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Hyperglycaemia and lipid differentially impair mouse oocyte developmental competence

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Abstract. Maternal diabetes and obesity are characterised by elevated blood glucose, insulin and lipids, resulting in upregulation of specific fuel-sensing and stress signalling pathways. Previously, we demonstrated that, separately, upregulation of the hexosamine biosynthetic pathway (HBP; under hyperglycaemic conditions) and endoplasmic reticulum (ER) stress (due to hyperlipidaemia) pathways reduce blastocyst development and alter oocyte metabolism. In order to begin to understand how both glucose and lipid metabolic disruptions influence oocyte developmental competence, in the present study we exposed mouse cumulus–oocyte complexes to hyperglycaemia (30 mM) and/or lipid (40 μM) and examined the effects on embryo development. The presence of glucosamine (GlcN; a hyperglycaemic mimetic) or increased lipid during *in vitro* maturation severely perturbed blastocyst development ($P < 0.05$). Hyperglycaemia, GlcN and hyperglycaemia + lipid treatments significantly increased HBP activity, increasing total *O*-linked glycosylation (*O*-GlcNAcylation) of proteins ($P < 0.0001$). All treatments also induced ER stress pathways, indicated by the expression of specific ER stress genes. The expression of genes encoding the HBP enzymes glutamine:fructose-6-phosphate amidotransferase 2 (*Gfpt2*) and *O*-linked β-*N*-acetylglucosaminyltransferase (*Ogt*) was repressed following lipid treatment ($P < 0.001$). These findings partially implicate the mechanism of *O*-GlcNAcylation and ER stress as likely contributors to compromised fertility of obese women.

Additional keywords: cumulus–oocyte complex, embryo, endoplasmic reticulum stress, hexosamine biosynthesis pathway, hyperlipidemia.

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Introduction

Maternal obesity, a condition associated with elevated plasma insulin, glucose and lipid, is a global health problem that affects an increasing number of women of reproductive age (Australian Institute of Health and Welfare (AIHW) 2012). Obesity is also a contributing factor to Type 2 diabetes, characterised by hyperglycaemia (elevated blood glucose level) and hyperlipidemia. These conditions have been associated with a higher risk of subfertility and pregnancy complications, including an increased risk of anovulation, pre-eclampsia, miscarriage and spontaneous abortion (Jungheim and Moley 2010). They are also associated with an increased incidence of congenital anomalies, macrosomia and stillborns (Sirimi and Goulis 2010). Moreover, these complications extend beyond neonatal health, into childhood and adulthood (Heerwagen *et al.* 2010; Dabelea and Crume 2011; O'Reilly and Reynolds 2013).

The periconception period (including the final stages of oocyte development and fertilisation) is particularly sensitive to the maternal metabolic environment. For example, zygotes collected from oviducts of diabetic mice and subsequently transferred to normoglycaemic recipients have retarded fetal growth and increased fetal abnormalities (Wyman *et al.* 2008). Hyperglycaemia during oocyte maturation and early development is associated with altered intracellular parameters, such as perturbed meiotic maturation and disrupted mitochondrial distribution (Colton *et al.* 2002; Chang *et al.* 2005), as well as apoptosis in follicular and granulosa cells (Chang *et al.* 2005).

The detrimental effects of hyperglycaemia on oocyte developmental competence are mediated, in part, by upregulation of the hexosamine biosynthesis pathway (HBP; Sutton-McDowall *et al.* 2006; Schelbach *et al.* 2010, 2012), a fuel-sensing pathway that metabolises glucose to uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc). Single UDP-GlcNAc molecules modify serine or threonine amino acids of proteins, a process known as β-*O*-linked glycosylation (*O*-GlcNAcylation), which acts in

an analogous manner to phosphorylation to regulate protein function (Butkinaree *et al.* 2010). Altered *O*-GlcNAcylation is one of the primary pathologies of diabetes in somatic cells and notably the primary mechanism behind the development of insulin resistance in Type 2 diabetes (Marshall *et al.* 1991; Yang *et al.* 2008).

Glucosamine (GlcN), a known hyperglycaemic mimetic, can be metabolised via the HBP by bypassing the rate-limiting enzyme of the HBP, namely glutamine:fructose-6-phosphate amidotransferase (GFPT; Nelson *et al.* 2000; Uldry *et al.* 2002); hence, it is a potent stimulator of HBP pathway activity. Previously, we demonstrated that GlcN supplementation during IVM results in increased *O*-GlcNAcylation in mouse COCs (Frank *et al.* 2014*a*) and perturbed oocyte developmental competence in cow, pig and mouse and decreased cleavage rates in the mouse (Sutton-McDowall *et al.* 2006; Kimura *et al.* 2008; Schelbach *et al.* 2012; Frank *et al.* 2013).

Hyperlipidaemia is also known to cause numerous perturbations in oocyte structure and developmental competence. Female mice fed a high-fat diet (HFD) have higher rates of anovulation, smaller and fewer mature oocytes, increased lipid accumulation, altered mitochondrial activity and decreased rates of oocyte nuclear maturation and fertilisation compared with oocytes derived from control mice (Igosheva *et al.* 2010; Jungheim *et al.* 2010; Wu *et al.* 2010; Luzzo *et al.* 2012). Increased lipid accumulation induces lipotoxicity, which causes damage to cellular organelles, particularly mitochondria and endoplasmic reticulum (ER). A biomarker of lipotoxicity is ER stress, characterised by the accumulation of misfolded proteins and consequently triggering the unfolded protein response (UPR; Alhusaini *et al.* 2010; Wu *et al.* 2012*a*).

UPR is an attempt by the cell to slow protein production and improve protein folding, characterised by the induction of several genes, including the markers activating transcription factor 4 (*Atf4*), activating transcription factor 6 (*Atf6*) and glucose-regulated protein 78 (*Grp78*; Malhotra and Kaufman 2007). In the absence of the UPR, cellular stress will likely culminate in apoptotic cell death. Cumulus–oocyte complexes (COCs) from mice fed an HFD were shown to have increased expression of ER stress marker genes *Atf4* and *Grp78*, similar to those matured in lipid-rich follicular fluid (Wu *et al.* 2010; Yang *et al.* 2012). Similar events happen in women, with increased *Atf4* expression observed in granulosa cells of obese women (Wu *et al.* 2010).

A link between the HBP and ER stress has been suggested in somatic cells (Srinivasan *et al.* 2009; Sage *et al.* 2010; Lombardi *et al.* 2012), with increasing activity through the HBP increasing the transcript and translocation of ER stress markers. However, little is known about how hyperglycaemia and lipid conditions impact reproductive function, in particular preimplantation embryo development. Furthermore, it is unclear whether these metabolic perturbations operate through the same or different mechanisms. The aim of the present study was to examine the impact of hyperglycaemia and lipid supplementation on mouse oocyte developmental competence. We hypothesised that combination of lipid and high glucose leads to the accumulation of the HBP product UDP-GlcNAc by increasing *O*-GlcNAcylation and activating the ER stress pathway.

Materials and methods

Mice

Female CBA/F1 mice were obtained at 21 days of age from the University of Adelaide (Waite campus) and kept in the Animal House at the Medical School, the University of Adelaide (North Terrace campus) under a 14 : 10-h light–dark cycle with *ad libitum* access to food and water. All animal experiments were approved by the University of Adelaide's Animal Ethics Committee (Medical) and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Isolation and in vitro *maturation of mouse COCs*

Immature, unexpanded COCs were isolated by puncturing the antral follicles of ovaries collected 46 h after i.p. injection of 5 IU pregnant mare's serum gonadotropin (PMSG; Folligon; Intervet, Boxmeer, The Netherlands). All COCs were collected in HEPES-buffered α-minimum essential medium (MEM) handling media (Life Technologies, Invitrogen, Carlsbad, CA, USA) supplemented with 4 mg mL^{-1} fatty acid-free bovine serum albumin (MP Biomedicals, Solon, OH, USA). Immature COCs were cultured in groups of 30 in 1.5 mL pre-equilibrated *in vitro* maturation (IVM) medium, overlaid with paraffin oil (Merck, Darmstadt, Germany). The base IVM medium (control) was bicarbonate-buffered α-MEM supplemented with 1% fetal bovine serum (FBS; Invitrogen), 50 mIU mL^{-1} recombinant human FSH (Puregon-Organon, Oss, The Netherlands) and 10 ng mL^{-1} recombinant human epidermal growth factor (EGF; R&D Systems, Minneapolis, MN, USA). Experimental treatments were as follows: (1) control (5.56 mM glucose); (2) high glucose (30 mM glucose); (3) glucosamine (2.5 mM GlcN plus 5.56 mM glucose); (4) lipid (40 μM of a commercially available lipid concentrate; GIBCO, Invitrogen); and (5) lipid and high glucose (40 μM lipid concentrate plus 30 mM glucose). COCs were cultured at 37°C in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2 for 8 or 16 h. We have previously demonstrated that large culture volumes are required to maintain hyperglycaemic concentrations of glucose due to the high metabolic rate of COCs (Frank *et al.* 2013). In the present study, 30 mM glucose was used to avoid depletion to more normoglycaemic levels during the course of maturation (Sutton-McDowall *et al.* 2010). The commercially available lipid concentrate was a solution composed of 1 mM of 10 lipids, dissolved in ethyl alcohol and Tween 80 (see Table 1 for the composition of the lipid concentrate).

Development of the lipid accumulation model

A dose–response experiment was performed to determine the concentration of the lipid concentrate (0, 5, 10 20, 40 μM) that resulted in increased lipid accumulation within the oocyte. After 16 h culture, COCs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h, washed in PBS and transferred to 1 μg mL^{-1} of the neutral lipid stain BODIPY 493/503 (Life Technologies, Invitrogen) in PBS for 1 h in the dark at room temperature. COCs were washed in PBS for 5 min and mounted on coverslips in 3 μL DAKO fluorescent mounting medium (Dako, Glostrup, Denmark). Images were captured

Table 1. Chemically defined lipid concentrate used in lipid treatment during *in vitro* maturation

Components	Concentration ($mg\,L^{-1}$)
Arachidonic acid	20
Cholesterol	220
DL-α-Tocopherol acetate	70
Ethyl alcohol 100%	0
Linoleic acid	10
Linolenic acid	10
Myristic acid	10
Oleic acid	10
Palmitic acid	10
Palmitoleic acid	10
Pluronic F-68	90 000
Stearic acid	10
Tween 80	2200

using a Fluoview FV10i confocal microscope (Olympus, Tokyo, Japan) using a green laser (excitation 480 nm, emission 515 nm); identical magnification, image and laser settings were used throughout experiments (see Fig. S1 available as Supplementary Material to this paper). Two replicates were performed.

Assessment of cumulus expansion index

Cumulus expansion was assessed after 16 h culture by an independent assessor, blinded to treatments, using a scale described previously (Vanderhyden *et al.* 1990), as follows: 0, no expansion of cumulus cells; +1, the outer most layers of cumulus cells expanded; +2, expansion of the entire outer half of cumulus cells; +3, all layers expanded except the corona radiatae; and +4, maximal expansion of all layers of cumulus cells. For each treatment group, a mean cumulus expansion index (CEI; over the range 0.0–4.0) was calculated. Three replicates were performed, averaging 25 COCs per treatment group and replicate.

IVF and assessment of embryo development

Following 16 h maturation, COCs were washed once in fertilisation medium (VitroFert; Cook Australia, Brisbane, Qld, Australia) and COCs were transferred to pre-equilibrated fertilisation drops overlaid with paraffin oil. Male mice, which had previously been assessed for mating ability (not less than 3 days prior), were used as sperm donors for IVF. Mice were killed by cervical dislocation and the epididymides and vasa deferentia were collected into warm (37°C) wash medium (VitroWash; Cook Australia), cleaned of excess fat and tissue and transferred into 1 mL fertilisation medium. Spermatozoa were extracted into the medium and allowed to capacitate at 37°C in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2 for 1 h. Capacitated spermatozoa (10 μL) were added to 90-μL fertilisation drops and COCs and spermatozoa were coincubated for 4 h at 37°C in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2. COCs were transferred to wash medium and cumulus cells were removed mechanically by repeat pipetting. Presumptive zygotes were washed in embryo culture medium (VitroCleave; Cook Australia) and placed into culture drops (four to seven per 10-μL drop) at 37°C in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2. Twenty-four hours after IVF (Day 2), the fertilisation rate was assessed and 2-cell embryos were transferred onto a fresh 20-μL drop of embryo culture medium. Embryo morphology was assessed on Day 5 (the end of the culture period, 96–100 h after fertilisation). Embryo development was assessed on Day 2 (expected 2-cell stage) and Day 5 (blastocysts or hatching blastocysts). Seven replicates were performed, with 50 COCs per treatment group and replicate.

Immunocytochemistry

Following IVM (16 h), COCs were fixed in 4% paraformaldehyde in PBS overnight. Whole COCs were adhered on Cell-Tak (BD Biosciences, San Jose, CA, USA)-coated slides and stained immunohistochemically using the primary antibody CTD110.6 (anti-*O*-GlcNAc antibody; Covance, Princeton, NJ, USA) for *O*-GlcNAc and propidium iodide (PI) for nuclear staining. Briefly, COCs were permeabilised for 30 min in 0.25% Triton X-100 (United States Biochemical, Salem, MA, USA), blocked for 2 h using 10% goat serum in PBS (Jackson Immuno Research Labs, West Grove, PA, USA) and incubated overnight at 4°C with 1 : 250 CTD110.6 in blocking solution. On Day 2, COCs were washed and incubated for 2 h at room temperature with 1 : 250 Alexa Fluor 488 goat anti-mouse IgM (Invitrogen) in blocking solution, washed twice in PBS–polyvinylpolypyrrolidone (PVP) and 30 min with PI before being mounted under a coverslip in 3 μL DAKO fluorescent mounting medium (Dako). A negative control with no primary antibody was also included and no autofluorescence was observed with the microscope settings used. Fluorescence intensity and localisation were examined using a Fluoview FV10i confocal microscope (Olympus) using the Alexa Fluor 488 filter for CTD110.6 (excitation 488 nm, emission 519 nm) and the PI filter (excitation 488 nm, emission 617 nm). Single optical sections were acquired from a median plane of zona in the oocytes. Three replicates were performed, with 10 COCs per treatment group. Localisation and intensity of CTD110.6-positive staining across the COC were determined using ImageJ software (NIH; http://imagej.nih.gov/ij/) by placing a box across the oocyte and cumulus cell image and measuring pixel intensity. The mean fluorescence intensity in each pixel column was reported and the mean ± s.e.m. calculated. Data are represented graphically as the intensity of fluorescence over pixel widths (Wu *et al.* 2010). Total *O*-linked-glycosylated protein was determined as the sum of total fluorescence in the boxed area.

RNA isolation and real-time reverse transcription–polymerase chain reaction

Following 8 or 16 h IVM, groups of 90–100 COCs were collected in a minimal volume of medium, snap frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from COC using the TRIzol reagent (Invitrogen) method, followed by DNase treatment using an Ambion kit (Invitrogen) as per the manufacturer's instructions. RNA concentration and purity were quantified using a Nanodrop ND-1000 Spectrophotometer (Biolab, Carmel, IN, USA) before reverse transcribing 600 ng RNA using random primers (Invitrogen) and Superscript III

Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Ribosomal protein *RPl19* (QIAGEN, Venlo, Limburg, The Netherlands) was used as a validated internal control for every sample. The X-box binding protein 1 (*Xbp1s*) primers were 5′-AGG CTT GGT GTA TAC ATG G-3′ (reverse) and 5′-GGT CTG CTG AGT CCG CAG GAG G-3′ (forward; Ozcan *et al.* 2009); the other primers were Quantitect Primer assays (QIAGEN). Real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed in quadruplicate using SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and a Rotor-Gene 6000 (Corbett, Valencia, CA, USA) real-time rotary analyser. Real-time RT-PCR data were analysed using the $2^{-(\Delta CT)}$ method and are expressed as the fold change relative to a calibrator sample, which was included in each run. Eight replicates were performed for the control treatment and four replicates were performed for all other treatments, with 90–100 COCs per treatment group and replicate.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). Differences between treatment groups were tested using a general linear model, followed by Bonferroni post hoc tests. Cleavage and blastocyst rates were arcsine transformed before analyses. Outcome parameters are reported as the mean ± s.e.m. Two-sided $P < 0.05$ was considered significant.

Results

Experiment 1: lipid and GlcN impair oocyte developmental competence

CEIs were examined before IVF. Hyperglycaemia and lipid treatments during IVM had no effect on cumulus expansion ($P > 0.05$; Table 2). On Day 2, the cleavage rate of COCs treated with high glucose + lipid was significantly lower than lipid treatment alone (81.86 ± 1.88% vs 89.86 ± 2.06%, respectively; $P < 0.05$). There were no significant differences in cleavage rates between the other treatment groups. The fertilised oocytes from COCs treated with GlcN or lipid yielded significantly fewer blastocysts on Day 5 than the control (48.49 ± 5.19% and 50.35 ± 4.93% vs 69.28 ± 3.29%, respectively; $P < 0.05$). The blastocyst rate in the high glucose + lipid treatment group tended to be lower than in the control group (Fig. 1; $P = 0.08$).

Experiment 2: O-GlcNAcylation localisation

Immunohistochemical localisation of *O*-GlcNAc with CTD110.6 revealed positive staining in the oocytes, with higher intensity in COCs treated with high glucose, lipid, GlcN and high glucose + lipid compared with control (Fig. 2*a*). Densitometry analysis of CTD110.6 revealed similar results, where GlcN treatment significantly increased *O*-GlcNAcylation-positive staining within the oocyte compared with the control group (Fig. 2*b*; $P < 0.05$). Increased *O*-GlcNAcylation was also observed in oocytes treated with high glucose. In contrast, the addition of lipid to high glucose diminished the extent of staining relative to glucose alone. No significant differences were observed in cumulus cells among treatment groups (Fig. 2*b*).

Table 2. Cumulus expansion indices following maturation (16 h) in the different treatment groups
Data are the mean ± s.e.m. GlcN, glucosamine

Treatment	Cumulus expansion index
Control	3.81 ± 0.14
Glucose	3.84 ± 0.10
Lipid	3.68 ± 0.03
Glucose + lipid	3.63 ± 0.07
GlcN	3.89 ± 0.07

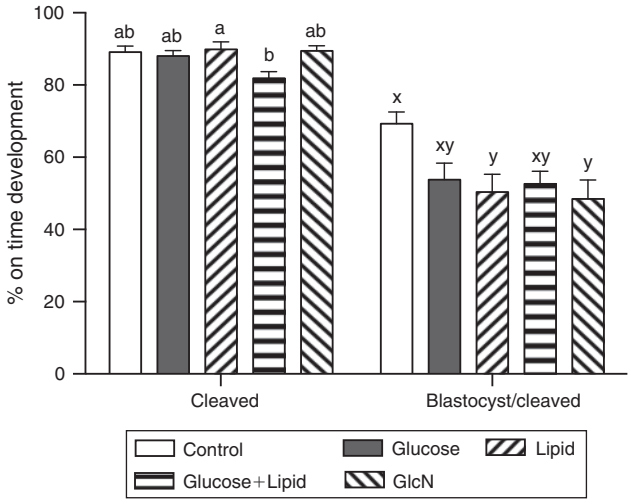

Fig. 1. Cleavage and blastocyst development following *in vitro* maturation in high glucose, lipids or glucosamine (GlcN). Data are the mean + s.e.m. Within each developmental stage, columns with different letters differ significantly ($P < 0.05$).

Experiment 3: expression of HBP enzymes and ER stress genes

The mRNA expression for HBP enzymes, namely *Gfpt1*, *Gfpt2*, *O*-linked β-*N*-acetylglucosaminyltransferase (*Ogt*) and hyaluronan synthase 2 (*Has2*), was determined following exposure to high glucose and lipid. After 8 h maturation, GlcN treatment resulted in 2.2-, 2.8- and 4.0-fold increases in *Gfpt1*, *Gfpt2* and *Has2* expression, respectively, compared with control (Fig. 3*a*, *b*, *d*). There were no other significant differences in gene expression between the other treatment groups (Fig. 3). After 16 h, there was a corresponding increased in *Gfpt1* (1.8-fold) and *Gfpt2* (1.4-fold) expression (Fig. 3*a*, *b*) following the GlcN treatment. Interestingly, the addition of lipid to the culture medium significantly decreased the expression of *Ogt* (an enzyme that adds the *O*-GlcNAcylation to proteins) and *Gfpt2* compared with control (Fig. 3*b*, *c*).

After 8 h maturation, supplementation of GlcN in the culture medium resulted in increased expression of the ER

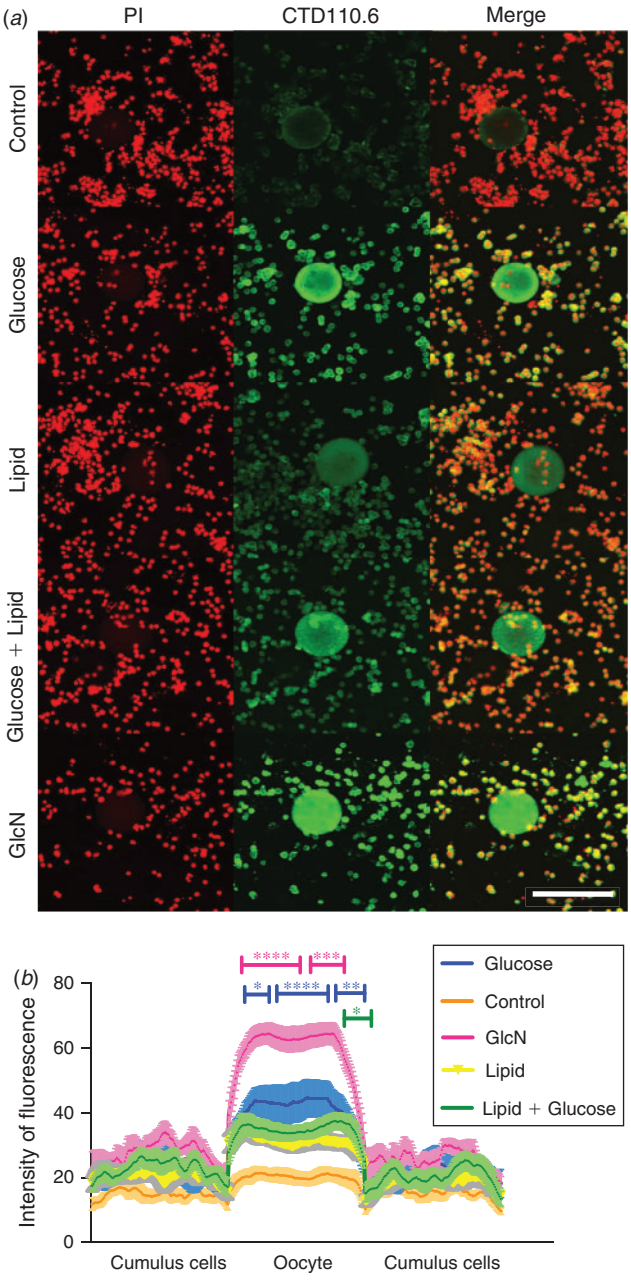

Fig. 2. (*a*) Localisation of β-*O*-linked glycosylation in cumulus–oocyte complexes (COCs) following *in vitro* maturation in the presence of high glucose and lipid. Propidium iodide (PI; red) shows nuclear staining, CTD110.6 (green) shows β-*O*-glycosylation (*O*-GlcNAc). The images were merged (merge). Scale bars = 100 µm. GlcN, glucosamine. (*b*) Intensity of CTD110.6 after culture with different treatments. COCs were collected after 16 h culture with control and glucose, fixed in 4% paraformaldehyde and incubated with CTD110.6 for *O*-GlcNAc and PI for nuclear staining. Data are the mean ± s.e.m. (lighter shading represents error bars). ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared with control.

stress gene *Grp78* (threefold) and *Atf4* (Fig. 4*a–c*). After 16 h, a similar trend was observed in gene expression levels, with increased *Xbp1* (1.8-fold) and *Grp78* (2.6-fold) expression following GlcN treatment (Figs 4*a*, c). Lipid- and high glucose + lipid-treated COC also exhibited significantly increased expression of *Xbp1* (1.8-fold). In addition, after 16 h, *Atf4* expression was increased in COCs treated with GlcN (2.3-fold) and high glucose (1.5-fold). Interestingly, *Atf4* expression was significantly reduced when lipid was added to the high-glucose culture medium. There were no significant differences in *Atf6* gene expression between treatment groups (Fig. 4*d*).

Discussion

There is a significant body of evidence that demonstrates poor preimplantation embryo development outcomes following hyperglycaemic or lipidaemic exposure during *in vivo* or *in vitro* development. The present study elucidates some of the cellular mechanisms by which high glucose and lipid conditions contribute to reduced oocyte quality and developmental competence. In the present study we used a chemically defined lipid concentrate that contains various non-esterified fatty acids (NEFAs) as a substitute for using single lipids, such as palmitic acid or lipid-rich follicular fluid, which have been used in other studies (Aardema *et al.* 2011; Wu *et al.* 2012*b*; Yang *et al.* 2012). This was because the lipid concentrate more closely resembles the NEFA detected in women with increased body mass index (Robker *et al.* 2009; Valckx *et al.* 2012) and follicular fluid contains hormones, growth factors and undefined proteins that may compromise development. Furthermore, we have investigated the role of high glucose and lipid on O-GlcNAcylation and ER stress during and at the end of IVM. Notably, hyperglycaemic conditions increase aberrant *O*-GlcNAcylation in oocytes and induce the expression of ER stress genes. Surprisingly, the presence of lipid in the culture medium represses the expression of genes encoding HBP enzymes, such as the rate-limiting enzymes Gfpt2 and Ogt.

The cleavage rate of COCs treated with the combination of high glucose and lipid was lower than treatment with lipid alone, whereas blastocyst development was significantly reduced following GlcN and lipid supplementation, but only tended to decrease following high glucose treatment. This discrepancy with high glucose treatment may be explained by the difference in culture media used: a much simpler medium was used in our previous study (Frank *et al.* 2013). The result with lipid treatment is consistent with several other studies demonstrating that lipid level is critical during embryo development (Leroy *et al.* 2005; Van Hoeck *et al.* 2011; Wu *et al.* 2012*b*; Yang *et al.* 2012). Interestingly, there was a tendency for decreased blastocyst development following culture with high glucose + lipid, suggesting that high levels of glucose do not have an additive effect in impairing oocyte developmental competence.

Increased *O*-GlcNAcylation of proteins is a hallmark of increased HBP activity, induced by either hyper- or hypoglycaemia. Following IVM, high glucose or GlcN significantly increase *O*-GlcNAcylation levels, in line with GlcN being a potent stimulator of HBP UDP-GlcNAc production (~40-fold, as measured in adipocytes; Marshall *et al.* 1991). Excess flux with either glucose or GlcN through HBP has been shown previously to reduce embryo development and this phenomenon was reversed using an O-linked N-acetylglucosamine trasnferase (OGT) inhibitor, reflecting their relative potential

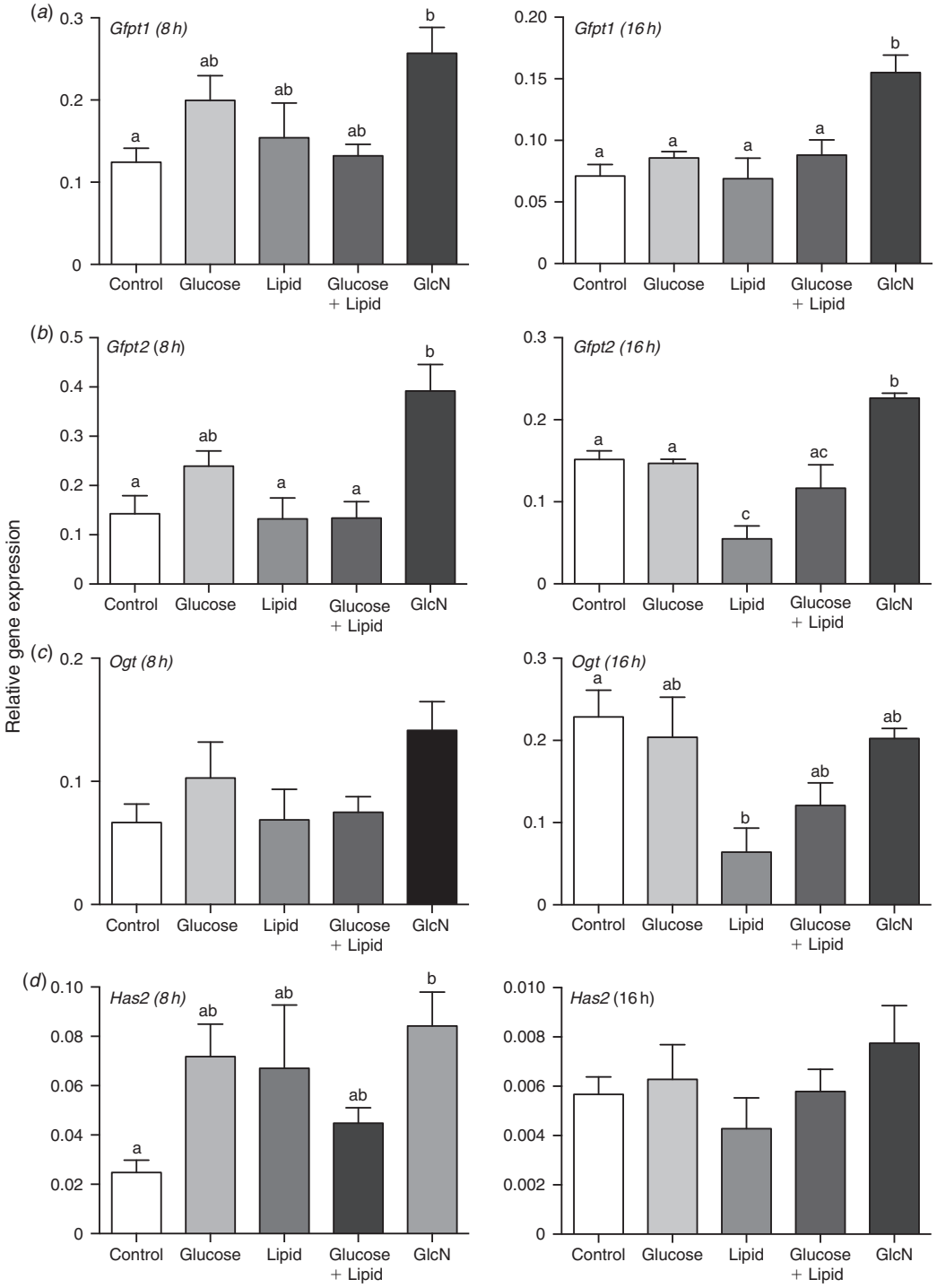

Fig. 3. Expression of mRNA of enzymes in the hexosamine biosynthetic pathway (HBP) after 8 h (left panels) and 16 h (right panels) *in vitro* maturation. Cumulus–oocyte complexes (COCs) were collected after 8 h and 16 h of culture with different treatments. Total RNA was isolated, reverse transcribed and expression of mRNA of enzymes in the HBP analysed by reverse transcription–polymerase chain reaction. (*a*) Glutamine:fructose-6-phosphate transaminase 1 (*Gfpt1*), (*b*) glutamine:fructose-6-phosphate transaminase 2 (*Gfpt2*), (*c*) *O*-linked glycosyltransferase (*Ogt*) and (*d*) hyaluronan synthase 2 (*Has2*). Data are the mean ± s.e.m., expressed as a fold change relative to *Rpl19*. Columns with different letters differ significantly ($P < 0.05$). GlcN, glucosamine.

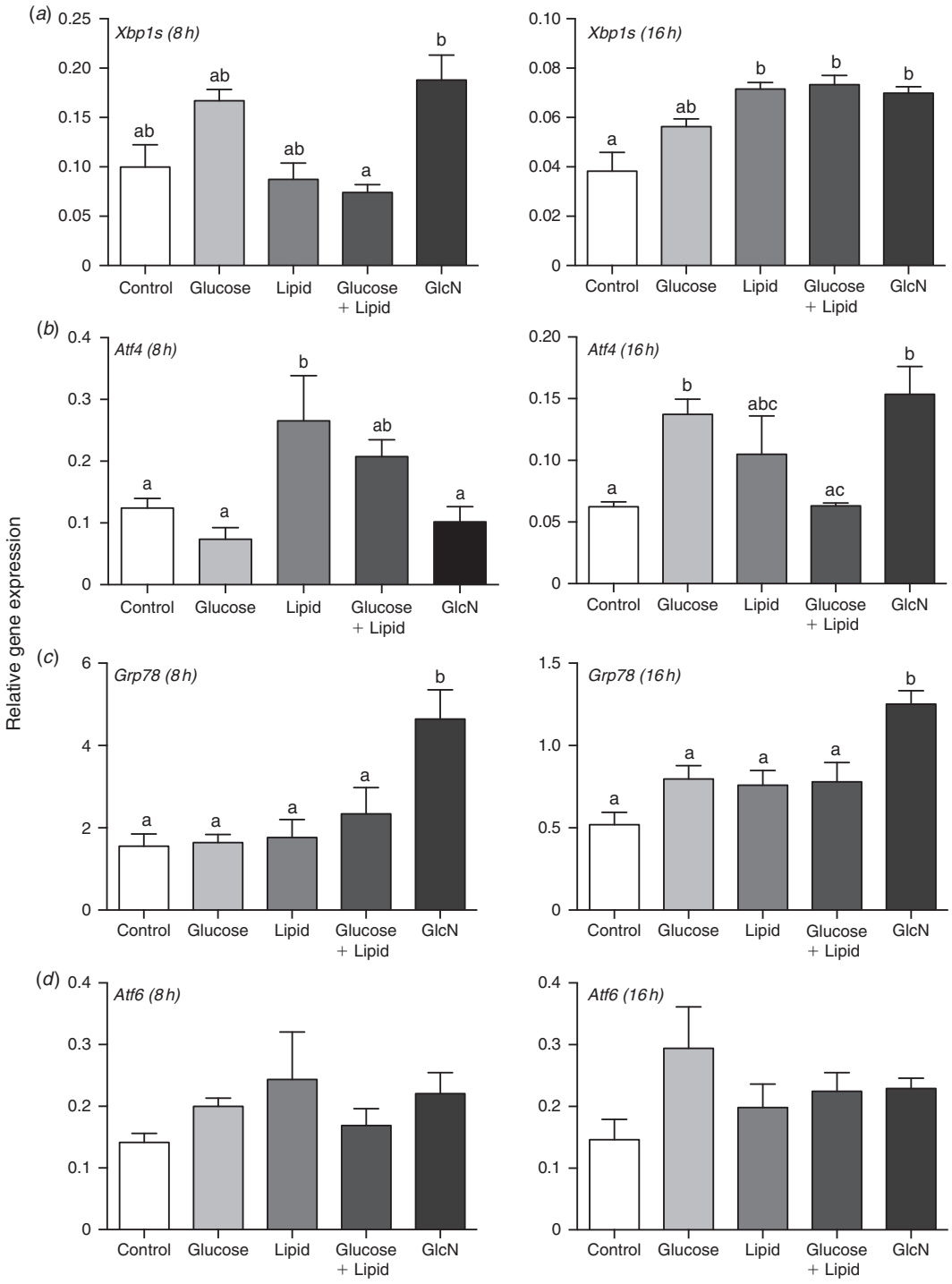

Fig. 4. Expression of endoplasmic reticulum (ER) stress marker genes after 8 h (left panels) and 16 h (right panels) *in vitro* maturation. Cumulus–oocyte complexes (COCs) were collected after 8 h and 16 h of culture with different treatments. Total RNA was isolated, reverse transcribed and expression of ER stress marker genes analysed by reverse transcription–polymerase chain reaction. (*a*) X-Box-binding protein-1 (*Xbp1*), (*b*) activating transcription factor 4 (*Atf4*), (*c*) glucose-regulated protein 78 (*Grp78*) and (*d*) activating transcription factor 4 (*Atf6*). Data are the mean ± s.e.m., expressed as a fold change relative to *Rpl19*. Columns with different letters differ significantly ($P < 0.05$, one-way ANOVA followed by a Bonferroni post hoc test). GlcN, glucosamine.

to stimulate HBP and UDP-GlcNAc production (Pantaleon *et al.* 2010; Frank *et al.* 2014*a*, 2014*b*). The addition of lipid to the medium did not increase *O*-GlcNAcylation levels. This phenomenon could be explained by the fact that opposing interactions between unsaturated fatty acids (such as oleic acid) are able to inhibit the pro-apoptotic effect of their counterpart-saturated palmitic and stearic acid (Němcová-Fürstová *et al.* 2011).

There were no additive effects of high glucose and lipid on β-*O*-GlcNAcylation. Indeed, intensity within the oocyte the in lipid and high glucose group was reduced overall compared with high glucose-treated COCs. Under the *in vitro* conditions used herein, lipid appears to play a role in downregulating cellular responses to hyperglycaemia. Hyperglycaemia is a characteristic of both types of diabetes, yet hyperlipidaemia is only associated with Type 2 diabetes. Although the present study used an *in vitro* model, we have also found that systemic administration of GlcN during the pericompaction period has significant effects on fetal survival and abnormalities, which were most evident in lean rather than obese mice, indicating the possibility that high lipid and glucose levels may interact and regulate fuel-sensing pathways, specifically the HBP (Schelbach *et al.* 2013).

Compared with the control group, GlcN treatment significantly increased mRNA expression of the rate-limiting enzymes of the HBP, namely *Gfpt1* and *Gfpt2*, after 8 and 16 h culture. Although high glucose treatment did not affect the gene expression of HBP enzymes (possibly due to glucose regulation of these enzymes being at the substrate availability and post-translational level), expression of Gfpt2 and Ogt (the enzymes that modify proteins with GlcNAc) was downregulated in the lipid-treated compared with control group, supporting the notion that lipids interact with glucose-sensing pathways. Elevated fatty acids have been shown to interfere with glucose by inhibiting glucose-induced insulin secretion and β-cell oxidation in rat pancreatic islets (Sako and Grill 1990; Zhou and Grill 1994). Moreover, free fatty acid supplementation decreases the expression of solute carrier family 2 (facilitated glucose transporter), member 2 (SLC2A2) and glucokinase (Gremlich *et al.* 1997). Therefore, downregulation of HBP enzymes in the presence of lipids may be due to impaired glucose transporter activities, reducing glucose transportation down the pathway and decreasing the production of UDP-GlcNAc, the end-product of HBP. Alternatively, the level of UDP-GlcNAc may also be limited by the known feedback inhibition of *Gfpt* by UDP-GlcNAc (Kornfeld 1967). All these interactions of lipid with glucose further support the idea that nutrient-sensing pathways are interconnected in the ovary, COC and other ovarian cells. Although O-GlcNAcylation staining revealed maximal staining in the oocyte, the differences in gene expression are likely to be within the oocyte and surrounding cumulus cells. Oocytes exhibit low transcriptional activity; however, bidirectional communication between the oocyte and cumulus cells is essential for oocyte viability (Eppig 1991; Albertini *et al.* 2001). So, changes in cumulus cells should be reflected in the oocytes.

The induction of ER stress is a well-characterised response of lipotoxicity (Borradaile *et al.* 2006; Malhi and Gores 2008). Consistent with previous studies, *Xbp1* and *Atf4* were specifically upregulated in COC following lipid treatment (Wu *et al.* 2012*b*; Yang *et al.* 2012). GlcN treatment significantly increased *Xbp1*, *Atf4* and *Grp78* expression, suggesting that the COC is undergoing a distinct UPR response or that the presence of GlcN influences the stress response. Interestingly, high glucose significantly increased the expression of *Atf4* after 16 h culture, which was downregulated in the high glucose and lipid treatment group, further emphasising that lipid appears to inhibit the effects of hyperglycaemia. In contrast, *Atf6* expression remained unaffected by all treatments at both time points. Activation of *Atf6* requires a dissociation step from its inhibitory regulator, binding immunoglobulin protein (BiP). Binding of *Atf6* to BiP was reported to be very stable (Shen *et al.* 2005) and could explain why there was no activation of this pathway in response to the treatments.

In summary, the findings of the present study demonstrate that lipid at levels of 40 μM (and GlcN) induce ER stress and that high glucose (and GlcN) increase *O*-GlcNAcylation but that the two treatments do not potentiate each other in these pathways. Furthermore, the present study suggests the possibility of lipid downregulating the detrimental consequences of hyperglycaemia on oocyte health. This study also indicates *O*-GlcNAcylation and ER stress as likely contributors to the reduced fertility observed in obese women. This provides new leads for further investigations into possible treatment strategies and interventions that may improve pregnancy and fetal outcomes in obese women and those with associated co-morbidities (e.g. diabetes).

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