to 400 μM, compared to control (Fig. 6A and C). As expected, lipid droplet formation was time dependent, detectable after 3 h of incubation with oleate and increased further with additional time of exposure (Fig. 6B and D). In contrast, palmitate neither increased nor decreased lipid droplet number (Fig. 6A, B, C and D) or diameter (data not shown) under any condition assayed, compared to vehicle control. Moreover, cells exposed to equimolar levels of oleate and palmitate yielded lipid droplet formation in a dose- and time-dependent manner (Fig. 6A, B, C and D). These data indicate that lipid droplet formation in syncytiotrophoblasts is induced by oleate, but not palmitate.

Discussion

The data show that palmitate is lipotoxic for syncytiotrophoblasts and induces apoptosis in these unique cells through a caspase-mediated pathway involving ER stress, unfolded protein response and increased expression of the transcription factor CHOP. Our data also show that oleate is not lipotoxic, either alone or in combination with palmitate, and this unsaturated fatty acid provides protection from lipotoxicity by formation of lipid droplets in syncytiotrophoblasts.

Our results indicate that palmitate-mediated induction of prolonged ER stress induces apoptosis of trophoblasts, in agreement with the findings in other systems (Miller *et al.* 2005, Karaskov *et al.* 2006,

Wei *et al.* 2006, Lai *et al.* 2008). However, mechanisms in addition to, or instead of, elevated ER stress have been proposed for palmitate-induced lipotoxicity in non-trophoblast systems, including ceramide synthesis and formation of mitochondrial reactive oxygen species (Yuzefovych *et al.* 2010) and alterations in calcium homeostasis (Egnatchik *et al.* 2014). We recognize that ER stress is likely not the sole mechanism of palmitate-mediated lipotoxicity of syncytiotrophoblasts. Further work will be required to investigate these important issues.

Also, as an area for future investigation, it will be of interest to determine whether non-stoichiometric (sub-equimolar) levels of oleate may be able to reduce saturated fat-induced lipotoxicity in trophoblasts, as has been found in some other systems (Mantzaris *et al.* 2011, Leamy *et al.* 2014). The ratios of saturated vs unsaturated fatty acids on *in vitro* and *in vivo* lipotoxicity also merit further investigation.

In this study, we examined the effects of one saturated (palmitate) and one unsaturated (oleate) fatty acid on trophoblast viability. These were chosen because they are the most prevalent fatty acids present in the circulation and due to the need to keep the system experimentally tractable. However, the *in vivo* situation is clearly more complex, with multiple types of fatty acids being present. Several previous studies (Akazawa *et al.* 2010, Thorn & Bergsten 2010, Mantzaris *et al.* 2011, Nemcova-Furstova *et al.* 2011, Kwon *et al.* 2014) that investigated different dietary fats have found that the other prevalent dietary saturated fat, stearate and several dietary
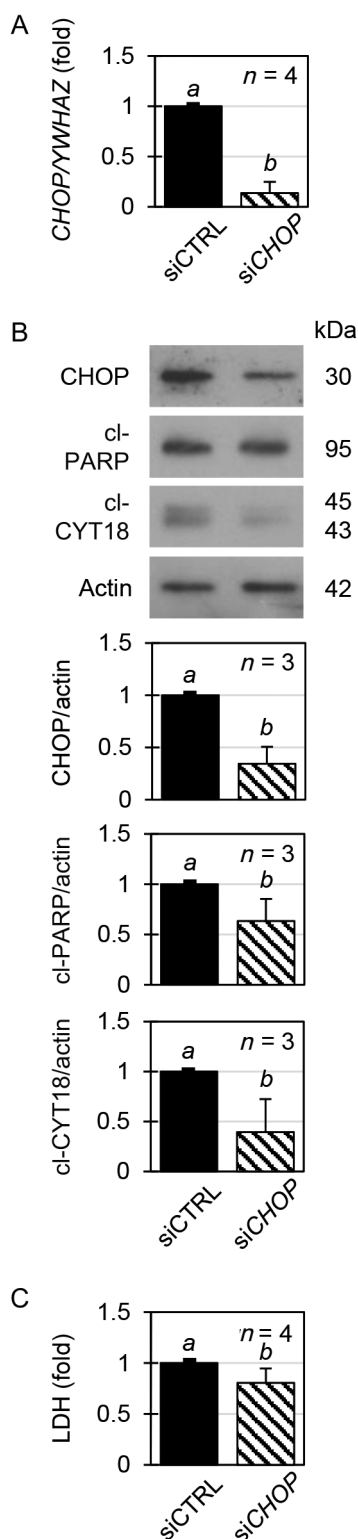
unsaturated fats, including linoleate, palmitoleate, eicosapentaenoic acid and docosahexaenoic acid, have lipotoxic or lipoprotective effects respectively, similar to what was observed in our study. Thus, it is important to note that the ratio of saturated to unsaturated serum fatty acids, rather than just the total levels of fatty acids, may be a very important in regard to lipotoxicity. Our work suggests that detailed evaluation of the relative serum levels and ratios of saturated vs unsaturated fatty acids in lean vs obese pregnant women would be worthwhile.

Our data also show that oleate, with or without the addition of palmitate, increased lipid droplet formation in syncytiotrophoblasts. We speculate that this offers a potential mechanism for the cytoprotective effect of oleate against palmitate-induced lipotoxicity. Several other studies of how unsaturated fats protect against saturated fat lipotoxicity have suggested several mechanisms (Guo *et al.* 2007, Kwon *et al.* 2014, Palomer *et al.* 2014), including an increase in neutral lipid formation in the form of triglycerides (Listenberger *et al.* 2003) via induction of the enzyme diacylglycerol acyltransferase (Soumura *et al.* 2010, Thorn & Bergsten 2010), thus preventing formation of compounds such as diacylglycerol, which can cause cellular damage through the induction of ER stress. Indeed, although we have shown several downstream markers of ER stress, including XBP1, BiP and CHOP, we have not investigated upstream markers, such as ATF6 cleavage, PERK phosphorylation or eukaryotic initiation factor 2 alpha (eIF2α), which merit investigation of future work. We do note, however, that there is a significant amount of overlap in the downstream targets of all three UPR pathways (Tabas & Ron 2011, Hetz 2012, Urra *et al.* 2013).

Fatty acids are obviously important for fetal development, as they provide a framework for all cell membranes and they are indispensable for most organ development, especially the brain. Notably, maternal blood oleate and palmitate constitute more than half of the nonesterified free fatty acids (FFA) in the maternal circulation (Villa *et al.* 2009). These two FFA differentially regulate trophoblast amino acid transport. Oleic acid stimulates system A amino acid transport through toll-like receptor 4 (Lager *et al.* 2013), and oleic acid increases the phosphorylation of multiple pathways to affect placental function, including ERK, mTOR, S6 kinase 1 and rpS6 signaling (Lager *et al.* 2014). Interestingly, palmitic acid does not affect amino acid transport but does reduce IκBα, predisposing to the inflammatory state typical of obese pregnant women

Figure 5 CHOP mediates palmitate-induced apoptosis. (A, B and C) Syncytiotrophoblasts were treated with 50 nM of siCTRL or si*CHOP* for 24 h then with 200 µM palmitate for 24 h. (A) Quantitative RT-PCR was performed and expression was normalized to *YWHAZ* as a stable reference gene. (B) Top panels, representative Western blots of CHOP, cl-PARP and cl-CYT18; bottom panels, quantified protein levels normalized to actin. (C) Media LDH levels were quantified as another marker of cell death and are expressed as fold change from control with siCTRL. Data are mean ± S.D. with the number of primary cultures indicated by *n*. Conditions with a common italicized letter do not differ as analyzed by Student's *t*-test ($P < 0.05$).
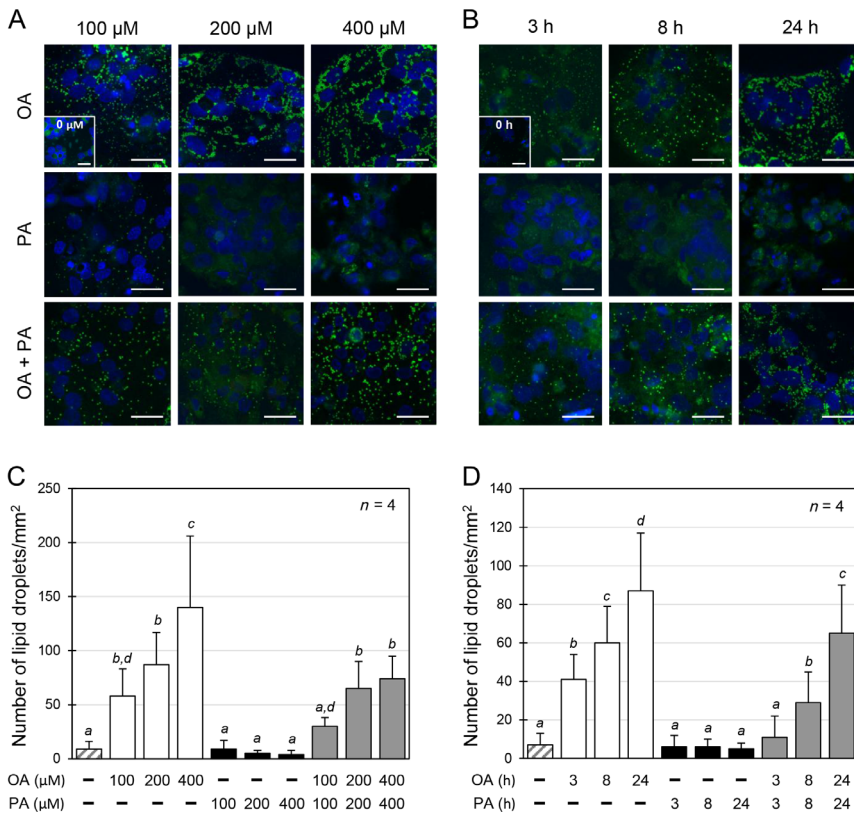
Figure 6 Free fatty acids affect lipid droplet formation. (A) Syncytiotrophoblasts were treated with oleate or palmitate at increasing concentrations or with equimolar concentrations of both lipids for 24 h. (B) Syncytiotrophoblasts were treated with 200 µM oleate, palmitate or 200 µM each for the indicated times. (A and B) Lipid droplets were stained with BODIPY and DNA was stained with TOPRO3. Inset figure in top left image is control condition with no added lipid. Scale bar is 25 µm. (C and D) Quantified lipid droplets are expressed as number of lipid droplets per unit area of syncytiotrophoblast. Data are mean ± s.d. with the number of primary cultures indicated by *n*. Conditions with a common italicized letter do not differ as analyzed by one-way ANOVA with Bonferroni *post hoc* test ($P < 0.05$).

(Lager *et al.* 2014). Collectively, the data show that oleate and palmitate, as prominent dietary FFA, have divergent effects on the trophoblast when considered alone, yet the ratio of these FFA is likely the ultimate determinant of the effect on trophoblast function *in vivo*.

Palmitate, but not oleate, is also cytotoxic in several non-placental cell types (Cnop *et al.* 2001, Listenberger *et al.* 2001, Miller *et al.* 2005, Ricchi *et al.* 2009), but the effect of these lipids on the viability of human syncytiotrophoblasts has not been previously investigated. The question of lipotoxicity is physiologically relevant as pregnant, obese women have elevated serum levels of FFA (Chen *et al.* 2010*b*), and maternal blood exposes syncytiotrophoblasts to ambient FFA levels. Importantly, obesity by itself is a risk factor for placental dysfunction that increases the risk for stillbirth (Aune *et al.* 2014); yet, no placental histopathology is attributed to the phenotype of obesity, independent of other disease states. Our study suggests that the effects of excess palmitate ingestion could enhance damage to villous trophoblasts *in vivo* and, if unrepaired, villous trophoblast transport functions could suffer and not be able to sustain the needs of the fetus. This speculation offers a testable hypothesis that would predict an increase in expression of CHOP in placentas of obese patients that had adverse outcomes. On the other hand, oleate induction of fat storage in lipid droplets may avoid trophoblast lipotoxicity but excess nutrients may support fetal overgrowth with the

accompanying risks of this condition. Importantly, we assayed trophoblasts for the effects of levels of fatty acids that are clearly within the range present in human serum. The 100 µM palmitate, or oleate, approximates those found in lean, healthy pregnant women, whereas 200–400 µM of these lipids are levels present in some obese pregnant individuals (Villa *et al.* 2009, Chen *et al.* 2010*b*). Clearly, the presence or absence of lipotoxicity *in vivo* will be modulated not only by the ratios of FFA but will be influenced by the other substrates present *in vivo*. For example, the toxic effects of saturated fats are exacerbated by exposure to elevated levels of glucose (El-Assaad *et al.* 2003, Prause *et al.* 2014).

We note that although it is possible that obesity is involved in ER-mediated lipotoxicity, it is unlikely the question is this simple. Notably, the effects of obesity on placental histopathology and function are poorly studied. One study compared placentas from women with high vs normal BMI (Higgins *et al.* 2013) found a modest, but significant, correlation of increased BMI with decreased trophoblast apoptosis. Moreover, Yung and coworkers (Yung *et al.* 2016) found no increase in placental ER stress in overweight women. Thus, the effects of obesity on placental ER stress, trophoblast apoptosis and placental function are as yet ill-defined and require further study. However, our data suggest that the most important factor in ER stress-mediated placental dysfunction may not be obesity *per se* but rather the ratios of saturated and unsaturated fatty acids.

Thus, in future studies, although stratification by BMI may be useful, it may be even more important to stratify women also to the levels, types and (perhaps most importantly) to the relative ratios of circulating saturated and unsaturated fatty acids.

Brass and coworkers (Brass *et al.* 2013) showed that uptake of oleic acid was lower in villi of male offspring, but higher in female offspring, of obese women, compared to lean women. This result raises a limitation of our study, as we did not plan experiments to identify sex effects of FFA exposure of trophoblasts. We thus cannot state whether or not differential oleate uptake is influenced by sex of the trophoblasts studied. A second limitation is that we investigated the effect of elevated fatty acids on *in vitro* cultured primary syncytiotrophoblasts. Although we believe our model has the benefit of using primary cells, we have not shown any direct effects on human placental villi *in situ*. Notably, there is not yet a clear placental histopathologic correlate identified for women with obesity, although recent studies have investigated this question (Roberts *et al.* 2011, Huang *et al.* 2014, Kovo *et al.* 2015). What these studies reveal is the presence of an increase in frequency of maternal placental vascular lesions in placentas from obese women, compared to lean controls. Unfortunately, vascular lesions in the basal plate is a non-specific marker. Our study has identified potential molecular and cellular targets for comparing the histopathology of placentas from obese and lean pregnancies. Specifically, we propose villi from obese vs lean women should be characterized for differences in markers for apoptosis (cl-PARP and cl-CYT18), ER stress (CHOP expression) and intracellular lipid accumulation (lipid droplet assessment). Such investigations may yield a more specific pathological correlate for signs of placental compromise in pregnancies of obese women.

Declaration of interest

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