Impaired mitochondrial function in human placenta with increased maternal adiposity

James Mele,* Srihalasubashini Muralimanoharan,* Alina Maloyan, and Leslie Myatt

Center for Pregnancy and Newborn Research, Department of Ob/Gyn, University of Texas Health Science Center San Antonio, San Antonio, Texas

Submitted 17 January 2014; accepted in final form 11 July 2014


The rate of maternal obesity continues to increase, with up to 60% of the pregnant population in the US being overweight and 25% being obese (BMI >30) (13, 15). Maternal obesity is associated with several adverse perinatal outcomes, including hypertensive disorders, gestational diabetes, fetal macrosomia, and perinatal death. Furthermore, the male fetus is at greater risk for an adverse outcome than the female fetus (10), with a growing body of evidence showing sexual dimorphism in placental function, including basal placental gene expression (53), and changes in gene expression in response to maternal inflammatory status (7, 41, 43) or change in maternal diet (29).

Maternal obesity also programs the offspring for disease in later life, including obesity, cardiovascular disease, metabolic syndrome, and diabetes (2, 6, 12). Much of the evidence supporting a fetal programming effect of obesity in humans is based on epidemiological data, and it is only recently that attempts have been made to identify molecular mechanisms underlying programming effects. The placenta functions as a key regulator of fetal growth by facilitating nutrient supply to and waste removal from the fetus, with alterations in placental function having the ability to mediate fetal programming (9, 64). Mitochondrial oxidative phosphorylation is a key energy source for placental function (3); however, the changes in placental mitochondrial function with various pregnancy complications (e.g., diabetes and obesity) remain understudied.

Mitochondrial dysfunction associated with obesity has been studied predominantly in highly metabolic tissues, including adipose, heart, liver, and skeletal muscle. An increase in reactive oxygen species (ROS) and reduction in the oxidative capacity of brown adipocytes results in impaired thermogenesis and has been linked to diet-induced obesity (11). Wilson-Fritch et al. (63) demonstrated downregulation of approximately 50% of gene transcripts encoding mitochondrial proteins in adipose tissue in a rodent model with the onset of obesity. Several studies have shown that the increasing metabolic activity of placental mitochondria results in excessive production of ROS leading to oxidative stress, which may be exaggerated in pregnancies complicated by preeclampsia, intrauterine growth restriction (IUGR), and maternal obesity (24, 27, 59, 61, 62). However, besides being a major source of ROS and oxidative stress, mitochondria also appear to be highly susceptible to ROS attack (57). Proteins, lipids, and nucleic acids can be altered by ROS, resulting in covalent changes that affect mitochondrial structure and function (52). Thus, mitochondrial abnormalities and ROS formation could be part of a vicious cycle and represent a central mechanism of placental dysfunction in disease states.

In this study, we addressed the hypothesis that increasing maternal adiposity and fetal sex difference affect mitochondrial function in human placenta.

MATERIALS AND METHODS

Ethical Approval and Study Participants

Placentae were collected from the Labor and Delivery Unit at University Hospital San Antonio under a protocol approved by the Institutional Review Board of the University of Texas Health Science Center San Antonio, with informed consent from the patients.

Materials

Oligomycin, FCCP [4-(trifluoromethoxy) phenylhydrazone], rotenone, and antimycin A were obtained from Sigma and dissolved in DMSO as 2.5-mM stock solutions. The human oxidative phosphorylation (OXPHOS) antibody cocktail (MS601) was purchased from Abcam.
Collection of Placental Tissue

Placentae were collected immediately following delivery by cesarean section at term without labor from otherwise uncomplicated pregnancies in women with a range of prepregnancy BMI from 18.5 to 44.7, grouped as lean (LN; BMI 18.5–24.9), overweight (OW; BMI 25–29.9), and obese (OB; BMI >30). Villous tissue was randomly sampled from five sites in the placenta, as described previously (31), flash-frozen in liquid nitrogen, and stored at −80°C.

Visualization and Quantification of ROS Generation and Hydrogen Peroxide Content in Placental Villous Tissue

Flash-frozen villous tissue sections (7 μm) from six placentas in each group were incubated with 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen) for 30 min at 37°C, and staining was performed as described (33). Dichlorodihydrofluorescein (DCF) staining was quantified using ImageJ (National Institutes of Health). Hydrogen peroxide levels were assayed using the Amplex Red kit (Invitrogen) in whole cell lysates from placental villous extracts according to the manufacturers’ protocol.

ATP Levels

ATP levels were determined in villous tissue lysates from placenta of male and female fetuses (n = 6 for each sex/BMI group) using the Enliten ATP assay (Promega) according to the manufacturer’s protocol.

Western Blots

Mitochondrial fractions for Western blotting were obtained from villous tissue using a standard differential centrifugation protocol (40) and suspended in isolation medium (0.25 M sucrose and 1 mM EDTA, pH 7.4) supplemented with a protease and phosphatase inhibitor cocktail (Sigma). Total protein concentrations in the fraction were determined using Bradford’s reagent (Bio-Rad). Mitochondrial fraction proteins (10 μg) were separated on 4–20% precast linear gradient gels (Bio-Rad), transferred to nitrocellulose membranes, and blocked with 5% (wt/vol) nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h. Membranes were incubated overnight at 4°C with primary antibody diluted in primary antibody diluted in 1% nonfat milk (wt/vol) in TBST and detected using an appropriate peroxidase-conjugated secondary antibody. Products were visualized by ECL chemiluminescence (Millipore). Band intensities were measured using the G-box system (Syngene).

Mitochondrial Biogenesis

Mitochondrial DNA copy number. Total genomic DNA was isolated from villous tissue from placenta of males and female fetuses (n = 6 from each sex/maternity BMI group) using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Mitochondrial DNA copy number was determined using real-time PCR,
using primers for mitochondrial 16S rRNA and nuclear β2-microglobulin as described (60).

Citrate synthase activity. The activity was measured in the villous tissue using a commercial kit from Sigma (CS0720–1KT) according to the manufacturer’s protocol. Placental tissue was homogenized using lysis reagent (C3228; Sigma).

Isolation and Culture of Primary Trophoblasts

Villous cytotrophoblasts (CTs) were isolated from placental tissue of women with a range of adiposity using trypsin/DNAse digestion and the Percoll gradient purification method, as we have described previously (28). CT cells were plated in Seahorse XF24 plates, cultured in the DMEM containing glucose (17 mM), and allowed to syncytialize for a period of 72 h. For the last 24 h, medium was switched to contain either 17 mM glucose or galactose (10 mM). The choice of 10 mM galactose was based on previously published studies on other cell lines (30, 48).

Assessment of Mitochondrial Function

Mitochondrial function of the cultured syncytiotrophoblasts was measured using a Seahorse XF24 analyzer (Seahorse Biosciences) as described (28). Oxygen consumption rates (OCR) were normalized to total cellular protein (Bradford method). Basal respiration was calculated from four baseline OCR readings. ATP coupled, maximum respiration, spare capacity, proton leak, and nonmitochondrial respiration was calculated from OCR readings following the injection of oligomycin (1 μM), FCCP (1 μM), and a mixture of rotenone (3 μM) and antimycin A (1.5 μM).

Statistical Analysis

Data are reported as means ± SE. Comparisons between two groups were performed using Student’s t-test. One or two-way analysis of variance (ANOVA) and Bonferroni post hoc test were used to compare data sets with more than two groups. *P < 0.05 was considered significant. The OCR parameters were analyzed by regression and correlation analysis against maternal BMI using Excel and GraphPad (version 5.0).

RESULTS

Clinical Characteristics of Study Patients

There were no significant differences in maternal or gestational age at delivery and placental weight between the women of differing BMI (Table 1). Maternal weight gain was significantly lower in OB women with female fetuses (P < 0.05). Most births were to Hispanic women (86.6%), followed by white (8%), African-American (4%) women, and Asian women (1.3%). By experimental design, maternal BMI differed significantly between the chosen groups (1-way ANOVA and Bonferroni analysis). In the LN group, the birth weights of female fetuses were significantly smaller than male fetuses (P < 0.05). In addition, female fetuses from OB mothers were significantly heavier compared with female fetuses from LN mothers (P < 0.05).

Production of ROS Production in Villous Tissue

Production of ROS assessed by DCF staining was six- and 14-fold higher (P < 0.05) in placental villous tissue from fetuses of OW and OB women, respectively, compared with LN women (Fig. 1A and B). Since DCF reacts with a wide variety of ROS, we separated the samples by fetal sex and measured the level of hydrogen peroxide using the Amplex Red probe. Horseradish peroxidase-catalyzed resorufin fluorescence, an indicator of the presence of hydrogen peroxide, was increased significantly (P < 0.05) in placentas of male and female fetuses from both OW and OB women compared with lean women (Fig. 1C).

Effects of Maternal BMI on Placental ATP Levels

Since mitochondria are the main sources of ROS, we subsequently investigated whether an increase in production of
ROS is associated with mitochondrial dysfunction. We initially evaluated mitochondrial function by measuring the levels of ATP in placental tissue (Fig. 2A). The ATP content was twofold higher (P < 0.05) in the LN female placentas compared with males. However, a significant reduction in ATP levels was seen with increasing adiposity in placentas of both males and females.

**Effect of maternal adiposity on mitochondrial biogenesis.** To explore the mechanism that may account for the decline in placental ATP levels with increasing adiposity, we utilized a RT-PCR approach to measure changes in mitochondrial biogenesis (Fig. 2, B and C). Mitochondrial content estimated by citrate synthase activity (23) showed a significant reduction in male and female placentas of OB but not OW mothers (P < 0.05) compared with LN. However, mitochondrial DNA copy number was slightly but significantly decreased only in placentas of male fetuses from the OW group, whereas in OB mothers, placentas from both males and females showed a significant reduction in mitochondrial biogenesis (P < 0.05).

**Effect of maternal adiposity on expression of electron transport chain complexes.** To determine whether changes in placental ATP levels are related to altered protein expression of the mitochondrial electron transport complexes, we performed Western blotting on mitochondrial fractions using an antibody cocktail, recognizing epitopes of purified subunits of complexes I (NDUFB8), II (SDHB), III (UQCRC2), IV (MTCOX1), and V (ATP5a) (Fig. 3A). There were no fetal sex-dependent changes in expression of any of the five mitochondrial complexes in LN, OW, or OB women. However, the expression levels of subunits of complexes I–V placentas were significantly reduced (P < 0.05) in placentas of males and females from OW and OB women compared with LN women (Fig. 3, B–F).

**Placental Mitochondrial Energetics in Syncytiotrophoblast Culture**

We next determined the effects of increasing maternal adiposity on mitochondrial function in vitro using cultured syncytiotrophoblasts (Fig. 4). Linear regression and correlation analysis revealed a significant decrease in basal respiration, ATP-coupled respiration, maximum respiration, spare capacity, and nonmitochondrial respiration with increasing maternal adiposity (Fig. 4). Proton leak was unaffected by increasing maternal BMI (Fig. 4). No statistically significant differences were observed between primary trophoblast cultures from male or female fetuses; therefore, all data are combined.

**Metabolic Flexibility of Syncytiotrophoblasts is Compromised with Maternal Adiposity**

Culturing cells in galactose as the sole sugar source forces mammalian cells to rely on OXPHOS and is a strategy used previously to diagnose human mitochondrial disorders (47). Syncytiotrophoblasts from LN women cultured in galactose appeared to be metabolically flexible and demonstrated a twofold increase in basal mitochondrial oxygen consumption and ATP-coupled respiration rates compared with LN cells cultured in glucose (P < 0.05; Fig. 5A). Maximal respiration in these cells remained unchanged, leading to a 50% reduction in spare capacity. In contrast, syncytiotrophoblasts derived from OW and OB women, where respiratory parameters were already reduced, failed to increase their aerobic respiration when cultured in galactose-containing medium (Fig. 5, B and C), suggesting that they were compromised in oxidative metabolism. No differences were observed between syncytiotrophoblasts isolated from male and female placentas.

**DISCUSSION**

The placenta is an extremely metabolically active fetal tissue that produces large amounts of peptides and steroid hormones that both influence the mother’s metabolism to supply substrates to the fetus and also regulate fetal growth and development (65). Despite the utility of animal models (e.g., overnutrition) in examining the impact of maternal obesity on placental function (5), there is a paucity of data on the effect of maternal obesity on mitochondrial function in the human placenta. Reduction in the oxidative enzyme activity and the expression of mitochondrial respiratory complexes in skeletal muscle is reported in obese men and women (21) and as a risk factor for weight gain (56). Reduced expression of mitochondrial subunits was also found in white adipose tissue in response to genetic and nutritional obesity (8), short-term high-fat feeding (54), and type 2 diabetes (32, 42). We hypothesized that placental mitochondrial function would be compromised in response to increasing maternal adiposity.

**Fig. 3. Expression of placental mitochondrial complexes with increasing maternal adiposity.** A: representative Western blots for males (m) and females (f) are shown. B–F: quantification of expression of peptides from mitochondrial complexes I (CI), II (CII), III (CIII), IV (CIV), and V (CV) in placental mitochondrial fractions. Voltage-dependent anion channel (VDAC) was used as loading control. *P < 0.05 vs. LN group; n = 6/sex/group.
Mitochondria are the main source of endogenous ROS in most mammalian cell types (26). Of the oxygen consumed by mitochondria, up to 5% is converted to ROS as byproducts of oxidation-reduction reactions in the respiratory chain. Excessive ROS in the placenta is thought to play a central role in the pathogenesis of preeclampsia and IUGR (37). Our data confirm that maternal obesity creates a state of increased oxidative stress within the placenta, suggesting that mitochondria may be functionally impaired with increasing maternal adiposity.

Sex-specific adaptation of the placenta may be central to the differences in fetal growth and survival. Male fetuses reportedly try to maximize growth in utero, a strategy that places them at risk in an adverse environment and may lead to increased incidence of adverse perinatal outcomes, including preterm birth, placenta previa, and premature lung development; in contrast, females were shown to be more sensitive to maternal asthma than males (35, 36, 55). Females may adapt to the adverse intrauterine environment in an attempt to survive further maternal insults and ensure survival. Differences in placental cytokine expression, insulin-like growth factor pathways, and the placental response to cortisol in relation to an adverse maternal condition (asthma) during pregnancy may regulate the sexual dimorphic survival responses (17, 53). Recently, we have demonstrated a sexual dimorphism in proinflammatory cytokine production and apoptosis in the placentas from pregnancies complicated by preeclampsia (34). We also found an increase in the expression and DNA binding activity of NF-κB transcription factor in the preeclamptic placentas compared with normotensive placentas with much higher levels in placentas of males compared with females (34). The sexual dimorphic responses in placental mitochondrial energetics and function are not well defined. However, in other tissues (e.g., liver, heart, and astrocytes), differences in mitochondrial dynamics (i.e., fission and fusion), energy metabolism, biogenesis, and regulation of complex subunits by phosphorylation were postulated to be regulated by sex hormones (19, 22, 49). Further studies are required to understand the mechanism(s) underlying the sexual dimorphism in placental metabolism.

Measurement of ATP content in villous tissue revealed a sexually dimorphic response. In LN women, placentas of female fetuses showed significantly higher ATP content compared with male fetuses. However, as maternal adiposity increased, female and male placentas from OW and OB women failed to maintain ATP levels, indicating a mitochondrial dysfunction. Interestingly, a decrease in mitochondrial DNA

---

**Fig. 4.** Effect of increasing maternal BMI on mitochondrial respiration. Mitochondrial respiratory parameters were measured in syncytiotrophoblast cultures from women of a range of adiposity. Data were fitted using linear regression analysis (solid line). The coefficient of determination (r²) and P values are shown. Correlation analysis yielded significant correlations for basal respiration, ATP-coupled respiration, maximum respiration, spare capacity, and nonmitochondrial respiration with maternal BMI. The correlation of proton leak and maternal BMI was not significant. Oxygen consumption rate (OCR) measurements are normalized to total cellular protein content (pmol O₂/µg protein); n = 33 separate cultures from placentas of females (△) and males (■).

**Fig. 5.** Impairment of metabolic adaptation and reliance on mitochondrial energy generation with increasing maternal adiposity. Cytotrophoblasts were isolated from placentae of males and females from LN, OW, and OB women and allowed to syncytialize for 72 h. The medium was changed in the final 24 h to contain glucose (17 mM) or galactose (10 mM) as a carbohydrate source, facilitating the reliance on either glycolysis and mitochondrial or exclusively mitochondrial respiration, respectively. Mitochondrial function was determined, and the OCR parameters were calculated. *P < 0.05 vs. glucose; n = 8/BMI group. Basal, basal respiration; ATP, ATP-coupled respiration; Max, maximal respiration; Spare, spare capacity.
copy number was also fetal sex dependent, whereas citrate synthase activity, a marker of mitochondrial content, was reduced in placentas of both males and females from OB but not OW mothers. Mitochondrial “deficiencies” in the settings of obesity have been observed previously in the adipose tissue and skeletal muscle of obese rodents (39, 54, 58) and in the skeletal muscle of obese individuals (16, 45, 46).

To determine what affects ATP production in the placenta with maternal obesity, the expression of subunits encoding the complexes of electron transport chain was measured. All five mitochondrial complexes showed a tendency to decrease with increased adiposity, and there were no differences between male and female fetuses.

To further address the effect of maternal obesity on trophoblast respiration, we utilized an in vitro model of syncytiotrophoblast culture. Importantly, our data suggest that isolated trophoblasts can retain their in vivo phenotype in culture. Consistent with studies in other tissues (20), we found a decrease in mitochondrial function with increasing adiposity. The reduction in maximum respiration and spare respiratory capacity indicates that syncytiotrophoblasts from placentae of OW and OB women have an impaired cellular ability to meet energetic needs. In other cell types, such a reduction in energetic capacity renders them more susceptible to stressors (4). Similar findings of depressed mitochondrial oxygen consumption and decreased electron transport complex subunit mRNA expression have been observed in primary neurons of rodent models and skeletal muscle biopsies from obese and diabetic patients (20).

Cell culture medium containing galactose is often used to study the effect of mitochondrial toxins (14) and has been used to examine mitochondrial dysfunction in primary myotubes derived from diabetic patients (1). We found that galactose was able to increase the oxidative metabolism of primary trophoblasts derived from LN women. In contrast, trophoblasts derived from OW and OB patients were not able to increase their oxygen consumption rate when cultured in galactose, suggesting a reduction in metabolic flexibility of trophoblasts in response to maternal obesity.

Maternal obesity creates a unique in utero environment characterized by a failure to adequately store excess fatty acids, resulting in a chronic elevation of circulating fatty acids that can become cytotoxic (lipotoxicity) (18, 44, 50). Previously, free fatty acids were shown to affect mitochondrial respiration by increasing the production of ROS (51) and mitochondrial proton conductance (uncoupling) (38). Therefore, it is not surprising that mitochondrial abnormalities have been observed in the placentas of obese and overweight women. The functional consequences of mitochondrial deficiency and metabolic inflexibility on placental and fetal health remain to be clarified, but work is underway to study this important phenomenon. We speculate that mitochondrial dysfunction and decreased ATP content will lead to abnormal placental function contributing to perinatal complications and programming for metabolic disease in later life. Previously, it has been proposed that placental mitochondrial dysfunction may be critical in fetal programming of atherosclerosis (25).

**REFERENCES**

18. Jarvis E, Hauguel-de-Mouzon S, Nelson SM, Sattar N, Catalano PM, Freeman DJ. Lipotoxicity in obese pregnancy and its potential role in


