In response to oxidative stress, the expression of inflammatory cytokines and antioxidant enzymes are impaired in placenta, but not adipose tissue, of women with gestational diabetes

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Abstract

In response to oxidative stress, gestational diabetes mellitus (GDM) placenta releases less 8-isoprostane and tumour necrosis factor (TNF) α . The effect of oxidative stress on other cytokines and antioxidant gene expressions are unknown. The aim of this study is to further explore the antioxidant status and effect of oxidative stress in GDM tissue. Human placenta, omental and subcutaneous adipose tissue from women with and without GDM were exposed to hypoxanthine (HX)/xanthine oxidase (XO). Cytokine release was analysed by ELISA and cytokine and antioxidant gene expression by RT-PCR. Catalase (CAT) and glutathione reductase (GSR) mRNA expression was higher in GDM ($n=18$) compared with normal ($n=23$) placenta. There was no difference in glutathione peroxidase and superoxide dismutase mRNA expression. Antioxidant gene expression was unaltered between normal ($n=18$) and GDM ($n=10$) adipose tissue. HX/XO treatment significantly

stimulated cytokine release (13/16 cytokines) and cytokine mRNA expression, and decreased antioxidant gene expression (CAT and GSR) in human placenta from normal pregnant women. In GDM placenta, HX/XO only significantly increased the release of 3/16 cytokines, while there was no effect on antioxidant gene expression. In normal and GDM adipose tissues, HX/XO increased proinflammatory cytokine and 8-isoprostane release, while there was no change in antioxidant gene expression. GDM placenta is characterised by increased antioxidant gene expression, and is less responsive to exogenous oxidative stress than tissues obtained from normal pregnant women. This may represent a protective or adaptive mechanism to prevent damage from further oxidative insult *in utero* as indicated by increased tissue antioxidant expression.

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Introduction

Gestational diabetes mellitus (GDM) is a glucose intolerance of varying severity with onset or first recognition during pregnancy that complicates ~2–4% of pregnancies (Beischer *et al.* 1996). Both patients with GDM, and their offspring, have greater risk of developing type 2 diabetes later in life (Lee *et al.* 2007). GDM is considered a pre-diabetic state or a transient unmasking of the metabolic syndrome, offering a unique opportunity to study abnormalities that may appear very early in the development of type 2 diabetes (Kautzky-Willer *et al.* 1997). Increased biomarkers of oxygen radical damage and an impairment of antioxidant defence have been identified in individuals with type 2 diabetes (Baynes 1991, West 2000) and in women with GDM (Kinalski *et al.* 2001, Coughlan *et al.* 2004a, Lappas *et al.* 2004, Biri *et al.* 2006). We have previously demonstrated that, in GDM placenta, antioxidant activity is increased (Coughlan *et al.* 2004a), and

have a reduced capacity to respond to oxidative stress in terms of 8-isoprostane (marker of lipid peroxidation) and tumour necrosis factor α (TNF α) release (Coughlan *et al.* 2004b). We concluded that GDM placenta may be pre-conditioned by transient intracellular oxidative stress, which attenuates its responsiveness to further oxidative insult. However, the effect of oxidative stress on antioxidant gene expression and other inflammatory cytokines is not known. Furthermore, the antioxidant status and effect of oxidative challenge on other GDM tissues is also yet to be elucidated.

Oxidative stress refers to a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defences (West 2000). Abnormally high levels of free radicals and the simultaneous decline of antioxidant defence mechanisms can damage cellular lipids, proteins or DNA inhibiting their normal function. Because of this, oxidative stress has been implicated in a number of human diseases. In GDM placenta, there is an increase in oxidative

stress and lipid peroxidation compared with normal pregnant women (Trocino *et al.* 1995, Coughlan *et al.* 2004a, Lappas *et al.* 2004), but there also appears to be a concomitant increase in antioxidant enzyme activity that compensates for the increased oxidative stress (Kinalski *et al.* 2001, Chaudhari *et al.* 2003, Coughlan *et al.* 2004a, Peuchant *et al.* 2004, Madazi *et al.* 2008). It is, however, not known if this increased antioxidant capacity is due to a reduction in gene expression in GDM placenta or the result of the increased biological oxidation that leads to a loss of antioxidant protein function. As there is no data available on antioxidant enzyme gene expression of placenta and other GDM tissues, the aim of this study is to compare the mRNA expression of endogenous antioxidant proteins in placenta, and subcutaneous and omental adipose tissue from healthy pregnant women and women with GDM.

Oxidative stress due to excessive ROS and weakened antioxidant defences is causally associated with inflammation and inflammatory mediators (Singh *et al.* 2005). Our previous studies have shown that xanthine oxidase (XO), which is one of the sources of free radical formation in diabetes (Desco *et al.* 2002), increases 8-isoprostane and TNF α release in normal placenta but not in GDM placenta (Coughlan *et al.* 2004b). To further elucidate the mechanisms that mediate GDM-induced differences in placental tissue in response to oxidative stress, we tested the effect of XO on antioxidant capacity and a panel of inflammatory cytokines in placenta obtained from normal pregnant women and women with GDM. Additionally, to elucidate if an oxidative challenge elicits similar effects in other GDM tissues, the effect of XO treatment on subcutaneous and omental adipose tissue antioxidant gene expression and cytokine release from women with and without GDM will also be investigated. It should be noted, that in our previous studies, the experiments were performed in the presence of very high oxygen concentrations (95% O₂), which we previously have shown to induce inflammation, oxidative stress and apoptosis (Reti *et al.* 2007). Therefore, in this study, placenta will be incubated in the 8% O₂ (as the *in utero*-foetal organs are exposed to relatively hypoxic tensions (Evans *et al.* 1986)), and adipose tissue in the presence of 21% O₂.

Materials and Methods

Tissue collection and preparation

Human placenta, subcutaneous adipose tissue (from the anterior abdominal wall) and omental adipose tissue were obtained from a total of 40 pregnant women (23 healthy pregnant women and 17 with GDM) at the time of term Caesarean section before the onset of labour (Table 1). Indications for Caesarean section included repeat Caesarean section or breech presentation. Women with any adverse underlying medical condition (i.e. including asthma,

preeclampsia and pregestational diabetes) were excluded. All pregnant women were screened for GDM, and women participating in the normal group had a negative screen. Women with GDM were diagnosed according to the criteria of the Australasian Diabetes in Pregnancy Society by either a fasting venous plasma glucose level of ≥ 5.5 mmol/l glucose, and/or ≥ 8.0 mmol/l glucose 2 h after a 75 g oral glucose load at ~ 28 weeks of gestation. All women with GDM were prescribed insulin in addition to dietary management. Approval for this study was obtained from the Mercy Hospital for Women's Research and Ethics Committee and informed consent was obtained from all participating subjects.

Tissues were obtained within 10 min of delivery and dissected fragments were placed in ice-cold DMEM (containing 5 mM glucose, 100 U/ml penicillin G and 100 μ g/ml streptomycin). A placental lobule (cotyledon) was removed from the central region of the placenta, the basal plate and chorionic surface were removed from the cotyledon, and villous tissue was obtained from the middle cross section. Placental tissues were blunt dissected to remove visible connective tissue and calcium deposits. Adipose tissue was thoroughly washed in ice-cold PBS to remove blood, and then cut into 2 mm² pieces. A piece of the tissue was snap frozen in liquid nitrogen and stored at -80°C until required for RNA extraction. The remaining tissue was placed in DMEM at 37°C in a humidified atmosphere of 5% CO₂, 8% O₂ for placenta and 21% O₂ for adipose tissue for 1 h. Explants were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (100 mg wet weight/well). The explants were incubated in 2 ml DMEM. The procedure for each tissue performed in duplicate for each treatment. To induce oxidative stress, explants were exposed to 0.5 mM hypoxanthine (HX) and 15 mU/ml XO in a humidified atmosphere of 5% CO₂ and 8% O₂ for placenta, and 5% CO₂ and 21% O₂ for adipose tissue for 24 h. The concentrations of HX and XO were based on the results obtained from our previously published studies (Coughlan *et al.* 2004a). After the 24 h incubation, tissue and medium was collected separately and stored at -80°C .

Table 1 Characteristics of the study group. Values represent mean $\pm$ S.E.M.

	Control patients (n=23)	GDM patients (n=17)
Maternal age (years)	31.8 $\pm$ 0.8	32.6 $\pm$ 1.0
Maternal BMI (kg/m ²) ^a	28.9 $\pm$ 1.5	30.0 $\pm$ 2.0
Gestational age at birth (weeks)	38.8 $\pm$ 0.1	38.2 $\pm$ 0.2
Foetal birth weight (g)	3408 $\pm$ 88	3314 $\pm$ 98
Fasting plasma glucose (mmol/l)	4.4 $\pm$ 0.1	4.9 $\pm$ 0.1*
1 h plasma glucose (mmol/l)	6.7 $\pm$ 0.4	10.4 $\pm$ 0.3*
2 h plasma glucose (mmol/l)	5.5 $\pm$ 0.2	8.8 $\pm$ 0.3*

NS, not significant. *P<0.05 versus normal patients.
^aBased on first antenatal visit at ~ 12 weeks.

Validation of explant cultures and viability

To determine the effect of treatment on cell membrane integrity, the release of the intracellular enzyme lactate dehydrogenase (LDH) into incubation medium was determined as previously described (Lappas *et al.* 2004). LDH release was investigated over the 24 h time course of tissue explants. Explants were incubated in either medium alone or medium containing 0.5 mM HX and 15 mU XO. Neither *in vitro* incubation nor experimental treatment significantly affected LDH activity in the incubation medium (data not shown). These data indicate that the concentrations used in this study did not affect cell viability.

Cytokine assays

For placenta After 24-h incubation, the explant incubation medium was collected and the release of cytokines was performed using the Bio-Plex suspension assay system and 17-plex human cytokine assay kit (Bio-Rad Laboratories). The system uses fluorescent-coded polystyrene beads, each of which is conjugated with a specific antibody and used to create a sandwich immunoassay. Sample and fluorochrome-conjugated antibody are allowed to react with the antibody-conjugated beads in micro plate wells. The assay utilised 50 µl incubation medium. The panel included pro- and anti-inflammatory cytokines as well as chemokines. The limit of detection varied for each cytokine and ranged from 0.2 to 3 pg/ml. For all assays, the manufacturer's specifications indicated that the intra- and interassay coefficients of variation are <10%. Cytokine assays were read using the Bio-Plex workstation (Bio-Rad Laboratories) and results analysed with Bio-Plex Manager (v3.0) software. All data were corrected for total protein and expressed as pg/mg protein. The protein content of tissue homogenates was determined using BCA protein assay (Pierce, Rockford, IL, USA), using BSA as a reference standard, as previously described (Lappas *et al.* 2004).

For adipose tissue After 24-h incubation, the explant incubation medium was collected and the release of TNFα, IL6 and IL8 was performed by sandwich ELISA according to the manufacturer's instructions (Invitrogen). ELISA assays were read using a Benchmark microplate reader (Bio-Rad Laboratories) and results analysed with Microplate Manager (v4.0) software. All data were corrected for total protein and expressed as pg/mg protein.

Figure 1 (A) Antioxidant gene expression in the placenta from healthy pregnant women ($n=23$) at term and women with GDM ($n=17$). (B) Antioxidant gene expression in the omental adipose tissue from healthy pregnant women ($n=18$) at term and women with GDM ($n=10$). (C) Antioxidant gene expression in the subcutaneous adipose tissue from healthy pregnant women ($n=18$) at term and women with GDM ($n=10$). 18S mRNA expression was used for the normalisation of the data. Gene expression is displayed as mean fold change ratio as calculated by the $2^{-\Delta\Delta C_t}$ method. All data is displayed as the mean $\pm$ S.E.M. * $P < 0.05$ versus normal antioxidant mRNA expression.

Table 2 Effect of hypoxanthine (HX)/xanthine oxidase (XO) on placental cytokine release

Analyte (pg/ml)	Normal (n=7)		GDM (n=7)	
	Basal	HX/XO	Basal	HX/XO
IL1β (IL1B)	5.6 ± 1.2	193.7 ± 61.3*	4.5 ± 1.3	60.2 ± 10.2 [†]
IL2	17.0 ± 1.9	23.9 ± 2.5*	12.8 ± 3.7	13.7 ± 3.3
IL4	12.5 ± 2.1	19.2 ± 2.1*	9.5 ± 3.2	10.5 ± 2.0
IL5	ND	ND	ND	ND
IL6 ^a	8.8 ± 2.3	37.4 ± 9.98*	6.0 ± 2.8	5.6 ± 1.3
IL7	22.8 ± 6.0	23.0 ± 4.8	14.3 ± 3.5	21.2 ± 9.4
IL8 ^a	29.6 ± 8.2	58.4 ± 9.5*	16.4 ± 5.8	13.6 ± 4.3
IL10	5.8 ± 1.0	20.8 ± 3.0*	3.9 ± 1.3	4.6 ± 1.2
IL12(p70)	17.6 ± 5.1	28.8 ± 2.9*	11.2 ± 2.3	16.4 ± 2.9
IL13	6.6 ± 1.0	5.8 ± 0.7	5.7 ± 1.4	7.2 ± 2.1
IL17	40.8 ± 7.8	86.4 ± 9.6*	27.3 ± 9.5	21.9 ± 4.9
G-CSF	955.2 ± 256.2	2144 ± 616*	865.0 ± 619.8	285.3 ± 102.6
GM-CSF	41.5 ± 5.5	61.3 ± 7.2*	29.1 ± 8.7	30.9 ± 5.7
IFNγ (IFNG)	454.0 ± 71.0	693.2 ± 82.2*	356.7 ± 109.2	387.3 ± 73.0
MCP1 (MCAF)	1523 ± 465	1745 ± 382	875 ± 145	1313 ± 373
MIP1β	298.2 ± 69.8	2673 ± 7408*	172.8 ± 45.4	472.8 ± 120.7 [†]
TNFα	209.1 ± 36.1	1023 ± 127*	169.8 ± 51.4	409.1 ± 72.9 [†]

**P* < 0.05 versus basal expression from normal placenta (paired Student's *t*-test). [†]*P* < 0.05 versus basal cytokine release from GDM placenta (paired Student's *t*-test). ND, not detected.

^aData in ng/ml.

8-Isoprostane release

The release of 8-isoprostane, prostaglandin (PG) E₂ and PGF_{2α} into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Cayman Chemical Company, Ann Arbor, MI, USA). All data were corrected for total protein and expressed as pg/mg protein.

RNA extraction and real time PCR

Total RNA was extracted from ~100 mg of tissue using Tri Reagent according to manufacturer's instructions (Sigma-Aldrich). RNA concentrations were quantified using a spectrophotometer (Smart Spec, Bio-Rad). RNA quality and integrity were determined via the A₂₆₀/A₂₈₀ ratio and agarose gels electrophoresis. One microgram of RNA was converted into cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacture's instructions. The cDNA was diluted tenfold, and 2 µl cDNA was used to perform RT-PCR using Sensimix Plus SYBR green (Quantace, Alexandria, NSW, Australia) and 100 nM the following primers: catalase (*CAT*) (Hs_CAT_1_SG QuantiTect Primer Assay, QT00079674); glutathione peroxidase (*GPX*) (Hs_GPX1_1_SG QuantiTect Primer Assay, QT00203392); glutathione reductase (*GSR*) (Hs_GSR_1_SG QuantiTect Primer Assay, QT00038325); superoxide dismutase (*SOD*) (Hs_SOD3_2_SG QuantiTect Primer Assay, QT01664327); *TNFα* (Hs_TNF_3_SG QuantiTect Primer Assay, QT01079561); *IL6* (Hs_IL6_1_SG QuantiTect Primer Assay, QT00083720); *IL8* (Hs_IL8_1_SG QuantiTect Primer Assay, QT00000322); and 18S (Hs_RRN18S_1_SG

QuantiTect Primer Assay, QT00199367). All primers were purchased from Qiagen. The specificity of the product was assessed from the melting curve analysis. RNA without reverse transcriptase during cDNA synthesis as well as PCRs using water instead of template showed no amplification. A positive control sample was also used in each run, on each plate.

Following baseline correction, the fluorescence threshold level was set during the geometric (exponential) phase of PCR amplification to generate the threshold cycle (C_t) value for each amplification curve. Average gene C_t values were normalised to the average 18S RNA C_t values of the same cDNA sample. Variations in gene expression were calculated by the comparative C_t method that compares test samples to a calibrator sample. This method uses results obtained for a uniformly expressed control gene to correct for differences in the amount of RNA present in the two samples being compared in order to generate a ΔC_t value. Fold differences were determined using the 2^{-ΔΔC_t} method (Livak & Schmittgen 2001).

Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics Plus version 3.1, Statistical Graphics Corp., Rockville, MD, USA). For expression data, Student's *t*-test was used to assess statistical significance between normally distributed data; otherwise, the non-parametric Mann-Whitney *U* (Wilcoxon) test was used. Paired Student's *t*-test was used to compare the effect of HX/XO on cytokine and antioxidant expression. Statistical difference was indicated by a *P* value of <0.05. Data are expressed as mean ± S.E.M.

Results

Participants

Demographic data of all participants involved in the investigation is shown in Table 1. There were no significant differences in maternal age, maternal BMI, foetal birth weight and gestational age between normal pregnant women and women with GDM. Fasting, 1- and 2-h plasma glucose concentrations at oral glucose tolerance test were significantly greater in women with GDM, compared with healthy pregnant women.

Antioxidant gene expression in placenta and adipose tissue

The mRNA expression of the antioxidant enzymes catalase (*CAT*), *GPX*, *GSR* and *SOD* were determined in placental tissue obtained from both women with normal glucose tolerance ($n=23$) and women with GDM ($n=17$). The gene expression of *CAT* and *GSR* were significantly higher in the placenta obtained from women with GDM compared to normal pregnant women (Fig. 1A). There was no significant difference in the mRNA expression of *GPX* and *SOD* in placental tissue between healthy pregnant and GDM participants (Fig. 1A).

The mRNA expression of the antioxidant enzymes *CAT*, *GPX*, *GSR* and *SOD* were determined in omental (Fig. 1B) and subcutaneous (Fig. 1C) adipose tissue obtained from both women with normal glucose tolerance ($n=18$) and women with GDM ($n=10$). There was no significant difference in the mRNA expression of *CAT*, *GPX*, *GSR* and *SOD* in both omental and subcutaneous adipose tissue between healthy pregnant and women with GDM.

Effect of HX/XO on placental cytokine and antioxidant expression

To investigate the effects of oxidative stress on inflammatory cytokines and antioxidant gene expression, an *ex situ* tissue explant incubation model was used in which placental was incubated in the absence (control) and presence of HX/XO. Antioxidant and cytokine gene expression was analysed by qRT-PCR, and cytokine release was measured by ELISA.

Table 2 presents a summary of the effect of HX/XO on cytokine release over 24 h from placenta from normal pregnant women and women with GDM. Only IL5 was

Figure 2 Effect of HX/XO on (A) *TNFα*, (B) *IL6*, and (C) *IL8* mRNA expression from human placenta. Human term placental obtained from both normal pregnant women and women with GDM was stimulated with 0.5 mM HX + 15 mU/ml XO for 24 h. 18S mRNA expression was used for the normalisation of the data. Gene expression is displayed as mean fold change ratio as calculated by the $2^{-\Delta\Delta C_t}$ method. All data is displayed as the mean $\pm$ S.E.M. ($n=5$ per group). * $P<0.05$ versus basal cytokine mRNA expression from normal placenta; ‡ $P<0.05$ versus basal cytokine mRNA expression from GDM placenta.

undetectable in all or most of the samples screened. This data was disregarded, as meaningful analysis of the data could not be made. Of all the other 16 cytokines assayed, there was no difference in the release between normal and GDM placenta under basal conditions. HX/XO significantly increased ($n=7$) the release of 13 cytokines in normal placenta. These cytokines were IL1B, IL2, IL4, IL6, IL8, IL10, IL12(p70), IL17, G-CSF, GM-CSF, IFNG, MIP1 β , TNF α . There was, however, no effect of HX/XO treatment on the release of IL7, IL13 and MCP1 (MCAF) from normal placenta. On the other hand, in GDM placenta, HX/XO induced a significant increase in the release of only three cytokines: IL1B, MIP1 β and TNF α ($n=7$). For IL1B, MIP1 β and TNF α , tissues obtained from women with GDM, however, were at least 2.5-fold less responsive to an oxidative challenge than tissues from normal pregnant women.

Having shown that the cytokine secretion in response to an oxidative challenge from control and GDM placenta was different, the next aim was to determine the effect of HX/XO treatment on gene expression. Figure 2A–C shows control and HX/XO-stimulated placental TNF α , IL6, and IL8 mRNA expression ($n=5$ per group). HX/XO significantly increased TNF α , IL6 and IL8 mRNA expression from normal placenta ($n=5$). However, in GDM placenta, only TNF α mRNA significantly increased in response to HX/XO treatment (Fig. 2A). GDM placental TNF α mRNA expression was threefold less responsive to an oxidative challenge than tissues from normal pregnant women. There was no effect of HX/XO treatment on IL6 (Fig. 2B) and IL8 (Fig. 2C) gene expressions from normal GDM placenta.

The effect of HX/XO treatment on antioxidant gene expression in normal and GDM placenta over 24 h is shown in Fig. 3A–D. In normal placenta, HX/XO significantly decreased ($n=6$) CAT (Fig. 3A) and GSR (Fig. 3C) gene expression in normal placenta, but not GDM placenta. There was no effect of HX/XO on GPX gene expression in normal and GDM placenta (Fig. 3B). HX/XO increased SOD mRNA expression in both normal and GDM placenta (Fig. 3D), however, this did not reach statistical significance.

Effect of HX/XO on adipose tissue cytokine and antioxidant expression

To investigate the effects of oxidative stress on inflammatory cytokines and antioxidant gene expression, an *ex situ* tissue explant incubation model was used in which subcutaneous

Figure 3 Effect of HX/XO on antioxidant enzyme mRNA expression from human placenta. Human term placental obtained from both normal pregnant women and women with GDM was stimulated with 0.5 mM HX + 15 mU/ml XO for 24 h. 18S mRNA expression was used for the normalisation of the data. Gene expression is displayed as mean fold change ratio as calculated by the $2^{-\Delta\Delta C_t}$ method. All data is displayed as the mean $\pm$ S.E.M. ($n=6$ per group). * $P < 0.05$ versus basal antioxidant mRNA expression from normal placenta.

and omental adipose tissues were incubated in the absence (control) and presence of HX/XO. Cytokine release was measured by ELISA and antioxidant gene expression was analysed by qRT-PCR.

Figure 4 Effect of HX/XO on (A) TNF α , (B) IL6, and (C) IL8 release from human omental adipose tissue. Human term omental adipose tissue obtained from both normal pregnant women and women with GDM were stimulated with 0.5 mM HX + 15 mU/ml XO for 24 h. All data is displayed as the mean $\pm$ S.E.M. ($n=6$ per group). * $P<0.05$ versus basal cytokine release from normal omental adipose tissue; ** versus $P<0.05$ basal cytokine release from GDM omental adipose tissue. ND, not detected. Similar results were obtained for subcutaneous adipose tissue (data not shown).

Control and oxidant (HX/XO)-stimulated TNF α , IL6, and IL8 release into the incubation medium for omental adipose tissue over 24 h is shown in Fig. 4A–C. Basal release of TNF α into the media was undetectable (Fig. 4A) and there was no difference in the release of IL6 (Fig. 4B) and IL8 (Fig. 4C) from normal or GDM omental adipose tissue under basal conditions. Adipose tissue explants that were exposed to an oxidative challenge (HX/XO) released significantly more TNF α , IL6 and IL8 compared with basal release in both normal ($n=6$) and GDM omental adipose tissue explants ($n=6$). There was no difference in the magnitude of the cytokine response to HX/XO between normal and GDM omental adipose tissue. Similar results were obtained for subcutaneous adipose tissue (data not shown).

In omental adipose tissue obtained from both normal and GDM women, HX/XO treatment had no effect on *CAT*, *GPX*, *GSR* and *SOD* gene expression ($n=5$ per group). The effect of HX/XO treatment on antioxidant gene expression omental adipose tissue (combined normal and GDM data) over 24 h is shown in Fig. 5. Similar results were obtained for subcutaneous adipose tissue (data not shown). The effect of HX/XO treatment on 8-isoprostane release was also investigated the data shown in Fig. 6. HX/XO treatment significantly increased 8-isoprostane release from omental adipose tissue obtained from both normal and GDM women. There was no difference in the magnitude of response to HX/XO between normal and GDM omental adipose tissue.

Figure 5 Effect of HX/XO on antioxidant enzyme mRNA expression from human omental adipose tissue. Omental adipose tissue was stimulated with 0.5 mM HX + 15 mU/ml XO for 24 h. 18S mRNA expression was used for the normalisation of the data. Gene expression is displayed as mean fold change ratio as calculated by the $2^{-\Delta\Delta C_t}$ method. As the antioxidant response between normal and GDM patients was the same, the data were combined data and displayed as the mean $\pm$ S.E.M. ($n=5$ normal and $n=5$ GDM). Similar results were obtained for subcutaneous adipose tissue (data not shown).

Figure 6 Effect of HX/XO on 8-isoprostane release from human omental adipose tissue. Omental adipose tissue was stimulated with 0.5 mM HX + 15 mU/ml XO for 24 h. All data is displayed as the mean $\pm$ S.E.M. ($n=3$ per group). * $P<0.05$ versus basal 8-isoprostane release from normal adipose tissue; ‡ $P<0.05$ versus basal 8-isoprostane release from GDM adipose tissue. Similar results were obtained for subcutaneous adipose tissue (data not shown).

Discussion

In this study, we have shown that the placenta obtained from women with GDM is characterised by increased antioxidant gene expression. In keeping with this, we have confirmed that GDM placenta is less responsive to an oxidative challenge than placental tissue from normal women. In normal placenta, HX/XO induced a significant increase in cytokine release (IL1B, IL2, IL4, IL6, IL8, IL10, IL12(p70), IL17, G-CSF, GM-CSF, IFN γ , MIP1 β and TNF α) and mRNA expression (TNF α , IL6 and IL8). This was associated with a decrease in *CAT* and *GPX* gene expression. In contrast, in GDM placenta, HX/XO induced a significant increase in the release of only three of the 16 cytokines assayed (IL1B, MIP1 β and TNF α). Additionally, in GDM placenta, there was no effect of HX/XO on antioxidant gene expression. In contrast to the results obtained for placenta, there is no difference in antioxidant gene expression in omental and subcutaneous adipose tissue obtained from normal and GDM patients. Furthermore, both the subcutaneous and omental adipose tissue from women with and without GDM responded similarly to an oxidative challenge. Specifically, HX/XO significantly stimulated proinflammatory cytokine release (TNF α , IL6 and IL8) from both normal and GDM adipose tissues. Although there was no effect of HX/XO on antioxidant gene expression, oxidative challenge did stimulate 8-isoprostane release equally in normal and GDM omental and subcutaneous adipose tissue.

In this study, the response of normal and GDM placenta to HX/XO was analysed in 16 cytokines. In response to oxidative stress, the release of 13 out of the 16 cytokines was significantly upregulated in placental extracts from normal

pregnant women, whereas in GDM placental extracts, the release of only three of the cytokine was significantly increased. Furthermore, for these three cytokines, the magnitude of cytokine response was higher from normal placental extracts. The placental cytokine results obtained in this study are consistent with our previous studies demonstrating that when the placental tissue was subjected to HX/XO system, TNF α release increased by 20-fold from normal pregnant women and only fourfold from women with GDM (Coughlan *et al.* 2004b). In contrast to our previous findings (Coughlan *et al.* 2004b), in this study HX/XO induced a significant increase in IL6 and IL8 release from normal placental extracts. It is of note that in this previous study, tissues were incubated under extremely hyperoxic conditions (95% O₂), whereas in the present study, placental tissue explants were performed in the presence of 8% O₂. Nevertheless, in GDM placental extracts, cytokine release was blunted in response to an oxidative challenge. The reduced cytokine response to oxidative stress by GDM placental extracts may be a consequence of decreased capacity to synthesise and thus release cytokines. In support of this, in this study, we have shown that HX/XO significantly increased cytokine mRNA expression in normal placental extracts, but not in GDM placental extracts. In marked contrast, adipose tissue obtained from women with and without GDM both respond to HX/XO by increasing TNF α , IL6 and IL8 cytokine release.

The cytokines that were induced by GDM represent early phase response genes that can then activate a number of intracellular signalling pathways including nuclear factor κ B (NFKB; Lappas *et al.* 2006). NFKB is a ubiquitous and inducible, transcription factor that is a central regulator of immune and inflammatory responses, cell adhesion, differentiation, redox metabolism and apoptosis (Courtois & Gilmore 2006). We have previously shown that in human placenta, NFKB is activated in response to oxidative stress (Lappas *et al.* 2003, Coughlan *et al.* 2004b), proinflammatory cytokines (Lappas *et al.* 2006), and adipokines (Lappas *et al.* 2005), and this is associated with increased expression of proinflammatory mediators (Lappas *et al.* 2003, 2006, Coughlan *et al.* 2004b, Courtois & Gilmore 2006, Lappas & Rice 2007). This positive regulatory loop may amplify and perpetuate local inflammatory reactions. However, and in keeping with the data shown in this manuscript, we have also previously demonstrated that GDM is associated with decreased NFKB activity and that HX/XO stimulated the activation of NFKB in placenta from healthy pregnant women but not women with GDM (Coughlan *et al.* 2004b).

Our previous studies demonstrate that when placental tissue was subjected to oxidative stress, 8-isoprostane release increased by twofold in normal pregnant women, but was unchanged in GDM (Coughlan *et al.* 2004b). Further to this, in this study we have shown that placental extracts from normal pregnant women, *CAT* and *GSR* mRNA expression were significantly decreased by HX/XO. There was, however, no effect of HX/XO on antioxidant gene

expression in GDM placental extracts. In contrast, although oxidative stress did not have any effect on antioxidant gene expression, there was increased 8-isoprostane release in adipose tissue obtained from both normal and GDM pregnant women. The observed changes in the antioxidant defence system are tissue specific, but it is evident that in both normal placenta, and normal GDM adipose tissue, induction of oxidative stress displaces the prooxidant–antioxidant balance of this defence system, by increasing the prooxidants while depleting the antioxidant capacities.

The ability of cells to accommodate oxidative stress may be enhanced by pre-exposure or pre-conditioning to a mild oxidative challenge, thus, inducing resistance to subsequent oxidative stress (Lu *et al.* 1993, Wiese *et al.* 1995, Lee & Um 1999, Lappas & Rice 2007). Changes in antioxidant concentrations in various tissues from experimental animal models of diabetes are reported, with the changes being related to the capacity of the tissues to adapt to oxidative stress. Some tissues, in response to diabetes, overexpress the genes for the antioxidant enzymes, whereas other tissues are more susceptible to oxidative damage (reviewed in Maritim *et al.* (2003)). It has also been proposed that in the early stages of diabetes there may be an initial elevation in antioxidant enzymes to counteract oxidative stress, whereas chronic diabetes continually depletes the sources of antioxidant enzymes. Thus, the blunted responses to oxidative stress by GDM placenta may be due to increased antioxidative capacity in these tissues. Although GDM is associated with increased *in vivo* oxidative stress, as evidenced by increased lipid peroxidation products in GDM placental tissue (Kinalski *et al.* 2001, Coughlan *et al.* 2004a, Lappas *et al.* 2004), studies on the tissue antioxidant enzyme activities in GDM placenta do not provide a consistent profile (Pustovrh *et al.* 2000, Kinalski *et al.* 2001, Coughlan *et al.* 2004a, Biri *et al.* 2006). However, in this study, we show that GDM placenta is associated with increased mRNA expression of *CAT* and *GSR*, the two antioxidant enzymes that decreased in response to HX/XO in normal placenta. In adipose tissue from normal and GDM patients, however, there was no difference in antioxidant gene expression. Oxidative stress can cause vascular dysfunction in the placenta, leading to foetal compromise (Myatt *et al.* 2000). Thus, elevation of ROS in GDM placenta may induce pathophysiological effects that contribute to adverse pregnancy outcome. However, the elevated antioxidant gene expression in GDM placental may represent an adaptive mechanism protecting the foetus from ROS-induced damage.

In summary, in normal placenta, and normal GDM adipose tissue, but not GDM placenta, HX/XO exerts prooxidative and proinflammatory actions by increasing the release of proinflammatory mediators and ROS. Our data support the hypothesis that placenta from women with GDM display a reduced capacity to respond to oxidative stress. The blunted response to oxidative stress by GDM placenta may be a consequence of enhanced gene expression of antioxidant enzymes. Thus, GDM placental tissues

have been pre-exposed and/or adapted to oxidative stress, and thus may be better prepared to accommodate an oxidative challenge.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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