Gestational diabetes impairs Nrf2-mediated adaptive antioxidant defenses and redox signaling in fetal endothelial cells in utero

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Abstract

In utero exposure to gestational diabetes mellitus (GDM) is associated with an increased risk of type 2 diabetes and cardiovascular disease in later life, yet the underlying mechanisms remain to be elucidated. We examined the effects of GDM on the proteome, redox status and nuclear factor erythroid 2-related factor 2 (Nrf2) mediated antioxidant gene expression in human fetal endothelial cells. Proteomic analysis revealed that proteins involved in redox homeostasis were significantly altered in GDM and associated with increased mitochondrial superoxide generation, protein oxidation, DNA damage and diminished glutathione synthesis. In GDM cells, the lipid peroxidation product 4-hydroxynonenal (HNE) failed to induce nuclear Nrf2 accumulation and mRNA and/or protein expression of Nrf2 and its target genes NAD(P)H:quinone oxidoreductase 1 (NQO1), Bach1, cystine/glutamate transporter xCT and glutamate cysteine ligase. Although methylation of CpG islands in Nrf2 or NQO1 promoters was unaltered by GDM, decreased DJ-1 and increased p~GSK3β levels may account for impaired Nrf2 signaling. HNE-induced increases in GSH and NQO1 levels were abrogated by Nrf2 siRNA in normal cells, and overexpression of Nrf2 in GDM cells partially restored NQO1 induction. Dysregulation of Nrf2 in fetal endothelium may contribute to the increased risk of type 2 diabetes and cardiovascular disease in offspring.

Keywords: Fetal endothelium; Nrf2-Keap1; NQO1; Bach1; GCLM; cystine/glutamate transporter; G6PD; DJ-1; GSK3β; Gestational diabetes
## Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>Bach1</td>
<td>BTB and CNC Homology 1</td>
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<td>DIGE</td>
<td>Differential in-gel electrophoresis</td>
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<tr>
<td>DJ-1</td>
<td>Parkinson’s disease associated protein</td>
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<tr>
<td>EpRE</td>
<td>Electrophile response element</td>
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<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
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<tr>
<td>GCLM</td>
<td>Glutamate-cysteine ligase regulatory subunit</td>
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<td>GDM</td>
<td>Gestational diabetes mellitus</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
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<td>GST</td>
<td>Glutathione-S-transferase</td>
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<td>HNE</td>
<td>4-hydroxynonenal</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<tr>
<td>Keap1</td>
<td>Kelch-like ECH associated protein 1</td>
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<tr>
<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NQO1</td>
<td>NAD(P)H-quinone oxidoreductase-1</td>
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<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
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<tr>
<td>PDIA3</td>
<td>Protein disulfide isomerase A3</td>
</tr>
<tr>
<td>Prx</td>
<td>Peroxiredoxin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>xCT</td>
<td>Cystine–glutamate amino acid transporter</td>
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Endothelial cells are primary targets of hyperglycemia induced oxidative damage (1), and elevated intracellular glucose levels increase mitochondrial superoxide generation, leading to activation of the polyol and hexosamine pathways and ultimately endothelial dysfunction (2). The risk of hypertension is increased in diabetic patients (3), and interestingly endothelial dysfunction correlates significantly with insulin resistance in young offspring of first degree relatives of patients with type 2 diabetes (4).

Gestational diabetes mellitus (GDM) is defined as glucose intolerance first diagnosed during pregnancy and affects ~3-10% of all births (5). Similar to type 2 diabetes, fetal exposure to GDM is strongly associated with a higher risk of insulin resistance in adulthood (6-8) with some but not all studies confirming elevated blood pressure and other metabolic risk factors in GDM offspring (9,10). Streptozotocin-induced diabetes in animal models has been reported to elevate blood pressure and reduce endothelium-dependent vasodilation in offspring (11), potentially a consequence of in utero fetal programing (5-7).

We previously reported that fetal umbilical vein endothelial cells (HUVEC) cultured from GDM pregnancies exhibit reduced rates of cell proliferation and protein and DNA turnover, which are maintained during culture in vitro (12). Our findings of a sustained membrane hyperpolarization, altered Ca$^{2+}$ signaling and insulin resistance provided further evidence for an altered phenotype of GDM HUVEC (12,13). Levels of reactive oxygen species (ROS) and lipid peroxidation products are elevated in cord blood (14) and the embryo (15) of pregnancies affected by diabetes. However, the consequences of increased placental oxidative stress in GDM (5) on the redox environment of fetal endothelial cells, a potential factor influencing the risk of vascular disease in later life, remain
The redox sensitive Nuclear Factor-E2-Related Factor 2 (Nrf2)/Kelch-like ECH Associated Protein 1 (Keap1) pathway plays a key role in transcriptional activation of antioxidant defense genes and restoration of vascular redox homeostasis (16,17). Nrf2 is normally targeted for proteasomal degradation via its cytosolic binding protein Keap1 (18) but in response to oxidative or electrophilic stress accumulates in the nucleus and binds to the antioxidant/electrophile response element (ARE/EpRE) in the promoter region of genes encoding phase II detoxifying enzymes, such as NAD(P)H quinone oxidoreductase 1 (NQO1) (19), and glutamate cysteine ligase (GCLM) and the cystine/glutamate transporter (xCT) involved in glutathione synthesis (16,20).

We sought to determine the effects of GDM on the redox status and Nrf2 antioxidant defenses in fetal endothelial cells challenged with the lipid peroxidation product 4-hydroxynonenal (HNE). We report the first proteomic analysis of HUVEC from normal and GDM pregnancies and demonstrate that GDM alters proteins involved in oxidative stress and Nrf2-linked phase II enzyme detoxification and GSH synthesis. We also identified markers of oxidative stress in GDM HUVEC, including increased mitochondrial superoxide generation, protein carbonylation and DNA damage, consistent with a pro-oxidative redox environment \textit{in utero} in GDM pregnancies. Although basal gene expression was altered negligibly by GDM, nuclear translocation of Nrf2 and induction of its target genes NQO1, Bach1, GCLM and xCT by the lipid peroxidation product HNE was abrogated in GDM cells. Our findings of decreased DJ-1 and increased p~GSK3β expression in GDM cells may contribute to the deficits in Nrf2 nuclear accumulation and signaling.

In view of accumulating evidence that pregnancies affected by GDM are associated with an increased risk of cardiovascular disease in later life (6,7), our study provides novel insights into the
mechanisms underlying dysregulation of redox homeostasis in fetal endothelium, which may contribute to endothelial dysfunction and type 2 diabetes in the offspring of GDM pregnancies (21).

**RESEARCH DESIGN AND METHODS**

Additional experimental data are provided in the Online Supplemental Materials.

**Materials.** Chemicals and tissue culture media were purchased from Sigma-Aldrich unless stated otherwise. 4-hydroxynonenal was from Alexis Biochemicals (San Diego, USA), Nucleospin 96 RNA Kit from Macherey-Nagel (Duren, Germany), QuantiTect reverse transcription and QuantiFast PCR reaction kits from Qiagen (Hilden, Germany), DNA fragmentation ELISA kit from Roche (Mannheim, Germany), TransAM Nrf2 binding activity assay kit from Active Motif (Carlsbad, USA). MitoSox Red (M36008) and Alexafluor goat anti-rabbit-488 (A11008) were from Invitrogen Molecular Probes (Carlsbad, USA). Nrf2 (SC-722, 110 kDa), NQO1 (SC-16464), Lamin A/C (SC-6254) and donkey anti-goat secondary antibody (SC-2020) from Santa Cruz (Santa Cruz, USA); DJ-1 (#2134) from Cell Signaling; G6PD (ab993) and p~GSK3β(Y216) (ab4797) from Abcam; α-tubulin (mab1864), goat anti-rabbit (abn117 HRP), goat anti-rat (abn192 HRP) and goat anti-mouse (abn106 HRP) secondary antibodies from Millipore (UK). GCLM was a gift from Terrance Kavanagh (University of Washington, USA) and AdNrf2 a gift from Jeffry Johnson (University of Wisconsin, USA).

**Patients and umbilical cords.** Umbilical cords were obtained from normal (n=55) and gestational diabetic (GDM, n=44) pregnancies from St Thomas’ Hospital (London, UK) with informed patient consent and Research Ethics approval. GDM was defined as glucose intolerance (fasting venous plasma glucose concentration ≥ 5.1 mmol/L and/or 2h venous plasma glucose concentration ≥ 8.5 mmol/L after 75g oral glucose tolerance test) first diagnosed during pregnancy. Clinical
characteristics of all subjects and pregnancies are summarised in Table I and Supplemental Table II summarizes clinical data for the proteomic analysis of normal and GDM HUVEC.

**Endothelial cell culture.** HUVEC were cultured in M199 containing 5 mM D-glucose as previously described (12). Experiments were conducted with passage 3 HUVEC serum-deprived in 1% FCS M199 for 4h prior to treatments. An endothelial phenotype was confirmed by a characteristic cobblestone morphology and von Willebrand factor immunostaining (12).

**Proteomic analysis of HUVEC from normal and GDM pregnancies.** Proteomic comparison of normal (n=5 donors) and GDM (n=5 donors) unstimulated HUVEC was conducted using differential in-gel electrophoresis (DIGE) combined with nano-liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described (22). Identified proteins were analyzed using the Ingenuity Pathway Knowledge Base (Ingenuity System, Mountain View, CA) to determine the most relevant interaction networks and biological functions. See Supplemental Table I for proteome profiling of normal versus GDM HUVEC and Supplemental Table II for clinical characteristics of the subjects included in the proteomic analysis.

Samples from different normal and GDM HUVEC cultures were paired for two-dimensional separation (23), fluorescent gel images captured using an Ettan DIGE Imager (GE Healthcare) and analyzed using Decyder software to detect differentially expressed proteins between normal and GDM samples. Spots exhibiting statistical differences ($P<0.05$, unpaired Student’s $t$-test) were excised for trypsin digestion and tandem mass spectrometry. Identification of proteins was performed using TurboSEQUEST software (Bioworks Browser version 3.3.1, Thermo Fisher) against a UniProt database with the following filter: for charge state 1, XCorr > 1.5; for charge state 2, XCorr > 2.0; for charge state 3, XCorr > 2.5.
**Intracellular GSH measurements.** HUVEC were treated with vehicle (0.08% v/v hexane) or HNE (20µmol/L) for 0-24 h before GSH extraction with 6.5% trichloroacetic acid on ice for 10 min, with total cellular glutathione (GSH) levels then measured using a fluorometric assay (16).

**DNA fragmentation assay.** DNA damage in HUVEC was assessed using a BrdU based DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit (Roche). HUVEC were incubated in 10µmol/L BrdU labeling solution for ~16h and treated for 6h with HNE (20µmol/L) or vehicle (0.08% v/v hexane) in 1% FCS M199. Cytosolic fractions containing BrdU labeled DNA fragments were extracted for ELISA, and DNA fragmentation expressed as the average optical density/mg protein.

**Protein oxidation.** Oxidation of proteins was assessed using an Oxyblot™ kit (Millipore, UK). Cell lysates were denatured and derivatized using 2,4-dinitrophenylhydrazine and carbonylated proteins detected by immunoblotting with an anti-DNP antibody relative to α-tubulin.

**Mitochondrial ROS generation.** Basal mitochondrial ROS levels were measured using MitoSox Red, a mitochondrial targeted derivative of hydroethidine, as previously described (24). Cells were loaded with MitoSox Red (5µmol/L) for 30 min and fluorescence (Ex/Em 560/625 nm) detected in 4% paraformaldehyde fixed cells using a Zeiss Axiovert 200 confocal microscope and quantified using Metafluor software and background fluorescence subtracted.

**Chemiluminescence detection of reactive oxygen species generation.** HUVEC were incubated at 37°C in Krebs buffer containing L-012 and challenged with vehicle or HNE in the absence or presence of the mitochondrial complex I inhibitor rotenone (1µM) (24). Luminescence was monitored over 40min after addition of L-012 in a microplate luminometer (Chameleon V, Hidex) and expressed per mg protein.
**Immunoblotting.** Whole cell and nuclear proteins were extracted using lysis buffer or a nuclear extraction kit (Active Motif) and denatured samples separated by gel electrophoresis and probed with primary antibodies using α-tubulin or Lamin C as reference proteins (17). Protein expression was determined by enhanced chemiluminescence with images captured in a Gel Documentation System (G-Box, Syngene Ingenius Bioimaging) and densitometry conducted using ImageJ software (NIH, USA).

**Nrf2 immunofluorescence.** Cells treated with HNE (20µmol/L) for 1-4h were fixed in PBS containing paraformaldehyde (4%), permeabilized with Triton X-100 (0.1%) and examined by immunofluorescence using an anti-Nrf2 primary and Alexa Fluor 488 secondary antibody. Nuclei were co-labeled with propidium iodide (1mg/mL, 10min). Cells were visualized in an inverted microscope (Nikon, Eclipse TE2000-U) and images captured using a CCD digital camera (Nikon, DXM1200F). Densitometry analysis is expressed as ratios of nuclear to cytosolic Nrf2 fluorescence intensity.

**Nrf2 binding activity assay.** Cells were treated with HNE (20µmol/L) for 2h, nuclear protein extracted and binding of nuclear Nrf2 to an ARE sequence (5’-GTCACAGTGACTCAGCAGAATCTG-3’) determined by ELISA (TransAM Nrf2 kit, Active Motif).

**Quantitative RT-PCR.** RNA was extracted and isolated using a Nucleospin RNA Kit (Macherey-Nagel), RNA integrity assessed by microcapillary electrophoresis using nano chip (RNA 6000 Nano Assay) and analyzed using an Agilent 2100 Bioanalyser system (Agilent Technologies, UK). Total RNA was reverse–transcribed using QuantiTect reverse transcription kit (Qiagen). Nrf2-linked and Nox4 (data not shown) gene expression was assessed using a QuantiFast
Probe PCR kit (Qiagen) in a real-time PCR system (Corbett Rotor-gene). mRNA levels were normalized to the geometric mean of three house-keeper genes: β-actin, ribosomal protein L13a and succinate dehydrogenase unit complex A. See Supplementary Table III for primer sequences.

**Nrf2 knockdown by siRNA and adenoviral overexpression of Nrf2.** Normal HUVEC were transfected with 40 pmol/24 well Nrf2 siRNA (Santa Cruz, USA) for 24h using a siRNA kit (Roche, UK) before treatment with HNE (20µmol/L) or vehicle for 20h, followed by measurements of Nrf2 and NQO1 protein or intracellular GSH levels. GDM HUVEC were transfected for 12h using adenoviral vectors (50 M.O.I) coordinating expression of AdGFP or active AdNrf2. Cells were then treated with HNE (20µmol/L) or vehicle and Nrf2 and NQO1 protein expression determined.

**Statistical analysis.** Data denote the mean ± S.E.M. of experiments with HUVEC isolated from 3-6 independent normal or GDM umbilical cords. Comparison of the proteome of normal (n=5) and GDM (n=5) HUVEC was conducted using an unpaired Student’s t-test. Comparisons of more than two conditions in the same experiment were evaluated using one-way or two-way ANOVA with Tukey’s or Bonferoni post-hoc test and \(P<0.05\) considered significant.

**RESULTS**

**Proteomic profiling of HUVEC from normal and GDM pregnancies.** The proteome of normal and GDM HUVEC was compared using DIGE (Fig. 1A), and differentially expressed proteins were identified by LC-MS/MS (Fig. 1B). As summarized in Supplemental Table I, GDM is associated with reduced levels of peroxiredoxin-1 (Prx1) and glutathione-S-transferase (GST) and increased levels of peroxiredoxin-5 (Prx5) and protein disulfide-isomerase A3 (PDIA3). Prx1 and GST are direct downstream target genes of the Nrf2/ARE pathway and decreased ratios indicate diminished
Nrf2 activity. Increased ratios for Prx5 and PDIA3, critical redox regulators in mitochondria and endoplasmic reticulum respectively, are indicative of increased oxidative stress that may affect antioxidant signaling (25,26).

When differentially expressed proteins (n=39, see Fig. 1B) were submitted to Ingenuity Pathway Analysis (Supplemental Fig. I), the computational algorithms generated a primary protein network highlighting oxidative stress, depletion of glutathione (GSH), phase II defense enzymes, PPARα/RXRα and Nrf2 mediated oxidative stress responses as key pathways altered in GDM HUVEC. These links were confirmed by a canonical pathway analysis showing that alterations in oxidative stress signaling were the major phenotypic changes detected in GDM cells (see Supplemental Table I).

**Increased mitochondrial ROS generation and protein carbonylation in GDM HUVEC.** As mitochondrial proteins were altered in GDM cells (Supplemental Table I), it is noteworthy that basal mitochondrial superoxide generation (Fig. 2A and B) and HNE stimulated superoxide generation (Fig. 2C and D) were increased in GDM HUVEC. The inhibition of HNE stimulated superoxide generation by rotenone and negligible changes in Nox4 expression (data not shown) suggests that mitochondria are the most likely source of increased ROS generation. To confirm that GDM was associated with oxidative modification of proteins, normal and GDM HUVEC lysates were immunoblotted for carbonylated proteins and, as shown in Fig. 2E, basal levels of protein oxidation were increased significantly in GDM cells.

**Increased DNA damage induced by 4-hydroxynonenal in GDM HUVEC.** GDM and diabetes are characterized by increased lipid peroxidation, and HNE is known to induce DNA damage (27,28). To determine whether GDM increased the sensitivity of fetal endothelial cells to DNA
damage, normal and GDM cells were treated with HNE (20µmol/L) or vehicle for 6h (Fig. 2D). Whilst basal DNA fragmentation was not altered, HNE-induced DNA damage was significantly elevated in GDM HUVEC, highlighting potentially compromised antioxidant defenses.

**GDM impairs HNE-induced adaptive increases in GSH synthesis and expression of xCT, GCLM and NQO1.** As depletion of GSH leads to an increased susceptibility to oxidative stress and apoptosis (21), we compared basal and HNE-stimulated GSH levels in normal and GDM cells. HNE elicited a biphasic adaptive response in GSH levels in normal HUVEC (Fig. 3A) with GSH (nmol/mg protein) initially decreasing to 52 ± 5 at 1.5 h (66 ± 5 in vehicle treated HUVEC, $P<0.05$) and then increasing to 112 ± 9 after 24 h (76 ± 5 in vehicle treated HUVEC, $P<0.01$). However, in GDM cells, basal and HNE-induced adaptive increases in GSH were significantly diminished (Fig. 3B and C). Adaptive increases in GSH involve induction of the Nrf2 regulated genes xCT and GCLM (17,20), and notably upregulation of xCT mRNA and GCLM protein and mRNA levels by HNE in normal cells was absent in GDM cells (Fig. 3D-F and Supplemental FIG II). In contrast, glucose-6-phosphate dehydrogenase (G6PD) expression and enzyme activity were unaffected by GDM (Supplemental FIG. II-C-E).

NQO1 is a key phase II defense enzyme regulated by Nrf2 (19), and induction of NQO1 in response to HNE was maximal after 12h in normal cells but notably abrogated in GDM cells (Fig. 3G and H). Similar to xCT and GCLM, NQO1 mRNA levels were not increased in GDM cells treated with HNE (Fig. 3I).

**Impaired Nrf2 activation by HNE in GDM HUVEC.** Given the deficits in GSH synthesis and absence of xCT, GCLM and NQO1 induction in GDM cells by HNE, we hypothesized that activation of Nrf2 may be compromised. Immunoblot analysis of nuclear enriched cellular fractions
confirmed that HNE increased nuclear Nrf2 levels in normal HUVEC maximally after 2h (2.2 ± 0.4 fold, \( P<0.05 \)) whereas nuclear translocation of Nrf2 was abrogated in GDM HUVEC. Moreover, HNE-induced binding of nuclear Nrf2 to an ARE consensus sequence was abolished in GDM cells (Fig. 4C). Immunofluorescence analysis of nuclear to cytosolic Nrf2 levels under basal and HNE-stimulated conditions further demonstrated that HNE induced nuclear accumulation of Nrf2 was abrogated in GDM cells (Fig. 4D).

To determine whether deficits in HNE-induced Nrf2 activation in GDM cells were due to alterations in Nrf2 expression, we measured basal and HNE induced Nrf2 mRNA and protein levels in normal and GDM cells. Basal Nrf2 expression was similar in both cell types, but HNE only enhanced total Nrf2 protein (12h) levels in normal cells (Fig. 4F). When we examined whether impaired Nrf2 activation in GDM was affected by either its cytosolic regulator Keap1 or nuclear transcriptional repressor Bach1 (29), basal mRNA levels were unaffected by GDM (Fig. 5A and B) and HNE only upregulated Bach1 mRNA in normal cells (Fig. 5B). Thus GDM cells may also have lost a key feedback mechanism to regulate Nrf2-linked gene expression via Bach1 (33).

As disruption of DJ-1, a Parkinson’s disease associated protein, leads to decreased Nrf2 protein stability and antioxidant enzyme expression (30), we measured basal DJ-1 expression in normal and GDM cells and found that DJ-1 protein levels were decreased in GDM cells (Fig. 5C). We further investigated whether GDM affects cellular levels of p~GSK3\(\beta\), known to enhance nuclear export of Nrf2 (31). Treatment of GDM HUVEC with HNE led to a significant and sustained increase in p~GSK3\(\beta\) expression, whereas p~GSK3\(\beta\) levels were unaffected in normal cells (Fig. 5D and E).

**Nrf2 siRNA abrogates HNE-induced increases in GSH and NQO1 levels in normal cells and Nrf2 overexpression upregulates NQO1 levels in GDM cells.** To confirm that adaptive increases
in GSH and NQO1 levels induced by HNE were mediated by Nrf2, we used Nrf2 siRNA to knockdown transcriptional activity in normal cells (Fig. 6A and B). Although Nrf2 gene silencing had negligible effects on basal GSH and NQO1 levels, it significantly attenuated HNE induced increases in GSH (Fig. 6C) and NQO1 expression (Fig. 6D), mimicking the phenotype of GDM HUVEC. Moreover, transient transfection of GDM HUVEC with an adenovirus coordinating overexpression of Nrf2 significantly increased basal Nrf2 and NQO1 expression in GDM cells (Fig. 6E and F), suggesting that impaired Nrf2 redox signaling in GDM HUVEC can be partially restored.

DISCUSSION

To date there are no reports of the effects of GDM on redox signaling in human fetal endothelium. The present study provides the first whole cell proteome analysis of the effects of GDM on fetal endothelial cells, characterizing phenotypic alterations in proteins involved in redox signaling. We further demonstrate that Nrf2 nuclear accumulation and Nrf2-mediated adaptive increases in GSH and induction of NQO1, xCT, GLCM and Bach1 expression by the lipid peroxidation product HNE are abrogated in GDM HUVEC. These findings are consistent with increased levels of protein oxidation, enhanced sensitivity to HNE-induced DNA damage and elevated mitochondrial ROS generation in GDM cells.

Previous studies reported decreased GSH levels in umbilical cord (14) and neonatal (15) blood from GDM pregnancies and correlated inflammatory markers such as CRP, ICAM-1, IL-6 in cord plasma with fetal triglycerides and/or neonatal fat mass (32). Moreover, adiposity in young children is strongly correlated with the extent of methylation of DNA encoding eNOS and retinoid X
receptor α in HUVEC at birth (33), suggesting that our comparison of DNA methylation (see Supplementary FIG. III) and redox regulation in normal and GDM HUVEC may provide valuable insights into epigenetic effects of GDM in utero.

Markers of oxidative stress involving both altered mitochondrial function and endoplasmic reticulum stress, as indicated by our proteomic data (see Supplementary Table I), were validated by our finding that mitochondrial ROS generation was markedly elevated in GDM cells. An elevated glucose load, leading to mitochondrial dysfunction and increased superoxide production, has been widely implicated in the development of glucose intolerance and vascular dysfunction in diabetes (2). Studies using a rodent model of intrauterine growth retardation have shown that elevated intrauterine stress and mitochondrial dysfunction are associated with type 2 diabetes and metabolic disease in offspring (25,34). In our study, fetal endothelial cells from GDM pregnancies exhibited increased basal protein carbonylation and enhanced DNA damage (Fig. 2) in response to physiologically relevant HNE concentrations that rarely exceed 100µM in cells and tissues (28).

Our proteomic network analysis revealed that GSH and phase II detoxification pathways were altered in GDM cells, suggesting that Nrf2 activation may be compromised in GDM. As HNE mediated adaptive increases in GSH and xCT and GCLM mRNA and/or protein levels were significantly decreased in GDM cells, these findings highlight the importance of Nrf2 and enzymes related to GSH synthesis in promoting cell survival and adaptation in oxidative stress (25). Although lower basal GSH levels in GDM HUVEC may contribute to the increased sensitivity to HNE-mediated damage, adaptive increases in Nrf2-linked gene expression in normal HUVEC appear to be independent of mitochondrial ROS generation (Fig. 2). In normal HUVEC, initial (1.5 h) depletion of intracellular GSH was paralleled by maximal Nrf2 nuclear translocation over 1-2 h,
suggesting that HNE-induced Nrf2 activation is a consequence of the altered cellular redox environment rather than a direct effect of acute ROS production \textit{per se}. Cardiomyocytes exposed to HNE exhibit higher levels of apoptosis in the absence of adaptive increases in GSH (35), findings consistent with our observation of increased HNE-induced DNA damage in GDM HUVEC. As HNE mediated induction of xCT, GCLM, NQO1 and Bach1 was abrogated in GDM cells, it seems unlikely that impaired activation of Nrf2 in GDM cells was due to down-regulation by Bach1 (29).

Disruption of DJ-1 leads to decreased Nrf2 stability and antioxidant enzyme expression (30), and reduced DJ-1 levels in GDM HUVEC may explain the lack of Nrf2-linked gene induction by HNE. Moreover, increased p-GSK3β levels in GDM HUVEC (Fig. 5) may also contribute to impaired Nrf2-mediated antioxidant gene expression in GDM HUVEC, since activated GSK3β has been reported to phosphorylate Fyn tyrosine kinase leading to enhanced nuclear export of Nrf2 and proteasomal degradation via the adaptor protein β-TrCP independent of Keap1 (36,37). As GSK-3β activity is increased in fibroblasts from rats with type 2 diabetes (38), it is possible that HNE stimulated nuclear accumulation of Nrf2 and induction of NQO1, xCT, GCLM and Bach1 in GDM endothelial cells are negatively regulated by GSK-3β activation and/or the loss of DJ-1.

Induction of NQO1 by HNE is primarily mediated by Nrf2 (19), and in this context we demonstrated that knockdown of Nrf2 in normal HUVEC abrogates HNE-induced increases in GSH levels and NQO1 expression, while overexpression of Nrf2 in GDM cells partially restores NQO1 levels (Fig. 6). NQO1 null mice exhibit insulin resistance accompanied by higher levels of glucose, triglycerides, lactate and pyruvate in liver (39), whereas induction of NQO1 suppresses oxidative stress and lipid peroxidation (19), underpinning the importance of NQO1 in cellular defense against oxidative stress.
There are conflicting reports concerning the activation of Nrf2 in endothelial cells exposed to hyperglycemia. Acute exposure of human dermal microvascular endothelial cells to high glucose (30 mM) causes negligible changes in nuclear accumulation of Nrf2 and NQO1 expression (40), whilst elevated glucose enhances Nrf2/ARE-driven luciferase activity and NQO1 mRNA levels in human coronary artery endothelial cells (41). However, in both these studies Nrf2 knockdown exacerbated glucose induced ROS generation. We found that elevated glucose (25 mM, 24h) elicits only marginal increases in ROS generation and Nrf2-mediated antioxidant responses in HUVEC and observed negligible differences between normal and GDM cells (data not shown). Although Nrf2 null mice are not diabetic, they exhibit significantly increased glucose intolerance, urine output and serum ketones and triglycerides following streptozotocin induced diabetes (39). Conversely, chemical inducers of Nrf2 markedly attenuate vascular dysfunction, glucose intolerance and prevent obesity and dyslipidemia in mice fed a high fat diet (42).

Epigenetic modifications have been implicated in altered prenatal programming (33,34,43), and may potentially modulate Nrf2-linked antioxidant responses in GDM endothelial cells. Although methylation of CpG islands in the Nrf2 promoter appear to inhibit transcriptional activity in Transgenic Adenocarcinoma Mouse Prostate (TRAMP) tumor cells (44), we found no significant differences in the methylation status of CpG islands in the promoters of Nrf2 (see Supplemental Fig. II) or NQO1 (data not shown) between normal and GDM cells. Serum levels of some miRNAs are decreased in mothers with GDM compared to normal pregnancies (45), and miRNAs may in part be involved in developmental priming of type 2 diabetes (46). By targeting “epigenetic machinery”, such as DNA methyltransferases and histone deacetylases (HDACs), miRNAs may affect Nrf2 mediated antioxidant gene expression and thereby influence the risk of cardiovascular disease.
Vascular Nrf2 levels and adaptive antioxidant defenses have been shown to decline with aging (24). As we have demonstrated that Nrf2 signaling is impaired in GDM fetal endothelial cells and others have reported that vascular cells in aged rodents (47) exhibit altered Nrf2 signaling, we hypothesize that the GDM endothelial redox phenotype may reflect in utero aging as a consequence of sustained oxidative stress. Premature aging in utero has been implicated in spontaneously hypertensive rats (SHR), in which a diabetic intrauterine environment was associated with a significantly reduced lifespan of offspring (48).

As illustrated in Fig. 7, we speculate that intrauterine exposure to maternal diabetes (GDM) alters the redox proteome of fetal endothelial cells, leading to impaired Nrf2-mediated antioxidant defenses. Nrf2 plays an important role in maintaining mitochondrial function (22,23) and protecting cells against ER stress (28). Persistent deficits in redox signaling in fetal endothelial cells in GDM will undermine cellular defenses against oxidative stress in utero, predisposing offspring to an increased risk of type 2 diabetes and cardiovascular disease in later life. The Nrf2 antioxidant defense pathway may therefore provide a therapeutic target for ameliorating oxidative stress associated with diabetes, aging and nephropathy (49).

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X.C., S.J.C., B.P., W.P. researched the data. X.P. and M.M. provided advice for the proteomic data analysis and interpretation. D.S. provided advice for qPCR measurements. G.E.M. and R.C.M.S. designed the research and all authors edited the draft manuscript. G.E.M. is the guarantor
of this work and assumes full responsibility for the integrity of the data and accuracy of the data analysis.

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Figure Legends

FIG. 1. Proteomic analysis of normal and GDM HUVEC using differential in-gel electrophoresis. A: Representative image of differential in-gel electrophoresis (DIGE). Proteins from unstimulated normal and GDM HUVEC are stained with Cy3 (red) and Cy5 (green), respectively. B: Fluorescent images of DIGE gels shown in panel A were analyzed using DeCyder 7.0 software to detect differentially expressed proteins in GDM HUVEC (n=5 pairs of HUVEC from different normal and GDM donors). Differentially expressed proteins were excised and identified by liquid chromatography tandem mass spectroscopy. The numbered spots show significant differences for 39 proteins (ratio of normal/GDM>1.1 or <1.1, P<0.05, unpaired Student’s t-test). Results are summarized in Supplemental Table I.

FIG. 2. Increased mitochondrial ROS generation, protein oxidation and HNE-induced DNA fragmentation in GDM HUVEC. Normal and GDM cells were loaded with MitoSox Red and mitochondrial reactive oxygen species (ROS) generation measured by fluorescence. A-B: Representative basal MitoSox Red fluorescence (grey scale) images in cells from normal (n=6) or GDM (n=6) donors, with average fluorescence obtained by densitometry from >100 cells on duplicate coverslips for each culture. C-D: Basal and HNE (20µmol/L) stimulated ROS generation in normal and GDM HUVEC measured by L-012 chemiluminescence in absence or presence of mitochondrial complex I inhibitor rotenone (1µmol/L). E: Basal protein carbonylation in DNPH-derivatized extracts expressed relative to α-tubulin in cells from normal (n=3) and GDM (n=3) donors. Representative immunoblot of normal versus GDM lysates run on a single membrane with non-relevant lanes omitted shown in Supplemental FIG. II A. F: Cells treated for 6h with HNE
(20µmol/L) or vehicle (0.08% v/v hexane) and DNA fragmentation measured in 4 replicates per culture from different normal (n=5) and GDM (n=5) donors. Absorbance expressed per mg protein. Data denote mean ± S.E.M., *P<0.05, **P<0.01, ***P<0.001.

**FIG. 3.** HNE stimulated adaptive increases in GSH levels and xCT, GCLM and NQO1 expression are abrogated in GDM HUVEC. Time-dependent changes in intracellular total glutathione (GSH) levels in normal (A) and GDM (B) HUVEC treated with HNE (20µmol/L) or vehicle. C: Basal and HNE stimulated GSH levels in normal and GDM HUVEC after 24h treatment with HNE (20µmol/L) or vehicle. D-F: Basal and HNE (20µmol/L) stimulated xCT mRNA (4h) and GCLM protein (12h) and mRNA (4h) expression in normal and GDM cells. G-I: Basal and HNE stimulated NQO1 protein (12h) and mRNA (4h) expression in normal and GDM cells. Data denote mean ± S.E.M. of measurements in cultures from normal (n=4-6) and GDM (n=4-6) donors, #P<0.05, ##P<0.01, *P<0.05, **P<0.01, ***P<0.001 vs normal HUVEC.

**FIG. 4.** Effects of GDM on basal and HNE stimulated Nrf2 nuclear translocation, ARE binding and expression. Normal and GDM HUVEC were treated with HNE (20µmol/L) or vehicle (0.08% v/v hexane). A: Representative nuclear immunoblot of nuclear Nrf2 with densitometric analysis relative to Lamin C shown in (B). C: Nrf2 binding to immobilized ARE on a TransAM ELISA plate with data expressed as absorbance values at 450nm. D: Quantification of Nrf2 immunofluorescence in normal and GDM HUVEC. Average nuclear:cytosolic Nrf2 fluorescence values from 100 cells per condition used for analysis. E-F: Basal and HNE stimulated Nrf2 mRNA and total protein expression in normal and GDM HUVEC. Data denote mean ± S.E.M. in cultures from different normal (3-6) and GDM (3-6) donors, *P<0.05, **P<0.01.
FIG. 5. Effects of GDM on DJ-1, Keap1, Bach1 and p-GSK3β expression. Basal and HNE stimulated protein (12h) and/or mRNA (4h) expression of DJ-1 (A), Keap1 (B) and Bach1 (C) in normal and GDM HUVEC. D-E: basal and HNE (20µmol/L) stimulated p-GSK3β protein levels in normal and GDM HUVEC. Data denote mean ± S.E.M. of measurements in cultures from different normal (n=4-6) and GDM (n=4-6) donors, *P<0.05, **P<0.01, ***P<0.001 vs normal HUVEC.

FIG. 6. Effects of Nrf2 knockdown in normal cells and Nrf2 adenoviral overexpression in GDM cells. Normal HUVEC were transfected for 24h with Nrf2 siRNA or scrambled siRNA and then treated with HNE (20µmol/L) or vehicle (0.08% v/v hexane) for a further 20h. A-B: Representative immunoblot and densitometric analysis of Nrf2 protein levels relative to α-tubulin. C: Basal and HNE-stimulated (24h) GSH levels in HUVEC lysates. D: Densitometric analysis of HNE-induced NQO1 protein expression in normal HUVEC transfected with Nrf2 siRNA. E-F: Representative immunoblot (non relevant lanes omitted) and densitometric analysis showing adenoviral overexpression of Nrf2 in GDM HUVEC partially rescues NQO1 expression. Data denote mean ± S.E.M. of measurements in independent cultures from 4 different normal or GDM donors, *P<0.05, ***P<0.01, ***P<0.001.

FIG. 7. Impaired Nrf2 activation and antioxidant gene expression in fetal endothelial cells in GDM. The Nrf2-Keap1 antioxidant defense pathway is compromised in fetal endothelial cells exposed to maternal diabetes in utero, potentially as a consequence of decreased DJ-1 and increased p-GSK3β expression. Decreased stabilization of Nrf2 by DJ-1 and/or enhanced nuclear export of Nrf2 by p-GSK3β renders cells more vulnerable to a disturbed redox balance. The altered fetal
endothelial redox phenotype may predispose offspring of GDM mothers to cardiovascular disease (CVD) and type 2 diabetes in later life.

Table 1. Clinical characteristics of normal and gestational diabetic pregnancies

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=55)</th>
<th>Gestational diabetes (n=44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>31 ± 1</td>
<td>35 ± 1###</td>
</tr>
<tr>
<td>Body mass index</td>
<td>24 ± 0.6</td>
<td>30.3 ± 1###</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>-</td>
<td>8.1 ± 0.4 ***</td>
</tr>
<tr>
<td>Oral glucose tolerance test (75g, 2h, mmol/L)</td>
<td>-</td>
<td>9.0 ± 0.4</td>
</tr>
<tr>
<td>HbA1C (mmol/mol)</td>
<td>-</td>
<td>43 ± 1.6</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>111 ± 2</td>
<td>118 ± 2</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>67 ± 1</td>
<td>75 ± 1</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>49% Caucasian</td>
<td>41% Caucasian</td>
</tr>
<tr>
<td></td>
<td>29% African</td>
<td>43% African</td>
</tr>
<tr>
<td></td>
<td>2% Asian</td>
<td>11% Asian</td>
</tr>
<tr>
<td></td>
<td>20% Other</td>
<td>5% Other</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>40.1 ± 1.1</td>
<td>38.7 ± 2</td>
</tr>
<tr>
<td>Caesarean delivery</td>
<td>18%</td>
<td>50%</td>
</tr>
<tr>
<td>Newborn gender (% Male)</td>
<td>49%</td>
<td>45%</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.4 ± 0.1</td>
<td>3.6 ± 0.1</td>
</tr>
</tbody>
</table>

Data denote mean ± S.E.M. ### P<0.001 relative to normal; *** P<0.001 versus theoretical mean of 5.1 mmol/L.
Fig. 1. Cheng et al.
Fig. 2. Cheng et al.
Fig. 3 Cheng et al.
Fig. 5  Cheng et al.
Normal fetal endothelial cells

- Oxidative stress (e.g. lipid peroxides)
  - Keap1
  - Nrf2 degradation
  - Nrf2 accumulation
  - DJ-1

- Antioxidant and phase II enzymes e.g. xCT, NQO1

Gestational diabetic fetal endothelial cells

- Susceptibility to CVD and insulin resistance
  - Oxidative stress (e.g. lipid peroxides)
  - Keap1
  - Nrf2 degradation
  - Nrf2 accumulation
  - DJ-1

- Antioxidant and phase II enzymes e.g. xCT, NQO1

Fig. 7. Cheng et al.