Fatty acids alter glycerolipid metabolism and induce lipid droplet formation, syncytialisation and cytokine production in human trophoblasts with minimal glucose effect or interaction

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ABSTRACT

The diabetic pregnancy is characterized by maternal hyperglycaemia and dyslipidaemia, such that placental trophoblast cells are exposed to both. The objective was to determine the effects of hyperglycaemia, elevated non-esterified fatty acids (NEFA) and their interactions on trophoblast cell metabolism and function.

Trophoblasts were isolated from normal term human placentas and established in culture for 16 h prior to experiments. Glucose utilisation, fatty acid oxidation and fatty acid esterification were determined using radiolabelled metabolic tracer methodology at various glucose and NEFA concentrations. Trophoblast lipid droplet formation including adipophilin mRNA expression, viability, apoptosis, syncytialisation, secretion of hormones and pro-inflammatory cytokines were also assessed.

Glucose utilisation via glycolysis was near maximal at the low physiological glucose concentration of 4 mM; whereas NEFA esterification into triacylglycerol and diacylglycerol increased linearly with increasing NEFA concentrations without evidence of plateau. Culture of trophoblasts in 0.25 mM NEFA for 24 h upregulated fatty acid esterification processes, inhibited fatty acid oxidation, inhibited glycerol release (a marker of lipolysis) and promoted adipophilin and lipid droplet formation, all consistent with upregulation of fatty acid storage and buffering capacity. NEFA also promoted trophoblast syncytialisation and TNF α , IL-1 β , IL-6 and IL-10 production without effects on cell viability, apoptosis or hormone secretion. Hyperglycaemia caused intracellular glycogen accumulation and reduced lipid droplet formation, but had no other effects on trophoblast metabolism or function.

NEFA have effects on trophoblast metabolism and function, mostly independent of glucose, that may have protective as well as pathophysiological roles in pregnancies complicated by diabetes and/or obesity.

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1. Introduction

The rapid increasing prevalences of overweight, obesity, gestational diabetes and type 2 diabetes are adversely affecting women of childbearing age and their pregnancy outcomes. In a recent study, obese pregnant women had significantly increased rates of hypertensive disorders of pregnancy, prolonged hospital stays,

caesarean section, neonates with birth defects and neonates with hypoglycaemia [1]. From the hyperglycaemia and adverse pregnancy outcomes study, it is evident that even mild degrees of hyperglycaemia are associated with greater risk of large for gestational age (LGA) babies, clinical neonatal hypoglycaemia and raised cord blood C-peptide [2]. Of major concern are the very high rates of serious pregnancy complications, such as perinatal death and congenital malformations, occurring in pregnancies of women with type 2 diabetes [3–5]. While multiple factors are likely to be involved, the dyslipidaemia very often associated with these high-risk pregnancies may be important.

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Normal maternal metabolism in pregnancy is characterized by low normal glycaemia and physiological hyperlipidaemia [6–8]. Gestational diabetes, type 2 diabetes and poorly controlled type 1 diabetes, however, are often associated with even higher blood lipids than in normal pregnancy [7,9,10]. Obese women also have abnormal lipid profiles in pregnancy [11]. Women with higher range non-esterified fatty acids (NEFA) in the 3rd trimester are more likely to be overweight or obese [12]. Furthermore, elevated NEFA have been shown to be associated with pre-term delivery [12], intrauterine growth retardation (IUGR), IUGR with pre-eclampsia [13] and fetal adiposity [14].

Diabetes is well known to affect placental structure causing, in particular, abnormalities in terminal villous maturity and vascularisation [15–18]. Also placentas of women without diabetes but with LGA babies have an increase in placental abnormalities [16]. Functionally, there is good evidence that the human placenta adapts to even mild diabetes by limiting placental transfer of glucose, which may provide some degree of protection to the fetus [19–21]. This may be partly explained by a reduction in the expression of GLUT1 [20,22], which is the main glucose transporter in human placenta. Obesity and the placenta have been less well studied. Obesity is associated with larger placental size and placental macrophage accumulation and inflammation [23]. Knowledge about fatty acid metabolism in human placenta in pregnancies of diabetic and obese women is limited [24–27]. A recent study suggested that lipid metabolism genes in placenta are altered in different patterns in gestational diabetes and type 1 diabetes [28].

It was proposed by Prentki and Corkey that the toxicity of NEFA would be enhanced in the context of hyperglycaemia, a process termed glucolipotoxicity [29], as elevated glucose, via increasing malonyl-CoA, alters the partitioning of NEFA away from oxidation (a means of detoxification) towards acylation and esterification processes [30], some of which may be toxic if in excess. In support of this concept, we have previously reported a marked synergistic effect of high glucose and saturated NEFA in inducing cell death by apoptosis in both rat INS 832/13 pancreatic β -cells and human islets [31]. It is unknown if the process of glucolipotoxicity has a role in placenta in diabetic pregnancy.

The current study was performed in human trophoblasts from normal term placentas in primary culture in order to investigate the potential for glucolipotoxicity as a pathogenic process within the placenta. The effects of elevated glucose, elevated NEFA and, importantly, the combination of both on trophoblast metabolism, morphology, viability and function were investigated.

2. Materials and methods

2.1. Primary human trophoblast culture

The project was approved by the ACT Health Human Research Ethics Committee and informed consent was obtained from all women providing placentas. The placentas were from women undergoing elective caesarean sections at term (>37 weeks gestation). All were submitted for macroscopic and histological assessment (for details refer to the Electronic Supplemental Material (ESM)) to exclude unexplained pathology and none was found.

Trophoblasts were isolated according to a previously described method [32] with few modifications as described in the ESM. The harvested cells were cultured in CMRL medium 1066 containing 5.5 mM glucose supplemented with 10% FBS, 25 mM HEPES, and antibiotic mix (50 μ g/ml penicillin, 50 μ g/ml streptomycin and 100 μ g/ml neomycin) (CMRL complete medium) and maintained at 37 °C in a humidified atmosphere and 5% CO₂ for 16 h prior to experiments. At this stage, trophoblast purity was assessed by haematoxylin and eosin staining and immunocytochemical assessment as described in the ESM. All placental cultures showed greater than 95% purity for trophoblasts (ESM-Fig. 1).

Two series of experiments were performed. The first related to trophoblast glucose and fatty acid metabolism at this 16 h time-point. The second related to the effects of culturing the trophoblast for a further 24–48 h in RPMI 1640 medium with the same additives as for CMRL complete (RPMI complete), in low (1 mM) or high

(10 mM) glucose concentrations and 0.5% fatty acid free bovine serum albumin (BSA) (Sigma, castle Hill, NSW, Australia) in the presence or absence of 0.25 mM NEFA (palmitate:oleate ratio, 1:1). Palmitate (C16:0) and oleate (C18:1) are the most prevalent NEFA in plasma and occur in a ratio of approximately 1:1 [33]. Various assessments of cell viability, metabolism and function were then performed.

2.2. Preparation of BSA-bound fatty acids

BSA-bound palmitate and oleate were prepared as described in the ESM.

2.3. Glucose utilisation via glycolysis

Trophoblasts (1×10^6 per well) were seeded in 6 well plates and cultures were established over 16 h as described above. Culture medium was removed and the cells were washed in Krebs Ringer Bicarbonate buffer with 10 mmol/l HEPES (KRBH; pH 7.4) containing 0.5% defatted BSA (KRBH/0.5%BSA) and 2 mM glucose. The cells were then incubated in 0.75 ml of KRBH/0.5%BSA containing 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mM glucose and either 0.2 μ Ci/ml [⁵-³H]glucose (10–20 Ci/mmol, Perkin–Elmer Life Sciences, Waltham, MA) (for cold glucose concentrations 0.5–2.0 mM) or 0.4 μ Ci/ml [⁵-³H]glucose (for cold glucose concentrations 4.0–16.0 mM) for 2 h at 37 °C in a 5% CO₂ humidified incubator. The ³H₂O is released from [⁵-³H]glucose at the enolase step of glycolysis, such that it allows for the measurement of glycolytic flux from glucose. Calculations are derived from the rate of ³H₂O production (refer to ESM for measurement) and the specific activity of glucose in the media.

2.4. Fatty acid oxidation and esterification

Trophoblasts (1×10^6 per well) were seeded into 6 or 12 well plates at a density 1×10^6 cells/well and cultures were established over 16 h, as described above. The 12 well plates were used for the second series of experiments. Culture medium was removed and the cells were washed in KRBH/0.5%BSA with 2 mM glucose.

For fatty acid oxidation, trophoblasts were incubated in KRBH/0.5%BSA containing 1 μ Ci/ml [⁹,10(n)-³H] palmitic acid (30–60 Ci/mmol, Perkin–Elmer Life Sciences), 0.1 mM cold palmitate and 1.0 mM L-carnitine (Sigma) at various glucose levels from 0.5 to 18.0 mM for 2 h at 37 °C in a 5% CO₂ humidified incubator. Fatty acid oxidation was calculated from the amount of ³H₂O produced (refer to ESM for measurement) and the specific activity of fatty acid in the media.

For fatty acid esterification, trophoblasts were incubated in KRBH/0.5%BSA containing 0.25 μ Ci/ml of [¹-¹⁴C] palmitate (40–60 Ci/mmol, Perkin–Elmer Life Sciences) and 0.1–0.4 mM cold palmitate at various glucose concentrations for 2 h at 37 °C in a 5% CO₂ humidified incubator. In a separate esterification experiment, trophoblasts were incubated in CMRL complete with 0.5% BSA at 5.5 or 15 mM glucose, 0.25 μ Ci [¹-¹⁴C] palmitate and 0.2 or 0.4 mM palmitate for 24 h. At the end of the incubation period for all experiments the cells were washed and harvested in cold PBS, pelleted and resuspended in 3 ml Folch reagent as previously described [34]. Total lipids were extracted and non-polar lipids were separated by thin-layer chromatography. Incorporation of labelled palmitate into phospholipids (PL), diacylglycerol (DAG) and triacylglycerol (TG) was quantified after scraping of bands from the plates and β -scintillation counting. Net esterification rates were calculated by correcting for the specific activity of fatty acid in the media.

2.5. Glycerol release

In order to assess lipolysis, glycerol release into the fatty acid esterification media (see above) over the course of the 2 h incubations was measured in indicated experiments. Glycerol concentrations in the media were determined by a coupled enzymatic reaction as previously described [35].

2.6. Trophoblast viability, proliferation, apoptosis and protein content assays

These methods are described in the ESM.

2.7. Assessment of cell aggregation, syncytia formation and oil red-O staining for lipid

These methods are described in the ESM.

2.8. Electron microscopy

Trophoblasts were cultured on sterilised collagen coated cover slips in the various conditions as indicated. Cell processing and examination by transmission electron microscopy (EM) is as described in the ESM.

2.9. Adipophilin mRNA expression

The method is described in the ESM.

Fig. 1. Short and medium term interaction of glucose with NEFA metabolism is minimal in human trophoblast cells in culture. Trophoblast cells were established in culture in CMRL complete over 16 h for all experiments. In short-term experiments, glucose utilisation (A), fatty acid oxidation (B, C) and fatty acid esterification into (D) triacylglycerol (TG), (E) diacylglycerol (DAG) and (F) phospholipids (PL) were measured in the cells over 2 h in KRBH (A) or KRBH/0.5%BSA (B–F) at various glucose and palmitate concentrations as indicated. In medium term experiments, fatty acid esterification into (G) TG, (H) DAG and (I) PL was measured in the cells over 24 h in CMRL complete medium containing 0.5% BSA at 5.5 (5.5 G) or 15.0 (15 G) mM glucose in the presence of 0.2 (0.2FA) or 0.4 (0.4FA) mM palmitate. Due to variation of rates of esterification between placentas, the data were corrected to 100% for the 0.2FA/5.5 G baseline condition. Data are means $\pm$ SEM of $n = 12$ determinations from 4 separate placentas (A), $n = 24$ determinations from 4 separate placentas (B, C), $n = 8–9$ determinations from 3 separate placentas (D–F) and $n = 9$ determinations from 2 placentas (G–I). FA- fatty acid. Two way ANOVA: $p < 0.0001$ palmitate effect (D, E, G, H, I), $p < 0.01$ palmitate effect (F), $p < 0.05$ glucose effect (H), $p < 0.01$ glucose effect (I). Bonferroni post hoc test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.10. Trophoblast hormone and cytokine secretion

Trophoblast secretion of human chorionic gonadotrophin (beta-HCG), progesterone, human placental lactogen and the cytokines IL-1 β , IL-6, IL-8, IL-10 and TNF α were performed as described in the ESM.

2.11. Statistical analysis

Values are expressed as means $\pm$ SEM. Statistical analysis was performed using two-way ANOVA or the Kruskal–Wallis test with, respectively, Bonferroni's or Dunn's post-test for multiple comparisons using GraphPad Prism v5.01 (GraphPad Software, San Diego, CA). For data not normally distributed, log transformations were performed prior to statistical analysis.

3. Results

3.1. Glucose utilisation via glycolysis by trophoblasts in culture is near maximal at 4 mM glucose

Glucose interacts with fatty acid partitioning via malonyl-CoA which is down-stream of glycolysis via the anaplerosis pathway [30]. In order to determine optimal glucose concentrations of the incubation media for the fatty acid partitioning experiments, we first determined the effects of the media glucose concentrations on glycolysis. The rate of glucose utilisation via glycolysis by

trophoblasts was 90% of maximal at 4 mM glucose and clearly maximum at 8 mM glucose (Fig. 1A). From the Lineweaver–Burk plot of the data (not shown), the V_{max} for glucose utilisation was 35 nmol/10⁶ cells/h with a low K_m of 0.6 mM glucose.

3.2. Glucose has no significant short-term effects on fatty acid oxidation or esterification processes in trophoblasts

The short-term effect of glucose on fatty acid oxidation was assessed at glucose concentrations of 0.5 and 5.0 mM (Fig. 1B) and 2.0 and 18.0 mM (Fig. 1C), respectively. It was expected that a greater effect of glucose would be seen in the lower glucose range experiment because of the low K_m of glucose for glucose utilisation in these cells (Fig. 1A). The glucose concentration of the incubation medium, however, had no effect on the rate of trophoblast fatty acid oxidation in either of the experiments (Fig. 1B and C). Fatty acid esterification into triglyceride (TG), diacylglycerol (DAG), and phospholipids (PL) was assessed at the glucose concentrations of 0.5 and 5.0 mM at increasing concentrations of cold palmitate from 0.1 to 0.4 mM (Fig. 1D–F). There was a trend for fatty acid esterification into TG and DAG to be increased at the 5.0 mM compared to the 0.5 mM glucose concentration (two-way ANOVA, $p = 0.08$ for both) only at the higher NEFA concentrations (Fig. 1D and E). Glucose had

no effect on fatty acid esterification into PL (Fig. 1F). Importantly, esterification into TG and DAG increased linearly with increasing concentrations of palmitate without evidence of plateau (Fig. 1D and E). Fatty acid esterification into PL, however, did appear to reach a maximum at the fatty acid concentration of 0.3 mM (Fig. 1F).

3.3. Glucose interacts with fatty acid esterification processes measured over 24 h in human trophoblasts, but only at the highest NEFA concentration

As a plateau effect of NEFA concentration on NEFA esterification into DAG and TG was not evident at 2 h, longer duration experiments were performed, as regulatory processes to prevent excess glycerolipid accumulation could develop gradually. Trophoblasts were cultured for 24 h at 5.5 or 15.0 mM glucose in the presence of 0.2 or 0.4 mM cold palmitate and [14 C] palmitate tracer. The glucose concentration of the culture medium enhanced 24 h fatty acid esterification processes into DAG and PL, with a trend for it to be increased into TG, this being most evident in cells exposed to the higher 0.4 mM palmitate concentration (Fig. 1G–I). The increased esterification of palmitate into TG and DAG in cells exposed to 0.4 mM compared to 0.2 mM palmitate for 24 h was in proportion to, or exceeded, the 2 fold increased palmitate concentration (Fig. 1G and H) such that there was no evidence for down-regulation of esterification into DAG and TG in response to longer term high NEFA exposure. Esterification into PL over 24 h was approximately 50–75% increased at the higher palmitate concentration suggesting some regulation into these complex glycerolipids (Fig. 1I).

3.4. Effects of glucose and NEFA on trophoblast aggregation and oil red-O staining for lipid

Subsequent experiments were performed on trophoblasts cultured for 24–48 h in RPMI complete medium containing 0.5% BSA at 1 or 10 mM glucose in the presence or absence of 0.25 mM NEFA (palmitate:oleate ratio 1:1). These concentrations were chosen after consideration of the glycolysis data (Fig. 1A). At 1 mM and 10 mM glucose, glycolysis was about half and fully maximal, respectively, such that down-stream flux into malonyl-CoA would likely differ between the 2 conditions. After 48 h, light microscopic examination of the cells in culture showed no obvious effect of the glucose concentration. Trophoblasts were predominantly found as single cells at 1 and 10 mM glucose in the absence of NEFA (Fig. 2A–upper panels and 2C). The NEFA mix, however, markedly induced cell aggregation with the suggestion of syncytia formation (Fig. 2A–upper panels and 2C). This effect of the NEFA was not altered by the glucose concentration. NEFA induction of syncytialisation was confirmed by immunostaining cells for desmoplakin that delineates the cell membrane together with DAPI staining of nuclei (Fig. 2B and D). Oil red-O staining showed the presence of lipid droplets by 6 h (not shown) and marked lipid droplet accumulation by 24 h only in cells cultured in the presence of NEFA (Fig. 2A–lower panels) without obvious glucose effect. There was, however, evidence of non-lipid staining vacuolation in the trophoblast cells cultured in 10 mM glucose suggestive of glycogen accumulation. Periodic acid Schiff (PAS) positive cells were seen, but this was variably removed with diastase, suggesting some non-specific staining. For this reason, quantitative assessment of glycogen by this method was not possible. The presence of glycogen at the higher glucose concentration was confirmed by EM (Fig. 3).

3.5. Effects of glucose and NEFA on trophoblast ultrastructure

Transmission EM was performed on trophoblasts cultured for 24 h at 1 or 10 mM glucose in the presence or absence of

0.25 mM NEFA (palmitate:oleate ratio 1:1). Glycogen lakes were abundant only in cells cultured at 10 mM glucose and lipid droplets and myelin figures were abundant only in cells incubated in the presence of NEFA (Fig. 3A–D). On blinded analysis, lipid droplets in the presence of NEFA were reported to be less abundant in cells cultured at 10 mM compared to 1 mM glucose (ESM-Table 1).

3.6. Trophoblast viability, apoptosis, protein content, beta-HCG, placental lactogen and progesterone secretion are not affected by NEFA or glucose

Trophoblasts were cultured in RPMI complete medium containing 0.5% BSA at 1 or 10 mM glucose in the presence or absence of 0.25 mM NEFA (palmitate:oleate ratio, 1:1) for 24–48 h. Cell viability as assessed by MTT assay, apoptosis as assessed by caspase-3/7 activity assay and protein content were not altered by any of the culture conditions (ESM-Fig. 2). Trophoblast beta-HCG, placental lactogen and progesterone secretion over 48 h were also not affected (ESM-Fig. 2).

3.7. Fatty acid metabolic partitioning in trophoblast cells is altered by culture in the presence of NEFA without interaction with glucose

In order to investigate whether longer term exposure of the trophoblasts to elevated glucose and/or NEFA altered fatty acid metabolism, trophoblasts were cultured for 24 h in RPMI complete medium containing 0.5% BSA at 1 or 10 mM glucose in the presence or absence of 0.25 mM NEFA (palmitate:oleate ratio, 1:1). Fatty acid partitioning into esterification and oxidation pathways, in addition to glycerol release, were then measured over 2 h in KRBH/0.5%BSA medium at 1.0, 5.0 and 15.0 mM glucose. Consistent with the short-term experiments at NEFA concentrations less than 0.3 mM, the glucose concentration in the KRBH/0.5%BSA incubation medium (1.0, 5.0 or 15.0 mM glucose) had no effect on any of the fatty acid partitioning parameters measured (Fig. 4A–E). Furthermore, culture of trophoblasts for 24 h in low (1 mM) or high (10 mM) glucose had no effect (Fig. 4A–E). The presence of NEFA in the culture medium for 24 h, however, altered all measured components of fatty acid partitioning. Treatment of the cells in culture with the 0.25 mM NEFA mix resulted a 50–100% increase in fatty acid esterification into TG (Fig. 4A), a 10–20% increase in fatty acid esterification into DAG (Fig. 4B), 20–50% reduction in fatty acid esterification into PL (Fig. 4C) and a 10–20% reduction in fatty acid oxidation (Fig. 4D). Glycerol release, generated by lipolysis of glycerolipids, was reduced up to 60% by treatment in culture with NEFA (Fig. 4E). The effects of NEFA on lipid partitioning were not altered by the glucose concentration of the medium.

3.8. Culture of trophoblast cells in the presence of NEFA upregulates the expression of adipophilin mRNA which is attenuated by higher glucose concentrations

In order to further assess the accumulation of lipid droplets in the presence of NEFA, the expression of the lipid droplet-associated protein adipophilin at the mRNA level was assessed. Within 6 h, NEFA in the presence of 1 mM glucose dramatically increased adipophilin expression (Fig. 4F). This effect, however, was markedly attenuated by a glucose concentration of 10 mM (Fig. 4F). This result is consistent with the rapid appearance of lipid droplets in the NEFA treated cells observed with red oil O staining. The glucose effect on NEFA-induced adipophilin is also consistent with the EM finding of less lipid droplets at the higher glucose concentration (ESM-Table 1).

Fig. 2. Light microscopic appearance of trophoblast cells in culture, staining of cells with oil red-O to demonstrate lipid and desmoplakin immunofluorescence staining to demonstrate syncytia formation. Trophoblast cultures were established in CMRL complete medium over 16 h and then cultured for a further 24–48 h in RPMI complete medium containing 0.5% BSA at 1 or 10 mM glucose in the presence or absence of 0.25 mM NEFA (palmitate:oleate ratio, 1:1). (A-upper panel) Representative photomicrographs of cells from 1 of 7 placentas cultured for 48 h in the conditions as indicated (x20). (A-lower panel) Representative photomicrographs of cells from 1 of 4 placentas harvested after 24 h culture in the conditions as indicated, fixed and stained with oil red-O as per Material and Methods (x40, inserts x1000). (B) Representative photomicrographs of cells cultured for 48 h in 10 mM glucose and 0.25 mM NEFA (palmitate:oleate ratio, 1:1) (10 G + FA) and 10 mM glucose without NEFA (10 G No FA) and immunostained for desmoplakin (green) together with DAPI staining of nuclei (blue) (x40). Syncytia cells (S) counted if more than one nucleus could be seen within a cell that had complete cell membrane demonstrable by immunostaining. (C) Cell aggregation from photomicrographs of trophoblasts in culture at 48 h as shown in the upper panel of (A) scored by a blinded observer. Mainly single cells, score = 1; mainly loose small groups, score = 2; mainly tight small groups, score = 3; mainly tight larger groups, score = 4. Culture conditions: 1 (1 G) or 10 (10 G) mM glucose in the presence or absence of 0.25 mM NEFA (palmitate:oleate ratio, 1:1) (+FA or No FA, respectively). (D) Syncytia count per high power field (x40) by blinded observer of desmoplakin immunostained trophoblasts after 48 h in culture. Means $\pm$ SEM of $n = 7$ separate experiments (C) and $n = 10$ randomly selected high power fields per condition from one experiment (D). Kruskal–Wallis test: $p < 0.0001$ (C), $p < 0.001$ (D). Dunn's multiple comparison post hoc test: $*p < 0.05$, $**p < 0.01$.

3.9. NEFA increase pro-inflammatory cytokine production in human trophoblasts

In order to determine if exposure of trophoblasts to elevated glucose, NEFA or the combination of both induced cytokine production by these cells, trophoblasts were cultured for 24 h in RPMI complete medium containing 0.5% BSA at 1 or 10 mM glucose in the presence or absence of 0.25 mM NEFA (palmitate:oleate ratio, 1:1). Production of TNF α , IL-1 β , IL-6 and IL-10 was detected in the culture medium after 6 h incubation and concentrations were higher at 24 h. Secretion of these cytokines was increased by the presence of NEFA, but was unaffected by the glucose concentration

(Fig. 5A–D). Trophoblasts secreted large amounts of IL-8. Secretion of IL-8, however, was not affected by the level of glucose or the presence of NEFA (data not shown).

4. Discussion

Elevated glucose and lipids have been implicated in the pathogenesis of fetal/neonatal complications of diabetic pregnancy. Whether their effects are independent of each other, or whether they act synergistically via a process termed “glucolipotoxicity” [29] is unknown. Our studies of primary human trophoblast cells in culture showed that a palmitate and oleate NEFA mix had marked

Fig. 3. Electron microscopic (EM) appearance of trophoblast cells established and cultured in conditions as described in Fig. 2 legend. Representative EM photomicrographs from 1 of 2 placentas cultured in (A) 1 mM glucose in the absence of NEFA, scale bar = 2 μ m, (B) 10 mM glucose in the absence of NEFA, scale bar = 2.0 μ m, insert scale bar = 0.5 μ m, (C) 1 mM glucose in the presence of 0.25 mM NEFA, scale bar = 5 μ m, insert scale bar = 1 μ m, and (D) 10 mM glucose in the presence of 0.25 mM NEFA, scale bar = 2 μ m. Nucleus (N), rough endoplasmic reticulum (R), mitochondria (m), glycogen lakes (G), lipid droplets (L) and myelin figures (MF).

effects on trophoblast cell morphology and behavior independent of the glucose concentration. NEFA were avidly incorporated into lipid droplets, caused trophoblast cells to fuse to form syncytia, and promoted pro-inflammatory cytokine secretion. There was little evidence for interaction of glucose and NEFA on the measured parameters except for lipid droplet formation. A glucose concentration of 10 mM compared to 1 mM attenuated the marked effect of NEFA to increase the expression of the lipid droplet-associated protein adipophilin and to form lipid droplets. Thus, dyslipidaemia may have a significant pathogenic role in the placentas of pregnant women with diabetes and obesity, with the majority of the effects independent of the glucose concentration. The effect of glucose to alter lipid droplet formation, however, could turn out to be a very important glucose/lipid interaction, as the elevated glucose may be inhibiting a mechanism by which trophoblasts buffer an elevated NEFA supply.

4.1. Glucose metabolism in human trophoblasts

We found glucose utilisation via glycolysis in the trophoblasts had a very low glucose substrate K_m of 0.6 mM and was almost maximal at the low physiological glucose concentration of 4 mM. This result is consistent with previous studies of perfused human placental cotyledons that showed no increase in lactate production for increases in the perfusate glucose concentration from 4.0 mM [19] and no increases in O_2 consumption from 5.5 mM [36]. Total glucose utilisation in both these perfused placenta studies, however, continued to increase with maternal glucose perfusate concentrations well above these levels [19,36]. This non-oxidative glucose utilisation may be into glycogen storage and/or into the pentose-phosphate pathway as previously suggested [36] (Fig. 6A). Hence, glycolysis in trophoblasts is likely to be running at near

maximum when maternal blood glucose is physiological and it will not be increased by hyperglycaemia. It follows that flux into glucose metabolic pathways below glycolysis, such as glucose oxidation via the tricarboxylic acid cycle, anaplerosis and lipogenesis, will also not increase with diabetic hyperglycaemia (Fig. 6A). The accumulation of glycogen on EM examination of trophoblasts cultured in 10 mM compared to 1 mM glucose, however, is consistent with glycogen synthesis being increased by hyperglycaemia.

4.2. Fatty acid oxidation in human trophoblasts

It has been generally believed that fatty acid oxidation is a minor contributor to placental energy supply [21], although this has recently been challenged [37,38]. In the current study, fatty acid oxidation in trophoblast cells was measured at a rate of about 0.6 nmol/ 10^6 cells/h which is consistent with results from a previous study [37]. At a concentration of 0.1 mM palmitate, fatty acid esterification into TG, DAG and PL combined was only about 0.25 nmol/ 10^6 cells/h, which is at variance with previous reports stating that fatty acid esterification is much more active than oxidation in placenta [21]. Considering that complete oxidation of palmitate can yield 129 ATP molecules and glucose metabolism to lactate, which is the predominant pathway of its metabolism in placenta, only produces two ATP, fatty acids may well make a significant contribution to placental energy needs. Use of fatty acids for placental energy requirements may preserve the available glucose supply for fetal requirements, particularly during fasting.

It is well established that an increase in glucose supply inhibits fatty acid oxidation and promotes fatty acid esterification in some tissues [30,39]. Metabolism of glucose can elevate malonyl-CoA, a signal of plenty, which is an allosteric inhibitor of

Fig. 4. Fatty acid partitioning and adipophilin mRNA expression in trophoblast cells is altered by pre-treatment of the cells for 24 h with NEFA. Trophoblast cultures were established in CMRL complete medium over 16 h. Cells were then cultured for a treatment period of 24 h (6 h for mRNA analysis) in RPMI complete medium containing 0.5% BSA at 1 (1 G) or 10 (10 G) mM glucose in the presence or absence of 0.25 mM NEFA (palmitate:oleate ratio, 1:1) (+FA or No FA, respectively). Fatty acid esterification into triacylglycerol (TG) (A) diacylglycerol (DAG) (B) and phospholipids (PL) (C), fatty acid oxidation (D) and glycerol release into the media (E) were then measured over 2 h in KRBH/0.5%BSA with 0.1 mM cold palmitate at 1.0, 5.0 or 15 mM glucose and palmitate metabolic tracers as indicated in Materials and Methods. Due to variation of rates of fatty acid oxidation and esterification between placentas, the data were corrected to 100% for the No FA/1.0 G treatment condition assessed at 1.0 mM glucose (A–D). (F) Expression of adipophilin at the mRNA level corrected for glyceraldehyde-3 phosphate dehydrogenase after 6 h in experimental culture conditions. Means $\pm$ SEM of $n = 6$ –8 determinations from 3 separate experiments (A, C), $n = 5$ determinations from 2 separate experiments (B), $n = 4$ determinations from 2 separate experiments (D), $n = 6$ determinations from 2 separate experiments (E) and $n = 8$ determinations from 4 separate experiments (F). Two way ANOVA: $p < 0.05$ 24 h treatment effect (B), $p < 0.0001$ 24 h treatment effect (A, C, D, E), $p < 0.0001$ FA effect (F), $p < 0.01$ FA and glucose interaction (F). Bonferroni post hoc test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

carnitine palmitoyltransferase 1, a rate limiting enzyme for mitochondrial fatty acid oxidation [30,39]. In the current study of trophoblasts, glucose had no effect on the rate of fatty acid oxidation, whether across the range of glucose concentrations that affect glycolysis rate (0.5–5.0 mM) or across a higher range (2.0–18 mM). It is possible that this is due to low levels of acetyl-CoA carboxylase in placenta [21], the enzyme involved in the generation of malonyl-CoA. (Fig. 6A and B).

4.3. Fatty acid esterification in human trophoblasts

Fatty acid esterification in trophoblasts was measured at physiological (0.1 mM, FA:BSA ratio 1.3), high physiological (0.2 mM, FA:BSA ratio 2.7) and supra-physiological (0.4 mM, FA:BSA ratio 5.4 (saturation occurs with ratio of 7)) NEFA concentrations. With the more

physiological NEFA concentrations, glucose either from 0.5 to 5.0 mM over 2 h or from 5.5 to 15.0 mM over 24 h had no effect on fatty acid esterification rates. Glucose did seem to increase fatty acid esterification at the highest NEFA concentration, particularly over 24 h, but this may have little physiological relevance. Most striking in these experiments was the finding that there was no evidence of a plateau in fatty acid esterification rates into DAG and TG, even over 24 h, with increases in the NEFA concentration to very high levels. There was, however, some suggestion of attenuation of esterification into PL.

4.4. NEFA induced lipid droplet formation in trophoblasts is attenuated by high glucose

Culture in the presence of fatty acids caused the appearance of lipid droplets within 6 h using oil red-O staining with confirmation

Fig. 5. Cytokine secretion from human trophoblast cells in culture is stimulated by NEFA but not glucose. Trophoblast cultures were established in CMRL complete medium over 16 h. Cells were then cultured for a treatment period of 24 h in RPMI complete medium containing 0.5% BSA at 1 (1 G) or 10 (10 G) mM glucose in the presence or absence of 0.25 mM fatty acid (palmitate:oleate ratio, 1:1) (+FA or No FA, respectively). The secretion of (A) tumor necrosis factor α (TNF α), (B) interleukin 1 β (IL1 β), (C) interleukin 6 (IL6), (D) interleukin 10 (IL10) into the incubation medium was measured by cytokine bead array assay at 24 h. Scatter dot plots showing means of $n = 10$ –12 experiments. Two way ANOVA with placenta matching: $p < 0.05$ FA effect (C, D), $p < 0.01$ FA effect (A, B). Bonferroni post hoc test: $*p < 0.05$.

by EM (Fig. 6B). This finding is consistent with a previous study in human trophoblasts in which a mix of oleic and linoleic acid, particularly in the presence of insulin in serum free medium, also induced the formation of lipid droplets in addition to enhancing the expression of adipophilin [40]. Considerable evidence now exists pointing to a role for peroxisomal proliferator-activated receptor γ (PPAR γ) and adipophilin expression in the promotion of lipid droplet formation and lipid metabolism in the trophoblast [41–44], implicating this transcription factor in this effect of NEFA. Our EM lipid droplet accumulation and adipophilin mRNA expression results indicate that elevated glucose attenuates this effect (Fig. 6B). Of note, similar results in primary human trophoblasts with respect to glucose/NEFA interactions and lipid droplet formation have recently been reported by another group [28]. The significance of the plentiful myelin figures seen on EM of cells cultured in the presence of NEFA is unknown but may be a response to cell injury. Lipid droplets, myelin figures and glycogen deposits have been observed in trophoblasts of placentas from 20 day heat-stressed rats [45].

4.5. NEFA alter their own partitioning to enhance placental fatty acid buffering

Culture of trophoblasts in the presence of NEFA for 24 h altered the subsequent intracellular partitioning of fatty acids. Fatty acid esterification into TG was substantially enhanced, esterification into PL was attenuated and there was a dramatic reduction in glycerol release, suggesting a reduction in intracellular lipolysis (Fig. 6B). These changes would be consistent with an effect of the fatty acids to upregulate their own partitioning into TG. As with the capacity of the placenta to buffer the fetus from excess glucose through storing it safely in the form of glycogen, these changes in

lipid partitioning would favour the safe storage of excess lipid supply within lipid droplets [46]. Fatty acids have previously been found to reduce placental lipoprotein lipase, an extracellular lipase that is involved in hydrolyzing TG from maternal circulating lipoproteins to allow uptake of the lipoprotein fatty acids into the cell [47], potentially another mechanism to protect the fetus from excess lipid (Fig. 6B).

With respect to intracellular lipolysis, the effects of fatty acids in trophoblasts to reduce glycerol release is at complete variance with the effect of oleate in a breast cancer cell line [48]. While the reduced glycerol release in trophoblasts in the presence of fatty acids could be due to reduced lipolysis, another potential explanation is upregulation of glycerol kinase activity. Little is known about the presence or activity of this enzyme in placenta. The regulation of lipolysis and importance of intracellular glycerolipid/fatty acid cycling within trophoblasts warrants attention. Also, the possibility that the trophoblast synthesizes and exports lipoprotein-like particles, for which there is a small amount of evidence [49], should be further investigated (Fig. 6B).

4.6. NEFA induce trophoblast syncytialisation and pro-inflammatory cytokine production

The exposure of the trophoblasts over 24 h to NEFA had a marked effect on promoting trophoblast aggregation and syncytialisation. This novel finding, however, was not associated with any alteration in the parameters of cell viability or growth. Also, within the time frame of these experiments, syncytialisation was not coupled with increases in the production of β -HCG, placental lactogen or progesterone. This is not consistent with the current wisdom amongst placentalogists. Another group, however, has recently found that β -HCG secretion is not necessarily linked to cell

Fig. 6. Models of trophoblast (A) glucose and (B) NEFA metabolism. Blue boxes relate to pathways assessed in the current study. Yellow boxes relate to pathways assessed by others and/or are relevant to the discussion of this work. (A) Glucose metabolic flux via glycolysis is near maximal at 4 mM glucose (1), but from previous studies we know that total glucose utilisation of placenta is not maximal until the glucose concentration is greater than 16 mM [19,36]. Elevated glucose in diabetes, therefore, may increase flux into pathways up-stream of glycolysis such as glycogen synthesis (2) and/or the pentose-phosphate pathway (3), but is unlikely to increase flux into mitochondrial oxidation or anaplerosis pathways (down-stream) (4), including to increase malonyl-CoA levels necessary for lipogenesis and to alter fatty acid partitioning via inhibition of fatty acid oxidation. (B) All arms of NEFA partitioning are active in the trophoblast and NEFA are avidly taken up into lipid droplets. Prolonged exposure to elevated NEFA results in lipid metabolism that may protect the fetus from excess supply. Elevated NEFA down-regulates lipoprotein lipase activity [47] such that NEFA entry to the trophoblast from lipoproteins can be curtailed. Elevated NEFA also upregulates NEFA esterification into TG, expression of the lipid droplet protein adipophilin and reduces lipolysis, all of which favours buffering of NEFA into lipid droplets. NEFA minimally down-regulates fatty acid oxidation. Of relevance to diabetes, elevated glucose has minimal effect on the NEFA partitioning pathways due to limited effects on malonyl-CoA levels (panel A), but may attenuate the formation of lipid droplets. The clearance of lipid from trophoblasts by synthesis and secretion of lipoproteins [28] may be of importance but has not been examined in this study. Gluc- glucose; G6P-glucose-6-phosphate; LPL-lipoprotein lipase; LC-CoA-long chain acyl-CoA; MTTP-microsomal triglyceride transfer protein; PA-phosphatidic acid; VLDL-very low density lipoprotein.

fusion in BeWo cells (personal communication, Kristina Orendi, Medical University of Graz). Of considerable interest was the finding that the NEFA mix stimulated trophoblast cytokine production, with no effect of the higher glucose concentration. Thus, maternal dyslipidaemia could have roles in promoting placental structural abnormalities and pro-inflammatory change as have been observed in placentas of gestational diabetic [50] and obese women [23].

4.7. Conclusion

The results of this work underscore the potential importance of dyslipidaemia in the pathophysiology of placental dysfunction and fetal abnormalities in diabetic pregnancy. The finding that NEFA enhance their own storage into lipid droplets is indicative of upregulation of a buffering mechanism to protect the fetus from excess lipid. The novel findings that NEFA promote syncytialisation and cytokine production may be indicative of harmful effects of dyslipidaemia via effects on placental structure and inflammation. It was remarkable how little effect glucose had on trophoblasts compared to the effects of NEFA. Glucose did not alter fatty acid partitioning as was predicted by the glucolipotoxicity hypothesis. Dyslipidaemia, therefore, may have greater pathogenic significance than hyperglycaemia at the level of this cell in diabetic pregnancy. Elevated glucose, however, did impede NEFA-induced lipid droplet formation, the importance of which warrants further examination.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.placenta.2009.12.013.

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