Diabetes and obesity during pregnancy alter insulin signalling and glucose transporter expression in maternal skeletal muscle and subcutaneous adipose tissue

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Abstract

Severe insulin resistance is a defining attribute of gestational diabetes mellitus (GDM). It is postulated that alterations in the insulin-signalling pathway and subsequent glucose disposal are the underlying cause of insulin resistance in patients with GDM. The purpose of this study was to profile the insulin-signalling pathway and intermediates in insulin-sensitive tissues. Subcutaneous adipose tissue and skeletal muscle were collected from normal glucose-tolerant (NGT) and insulin-controlled GDM in both non-obese and obese cohorts ($n=6-8$ per subgroup). Expression studies of the insulin-signalling pathway were performed using western blotting and quantitative reverse transcription-PCR. This study demonstrated altered mRNA expression of insulin receptor substrate (IRS)-1, IRS-2, glucose transporter (GLUT)-1, GLUT-4 and glycogen synthase kinase (GSK)-3 isoforms genes in adipose tissue in GDM women in comparison to NGT pregnant controls. In skeletal muscle, insulin-controlled GDM was associated with decreased IRS-1, phosphatidylinositol-3-kinase (PI3-K) p85 α , GLUT-1 and -4, GSK-3 isoforms and phosphoinositide-dependent kinase-1. Both adipose tissue and skeletal muscle from women with GDM displayed decreased IRS-1 and GLUT-4 and increased PI3-K p85 α protein expression. Both skeletal muscle and adipose tissue from obese women demonstrated lower GLUT-1 and -4 mRNA expression and diminished GLUT-4 protein expression in skeletal muscle only. Collectively, our results suggest that diabetes and obesity during pregnancy cause defects in insulin-signalling transduction in adipose tissue and skeletal muscle and may be the underlying cause of GDM.

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Introduction

The later part of normal human pregnancy is characterised by maternal hyperinsulinaemia and a progressive decline in insulin sensitivity, an adaptation required for adequate transport of nutrients between mother and foetus (Buchanan & Xiang 2005). When insulin secretion is inadequate and unable to compensate for the insulin resistance present in pregnancy, gestational diabetes mellitus (GDM) develops. In Australia, 5–8% of pregnant women develop GDM (Beischer *et al.* 1996), a maternal complication defined as any degree of glucose intolerance with onset or first recognition during pregnancy (Kuhl 1998). These women have increased risk of type 2 diabetes mellitus (T2DM) and heart disease later in life (Lee *et al.* 2008), and their offspring have greater incidence of perinatal complications and increased risk of obesity and diabetes in adulthood (Metzger 2007).

Blood glucose levels are regulated by the insulin-signalling pathway. The β -cells of the pancreas secrete insulin, which binds to the β -subunit of the insulin receptor (IR- β) resulting in autophosphorylation of its tyrosine residues and consequent tyrosine phosphorylation of downstream insulin receptor substrates (IRS). The activation of IRS substrates allows the recruitment of downstream effectors such as phosphatidylinositol-3-kinase (PI3-K; Pessin & Saltiel 2000). PI3-K regulates glucose uptake via translocation of glucose transporter (GLUT)-4 from the cytoplasm to the plasma membrane (Czech & Corvera 1999). A number of other genes have also been implicated in insulin signalling and glucose metabolism in peripheral tissues. These include glycogen synthase kinase (GSK)-3 (Cline *et al.* 2002, Hojlund *et al.* 2003, Patel *et al.* 2008), phosphoinositide-dependent kinase (PDK)-1 (Yamada *et al.* 2002, Hashimoto *et al.* 2006, Bayascas *et al.* 2008), insulin-like

growth factor-binding protein 1 (IGFBP1; Qiu *et al.* 2005) and phosphoenolpyruvate carboxykinase (PEPCK; Franckhauser *et al.* 2006).

There are only a few studies that have investigated endogenous levels of insulin-signalling intermediates in pregnancies complicated by GDM, and these have focused on obese women with GDM (Friedman *et al.* 1999, Catalano *et al.* 2002, Tomazic 2002). Nevertheless, these studies have found that GDM is associated with changes in insulin-signalling intermediates in skeletal muscle and adipose tissue and/or adipocytes (Friedman *et al.* 1999, Catalano *et al.* 2002, Tomazic 2002). Specifically, reports have demonstrated reduced IRS-1 and increased IRS-2 and PI3-K p85 α protein levels in both skeletal muscle and adipocytes from obese women with GDM in comparison to normal glucose-tolerant (NGT) obese pregnant controls (Catalano *et al.* 2002, Tomazic 2002). Furthermore, GLUT-4 and subsequent glucose uptake is lower in adipose tissue and skeletal muscle in women with GDM (Garvey *et al.* 1992, 1993).

Maternal obesity is a mounting problem worldwide and is significantly implicated in adverse pregnancy outcomes for both mother and offspring. Obese pregnant women have a three- to tenfold higher risk of diabetes compared to those of normal weight women (Sebire *et al.* 2001). Previous studies have demonstrated that although mothers can give rise to normal weight offspring, these offspring are still at risk of developing obesity and insulin resistance later in adulthood (Mingrone *et al.* 2008). Furthermore, the combination of obesity and GDM is thought to compound risk and complications for women during pregnancy. In recently published studies, we have shown that obesity during pregnancy causes decreased PI3-K p85 α and GLUT-4 mRNA expression and higher IRS-2 and decreased PI3-K p85 α protein expression in placental tissue (Colomiere *et al.* 2009).

To date, there is a paucity of data available examining the effect of diabetes in pregnancy with and without obesity on the insulin-signalling pathway in human skeletal muscle and adipose tissue. Moreover, studies are yet to establish the effects of obesity in pregnancy independently. Thus, the purpose of this study was to determine whether transduction of insulin-signalling pathway and glucose transport is hindered in maternal tissues from women with GDM (in the presence or absence of obesity). Furthermore, as studies have suggested obesity may have a negative impact on insulin sensitivity and secretion, we investigated insulin-signalling components in NGT obese patients to determine the sole affects of obesity on insulin signalling and glucose transport. Increased knowledge of the possible defects present in the insulin-signalling pathway may aid us to better understand the pathophysiology of GDM and maternal obesity in pregnancy.

Materials and methods

Patients and sample collection

Subcutaneous adipose tissue (anterior abdominal wall) and pyramidalis skeletal muscle (anterior to the rectus abdominis) (between 300 and 500 mg) were obtained from a total of 64 pregnant women (with institutional research and ethics committee approval) undergoing elective caesarean section (term >37 weeks gestation, indications for caesarean section were breech presentation and/or previous caesarean section). Women with any underlying medical conditions such as asthma, polycystic ovarian syndrome, pre-eclampsia and macrovascular complications were excluded. The tissues were collected and prepared as previously described (Lappas *et al.* 2004, 2005, 2007a). Briefly, dissections of adipose tissue and skeletal muscle were obtained within 10 min of delivery. Tissues were bluntly dissected to remove visible connective tissue, blotted dry on filter paper, and either snap frozen in liquid nitrogen and stored at -80°C until further analysis, or paraffin embedded for hematoxylin and eosin (H&E) analysis. H&E was performed on tissue collected to ensure that all connective tissue was removed from the adipose tissue and skeletal muscle, and that skeletal muscle was free of adipose tissue (data not shown). Women were screened for GDM and were diagnosed according to the criteria set by the Australasian Diabetes in Pregnancy Society (ADIPS) at 24–28 weeks gestation, by either a fasting venous plasma glucose level of 5.5 and/or 8.0 mmol/l glucose or higher 2 h after a 75 g oral glucose load. Body mass index (BMI; weight (kg)/height² (m²) was calculated based on measurements from patients' first antenatal visit (~ 12 weeks gestation). Non-obese pregnant women had a BMI of ~ 25 kg/m² and obese patients had a BMI of >30 kg/m².

Whole cell lysate preparation

Whole-protein lysate was prepared as previously described with minor amendments (Lappas *et al.* 2007b, Reti *et al.* 2007a). Adipose tissue and skeletal muscle lysates were prepared by homogenising ~ 100 mg tissue (2×20 s bursts) in 300 and 500 μl respectively of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Igepal, 0.1% SDS, 0.25% Na deoxycholate, 1 mM EDTA, pH 7.4, 1 mM AEBSE, 10 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM activated Na₃VO₄ and 1 mM NaF) with a metal blade tissue homogeniser (Ultraturrax, S25N 8G dispersing tool; Jenke and Kunkel GmbH and Co., Staufen, Germany). Tissue homogenates were incubated at 4°C for 1 h on a rotator. Homogenates were centrifuged at 15 000 g for 20 min. The supernatant was collected and respun. Whole-protein

lysates were assayed for protein concentration using BCA protein assay (Pierce Chemical Co., Rockford, IL, USA) with BSA as the reference standard (Lappas *et al.* 2003).

Plasma membrane protein extraction

Briefly, 100 mg adipose tissue and skeletal muscle were homogenised in 300 and 500 µl respectively of HEPES–sucrose buffer (25 mM HEPES, 250 mM sucrose, 1 mM AEBSF, 10 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM activated Na_3VO_4 and 1 mM NaF). Tissue homogenates were incubated at 4 °C for 1 h in a rotator. Homogenates were then centrifuged for 10 min at 1000 *g*. The supernatant was collected and was centrifuged for 20 min at 10 000 *g*. The supernatant was collected and centrifuged for 30 min at 25 000 *g*. The supernatant was collected and stored at –80 °C until further analysis. Protein concentrations were determined using Coomassie Protein Assay according to the manufacturer's instructions (Pierce Chemical Co). Coomassie Protein assay was used rather than BCA protein assay due to buffer incompatibility.

SDS-PAGE and western blot

SDS-PAGE and western blot were performed as described previously (Lappas *et al.* 2006, Reti *et al.* 2007b). Briefly, 30 µg adipose tissue and 50 µg skeletal muscle whole-cell lysate were separated on 7.5% gels and resolved proteins were transferred to polyvinylidene fluoride (PVDF) membrane. All antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). For detection of GLUT transporters, 50 µg adipose tissue and 80 µg skeletal muscle HEPES–sucrose samples were separated on 15% gels and resolved proteins were transferred to PVDF membranes. Molecular weights were identified by comparison with the motility of a protein standard. Whole-cell lysate blots were incubated with rabbit polyclonal anti-IR-β-C-19 (0.4 µg/ml), rabbit polyclonal anti-IRS-1 (A-19) (0.8 µg/ml) and rabbit polyclonal anti-IRS-2 (H-205) (0.8 µg/ml), diluted in blocking buffer (5% skimmed milk/TBS-T (0.05%)) for 48 h at 4 °C. HEPES–sucrose blots were incubated with rabbit polyclonal anti-GLUT-1 (H-43) (0.4 µg/ml) and rabbit polyclonal anti-GLUT-4 (0.2 µg/ml) diluted in blocking buffer II (5% BSA/TBS-T (0.05%)) for 24 h at 4 °C.

Blots were probed with goat anti-rabbit IgG HRP (0.02 µg/ml)-conjugated antibody in the corresponding blocking buffer for 1 h at room temperature. Proteins were detected using chemiluminescence kit according to the manufacturer's instructions (Luminol, Santa Cruz) and membranes were developed using the Chemidoc XRS (Bio-Rad).

All blots were stripped (62.5 mM Tris, pH 7.4, 2% SDS and 0.1 M β-mercaptoethanol) and reprobed with goat polyclonal actin (C-11; 0.4 µg/ml) in blocking buffer in order to ensure even loading and normalisation of blots.

Densitometry was performed on all blots to determine the density of the bands (OD/mm²) using Quantity One 4.6.6 image analysis program (Bio-Rad Laboratories). Separate western blots were run for each comparison (i.e. NGT (non-obese) versus GDM (non-obese); NGT (obese) versus GDM (obese)) and NGT subjects (NGT (non-obese) versus NGT (obese)). Expression of proteins was calculated as a ratio.

In-house PI3-K p85α and p110 ELISA

Total protein levels of PI3-K p85α and PI3-K p110 were quantified via in-house sandwich ELISA using antibodies purchased from Santa Cruz. Linearity of ELISA was determined with the use of placental lysate with an $r^2 \geq 0.98$. Briefly, microwell titre plates (Nunc, Maxisorp, Roskilde, Denmark) were incubated over 48 h at 4 °C with goat polyclonal anti-PI3K p85α (N-18) (2 µg/ml) and goat polyclonal anti-PI3K p110α (I-19) (2 µg/ml) in PBS. Plates were washed with wash buffer (PBS with 0.05% Tween, PBS-T) and blocked with blocking buffer (1% BSA/PBS-T). Plates were washed again and 30 µg adipose tissue or 50 µg skeletal muscle diluted in blocking buffer were added to the plates and incubated at room temperature for 1 h. Rabbit polyclonal anti-PI3K p85α (Z-8) (2 µg/ml) and mouse monoclonal anti-PI3K p110α (D-4) (2 µg/ml) diluted in blocking buffer were added to wells and incubated for 2 h at room temperature. Plates were washed with wash buffer followed by incubation with goat anti-rabbit IgG HRP-conjugated antibody (0.04 µg/ml) and goat anti-mouse IgG HRP antibody (0.04 µg/ml) for 1 h at room temperature. Final washes were performed and TMB (Sigma–Aldrich) was added to wells for colorimetric detection. Sulphuric acid (1.8 M) was added to stop the reaction and plates were read at 450 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories). Blanks (no sample), positive (placental lysate) and negative controls (placental lysate plus blocking peptide) were run on every plate to verify binding specificity. For negative controls, five times the volume of blocking peptide (sc-31969P and sc-7248P, Santa Cruz) for each antibody used for coating the plate was combined with 2 µg/ml antibody and incubated on a rotator overnight at 4 °C. The blocking peptide mixture was then added to the plate rather than coating antibody for the negative control samples. Total protein levels of PI3-K p85α and PI3-K p110 were semi-quantitatively calculated according to absorbance readings.

RNA extraction and real-time PCR

Total RNA was extracted from ~100 mg skeletal muscle and adipose tissue. Extraction was performed using TRI Reagent according to the 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 gel electrophoresis. One microgram of RNA was converted to cDNA using the iScript cDNA Synthesis Kit according to the manufacturer's instructions (Bio-Rad). One microlitre of cDNA was used to perform reverse transcription (RT)-PCR using Sensimix Plus SYBR green (Alexandria, New South Wales, Australia) and primers as listed below. β-actin was chosen as a suitable reference gene to normalise the mRNA expression and additionally to keep consistent with western blot data. The specificity of the product was assessed from melting curve analysis. All plates were run with a positive (placental cDNA) and negative (RNA without reverse transcriptase) control to ensure quality of run and confirm the absence of contamination. Pre-validated primers for IR (QT00082810), IRS-1 (QT00074144), IRS-2 (QT00064036), PI3-K p85α (QT,01005984) PI3K p110α (QT00014861), PI3K p110β QT00029148), GLUT-1 (QT00068957), GLUT-4 (QT00097902), GSK-3α (QT00075306), GSK-3β (QT00057134), IGFBP1 (QT00049427), PEPCK (QT00001197), PDK-1 (QT00063077) and β-actin (QT00095431) were purchased from Qiagen. The cycling conditions for RT-PCR were as follows: 95 °C for 3 min; 95 °C for 15 s (denaturation); 55 °C for 45 s (annealing); 72 °C for 45 s (extension); cycle 39 times; 95 °C for 1 min; 55 °C for 1 min; a melt curve analysis was programmed for 55 to 95 °C (1 °C intervals) and held for 20 s. Real-time PCR results were calculated according to the 2^{−ΔΔC_t} method previously described (Livak & Schmittgen 2001). NGT (non-obese) or NGT (obese) subgroups were used as calibrators.

Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics, STSC, Rockville, MD, USA). Unless otherwise stated, sample comparison was analysed by Student's *t*-test. When data were not normally distributed, Mann–Whitney *U* tests were used. Statistical difference was indicated by *P* value <0.05. Data are expressed as the mean ± S.E.M.

Results

The mRNA and protein expression of insulin-signalling components in adipose tissue and skeletal muscle from i) non-obese GDM compared to non-obese NGT patients, ii) obese GDM compared to obese NGT patients and iii) NGT non-obese compared to NGT obese patients (*n*=7–8 per subgroup unless otherwise specified) were compared in this study.

Participants

Demographic data of all participants involved in this study are summarised in Table 1. Maternal age did not differ among groups. Maternal BMI at 12 weeks was significantly higher in non-obese and obese GDM women compared to NGT controls. Fasting, 1 and 2 h OGTT readings were all significantly higher in both non-obese and obese GDM women in comparison to BMI-matched NGT pregnant controls. Gestational age of obese GDM women was significantly lower compared to obese NGT pregnant women. Obese NGT women had higher 12-week and term BMI, and fasting and 1 h OGTT readings when compared to non-obese NGT controls. Out of 59 participants, 12 had a family history of T2DM (mother, father or grandparents).

Table 1 Patient characteristics for study group

	Non-obese		Obese	
	NGT (<i>n</i> =16)	GDM-insulin (<i>n</i> =13)	NGT (<i>n</i> =15)	GDM-insulin (<i>n</i> =15)
12-week BMI (kg/m ²)	22.1±0.4	24.5±0.6*	34.8±0.9†	38.5±1.4*
Term BMI (kg/m ²)	27.0±0.5	29.1±1.0	39.3±1.2†	41.1±1.4
0 h OGTT (mmol/l)	4.2±0.1	4.9±0.5*	4.7±0.1†	5.7±0.3*
1 h OGTT (mmol/l)	6.2±0.3	10.4±1.0*	7.7±0.4†	11.1±0.5*
2 h OGTT (mmol/l)	5.5±0.3	8.6±0.8*	6.0±0.2	9.0±0.5*
Maternal age (years)	33.6±1.2	33.4±0.6	33.7±1.0	34.6±1.6
Birth weight (g)	3359±110	3205±115	3619±96	3546±120
Gestation (weeks)	38.8±0.2	38.4±0.2	38.8±0.2	38.1±0.2*
Gravida	3.0±0.4	2.8±0.3	2.4±0.3	3.2±0.4
Parity	2.2±0.2	2.2±0.2	1.9±0.2	2.3±0.3

Values represent mean ± S.E.M. BMI, body mass index; NGT, normal glucose tolerant; OGTT, oral glucose tolerance test. Student's *t*-test **P*<0.05 NGT versus GDM-insulin. †*P*<0.05 NGT (non-obese) versus NGT (obese).

Figure 1 Protein expression of IR-β (95 kDa), IRS-1 (180 kDa), IRS-2 (170 kDa), Akt (60 kDa), GLUT-1 (55 kDa) and GLUT-4 (43 kDa) in adipose tissue (a) and skeletal muscle (c) collected from a non-obese pregnant cohort ($n=7-8$ per group). Each bar represents the relative expression of the protein of interest $\pm$ S.E.M. A representative western blot image of two patients from each group has been included. All blots were stripped and reprobed with β -actin (adipose tissue) or actin (skeletal muscle) to normalise for protein load. Protein expression of PI3-K p85 α and PI3-K p110 in adipose tissue (b) and skeletal muscle (d) collected from the non-obese pregnant cohort ($n=8$ per group). Expression is measured via absorbance reading at 450 nm. Student's t -test, * $P<0.05$ NGT versus GDM.

Expression studies

The effects of GDM in pregnancy on insulin signalling and glucose transport in adipose tissue and skeletal muscle in a non-obese cohort

Quantitative RT-PCR performed on subcutaneous adipose tissue biopsies showed altered gene expression in GDM women when compared to NGT non-obese subjects. Specifically, decreased IRS-1 and GLUT-4 and increased IRS-2, GLUT-1, GSK-3 α , GSK-3 β , IGFBP1 and PDK-1 mRNA expression were seen in GDM women compared to NGT subjects. No differences were found in mRNA expression of IR, PI3-K p85 α , PI3-K p110 α , PI3-K p110 β and PEPCK. Protein expression of IR-β and IRS-1 was also lower, while PI3-K p85 α and Akt protein expression were higher in GDM women compared to NGT controls. Protein expression of IRS-2, GLUT-1 and -4 transporters was unaltered between the two groups.

For skeletal muscle, there was lower IRS-1, PI3-K p85 α , GLUT-1, GLUT-4, GSK-3 α , GSK-3 β and PDK-1 mRNA expression in GDM women compared to NGT controls. The mRNA expression of IR, IRS-2, PI3-K p110 α , PI3-K p110 β , IGFBP1 and PEPCK remained unchanged between the two groups. At a protein level, skeletal muscle IRS-1, Akt and GLUT-4 were lower, and IRS-2

and PI3-K p85 α were higher in GDM women compared to NGT controls. IR-β and GLUT-1 protein expression remained unchanged in between the two groups.

The effects of GDM in pregnancy on insulin signalling and glucose transport in adipose tissue and skeletal muscle in an obese cohort

In adipose tissue biopsies, RT-PCR analysis showed lower IRS-1 and GLUT-4 mRNA expression in GDM women when compared to NGT controls (Fig. 1). No changes were noted in IR, IRS-2, PI3-K p85 α , PI3-K p110 α , PI3-K p110 β , GLUT-1, GSK-3 α , GSK-3 β , IGFBP1, PEPCK and PDK-1 mRNA expression between the two cohorts. Adipose tissue protein expression of IR-β and IRS-1 was lower in GDM women, while IRS-2, Akt and PI3-K p85 α expression were increased respective to NGT controls. An unexpected increase in GLUT-4 protein expression was noted in GDM women, while GLUT-1 protein expression was significantly decreased when compared to NGT controls.

Skeletal muscle mRNA expression of GLUT-4 and GSK-3 α was significantly lower in GDM women compared to NGT obese controls. No changes in mRNA expression of IR, IRS-1, IRS-2, PI3-K p85 α ,

Figure 2 Protein expression of IR-β (95 kDa), IRS-1 (180 kDa), IRS-2 (170 kDa), Akt (60 kDa), GLUT-1 (55 kDa) and GLUT-4 (43 kDa) in adipose tissue (a) and skeletal muscle (c) collected from an obese pregnant cohort ($n=7-8$ per group). Each bar represents the relative expression of the protein of interest $\pm$ s.e.m. A representative western blot image of two patients from each group has been included. All blots were stripped and reprobed with β -actin (adipose tissue) or actin (skeletal muscle) to normalise for protein load. Protein expression of PI3-K p85 α and PI3-K p110 in adipose tissue (b) and skeletal muscle (d) collected from the obese pregnant cohort ($n=8$ per group). Expression is measured via absorbance reading at 450 nm. Student's t -test, $*P<0.05$ NGT versus GDM.

PI3-K p110 α , PI3-K p110 β , GLUT-1, GSK-3 β , IGFBP1, PEPCK and PDK-1 were noted between the two groups. Furthermore, in GDM women, skeletal muscle protein expression of IR-β, IRS-1, Akt and GLUT-4 was reduced, while PI3-K p85 α was increased compared to NGT controls. Skeletal muscle GLUT-1 transporter protein expression remained unchanged between the two groups.

The effects of obesity in pregnancy on insulin signalling and glucose transport in adipose tissue and skeletal muscle in a NGT cohort

Adipose tissue biopsies obtained from obese subjects displayed higher IRS-2 mRNA expression and lower GLUT-1 and -4 mRNA expression when compared to non-obese controls (Fig. 2). IR, IRS-1, PI3-K p85 α , PI3-K p110 α , PI3-K p110 β , GSK-3 α , GSK-3 β , IGFBP1, PEPCK and PDK-1 gene expression remained unchanged between the two cohorts. Higher protein expression of PI3-K p85 α and lower GLUT-1 expression in the obese cohort were found compared to non-obese controls (Table 2). Adipose tissue protein expression of IR-β, IRS-1 and IRS-2 was unaltered between non-obese and obese cohorts (Fig. 3).

Skeletal muscle mRNA expression of GLUT-1 and -4 and GSK-3 β was lower, while PEPCK mRNA expression was higher in obese women compared to non-obese controls. However, there were no differences in skeletal muscle mRNA expression of IR, IRS-1, PI3-K p85 α , PI3-K p110 α , PI3-K p110 β , GSK-3 α , IGFBP1 and PDK-1 between the two groups. Lower skeletal muscle IRS-1 and GLUT-4 expression and higher IRS-2 protein expression were found in obese subjects when compared to non-obese controls. No changes were found in IR-β, PI3-K p85 α , PI3-K p110 and GLUT-1 protein expression between the two groups.

Discussion

The data presented in this study provide evidence that diabetes and obesity during pregnancy cause defects in the insulin-signalling pathway in both adipose tissue and skeletal muscle. The action of insulin through the transduction of the insulin-signalling pathway mediates a wide variety of cellular responses including glucose transport via GLUTs, glycogen synthesis, protein synthesis and lipid metabolism. Thus, defects in the

Table 2 mRNA expression of insulin-signalling intermediates

	NGT (non-obese) (n=8)	GDM-insulin (non-obese) (n=8)	NGT (obese) (n=8)	GDM-insulin (obese) (n=8)
In adipose tissue				
IR-β	4.26 ± 1.65	2.07 ± 0.62	1.91 ± 0.78	1.39 ± 0.47
IRS-1	7.18 ± 0.93	4.44 ± 0.63*	8.20 ± 1.24	5.39 ± 0.81*
IRS-2	2.21 ± 0.24	3.54 ± 0.61*	3.61 ± 0.61 [†]	2.48 ± 0.51
PI3-K p85α	1.56 ± 0.15	2.80 ± 0.86	3.75 ± 1.25	1.64 ± 0.24
PI3-K p110α	1.20 ± 0.17	1.98 ± 0.51	1.58 ± 0.38	1.30 ± 0.37
PI3-K p110β	2.12 ± 0.43	3.12 ± 0.72	2.31 ± 0.50	2.10 ± 0.37
GLUT-1	0.50 ± 0.05	0.65 ± 0.06*	0.36 ± 0.05 [†]	0.47 ± 0.05
GLUT-4	0.91 ± 0.10	0.36 ± 0.08*	0.60 ± 0.07 [†]	0.26 ± 0.04*
GSK-3α	0.19 ± 0.03	0.41 ± 0.08*	0.17 ± 0.03	0.18 ± 0.03
GSK-3β	0.75 ± 0.20	1.36 ± 0.21*	0.94 ± 0.29	0.87 ± 0.24
IGFBP1	3.86 × 10 ⁻⁴ ± 1.59 × 10 ⁻⁴	0.01 ± 5.86 × 10 ⁻³ *	4.36 × 10 ⁻³ ± 2.21 × 10 ⁻³	5.76 × 10 ⁻³ ± 1.80 × 10 ⁻³
PEPCK	1.30 ± 0.72	2.70 ± 1.15	1.44 ± 0.79	1.65 ± 0.87
PDK-1	0.30 ± 0.09	0.78 ± 0.16*	0.36 ± 0.17	0.60 ± 0.17
In skeletal muscle				
IR-β	23.16 ± 2.11	24.43 ± 3.95	29.01 ± 5.40	19.99 ± 2.36
IRS-1	71.05 ± 17.73	39.89 ± 5.22*	52.78 ± 10.22	80.67 ± 30.35
IRS-2	2.63 ± 0.26	3.34 ± 0.40	3.21 ± 0.60	2.14 ± 0.32
PI3-K p85α	8.35 ± 1.39	5.16 ± 1.30*	6.31 ± 1.06	4.07 ± 1.06
PI3-K p110α	12.78 ± 2.94	11.74 ± 1.30	12.19 ± 1.90	11.34 ± 2.01
PI3-K p110β	6.19 ± 0.71	9.21 ± 3.28	9.21 ± 2.37	5.29 ± 1.10
GLUT-1	0.79 ± 0.15	0.39 ± 0.06*	0.44 ± 0.09 [†]	0.32 ± 0.04
GLUT-4	16.45 ± 2.25	9.61 ± 1.48*	11.46 ± 1.61 [†]	5.61 ± 0.57*
GSK-3α	2.71 ± 0.64	0.99 ± 0.33*	3.15 ± 1.27	0.78 ± 0.28*
GSK-3β	4.99 ± 0.94	1.96 ± 0.46*	2.65 ± 0.51 [†]	2.13 ± 0.71
IGFBP1	1.54 × 10 ⁻³ ± 6.22 × 10 ⁻⁴	4.04 × 10 ⁻³ ± 1.38 × 10 ⁻³	1.20 × 10 ⁻² ± 7.86 × 10 ⁻³	3.25 × 10 ⁻³ ± 1.97 × 10 ⁻³
PEPCK	1.95 × 10 ⁻² ± 1.38 × 10 ⁻²	2.61 × 10 ⁻² ± 7.84 × 10 ⁻³	0.11 ± 0.04 [†]	0.27 ± 0.23
PDK-1	4.95 ± 0.98	2.44 ± 0.82*	4.72 ± 1.53	2.79 ± 0.93

Values represent mean ± S.E.M. NGT, normal glucose tolerant. Student's *t*-test **P* < 0.05 NGT versus GDM-insulin. [†]*P* < 0.05 NGT (non-obese) versus NGT (obese).

insulin-signalling pathway may negatively impact on downstream pathways involved in glucose transport, glycogen synthesis and lipid metabolism.

Our study has demonstrated that, compared to NGT women, altered adipose tissue insulin signalling is present at multiple sites in lean GDM women, obese GDM women and minimal changes in obese NGT women. In our non-obese cohort, we demonstrate decreased mRNA expression of IRS-1 and increased mRNA expression of IRS-2 and PDK-1 in adipose tissue from GDM women compared to NGT controls. Further to this, lower protein expression of IR-β and IRS-1 and higher Akt and PI3-K p85α expression were found in adipose tissue from GDM women. Similar protein expression was noted in our obese GDM cohort with the addition of higher IRS-2 protein expression. IRS-1 mRNA expression was lower in adipose tissue from obese GDM women compared to BMI-matched controls. Furthermore, in our NGT cohort, obese pregnant women displayed higher IRS-2 mRNA expression compared to non-obese controls.

In skeletal muscle samples, lower IRS-1 and PI3-K p85α expression were found in non-obese GDM women. In addition, higher IRS-2 and PI3-K p85α expression and

lower IRS-1 and Akt protein expression were noted in GDM women from the non-obese cohort. Similar results were seen in the obese GDM women with the addition of lower IR-β protein expression. Skeletal muscle from NGT obese women had lower IRS-1 and higher IRS-2 expression. Overall, the results obtained suggest impaired insulin signalling in adipose tissue and skeletal muscle in women with GDM (both non-obese and obese) and to a lesser extent in women with maternal obesity. These results are in support of previous data obtained (Friedman *et al.* 1999, Catalano *et al.* 2002, Tomazic 2002). Collectively, these data suggest that both diabetes and obesity can impact on components of the insulin-signalling pathway. Defects in these proteins may alter downstream functions such as glucose uptake.

There is much evidence to suggest that adipose tissue plays a central role in the development of insulin resistance (Hajer *et al.* 2008) where adipose tissue itself is insulin responsive and contributes directly, although quantitatively less than skeletal muscle, to whole-body glucose disposal (Stump *et al.* 2006). Skeletal muscle plays an important role in glucose metabolism as it mediates 75% of all insulin-mediated glucose disposal under normal physiological conditions

Figure 3 Protein expression of IR-β (95 kDa), IRS-1 (180 kDa), IRS-2 (170 kDa), Akt (60 kDa), GLUT-1 (55 kDa) and GLUT-4 (43 kDa) in adipose tissue (a) and skeletal muscle (c) collected from NGT pregnant cohort ($n=7-8$ per group). Each bar represents the relative expression of the protein of interest $\pm$ s.e.m. A representative western blot image of two patients from each group has been included. All blots were stripped and reprobed with β -actin (adipose tissue) or actin (skeletal muscle) to normalise for protein load. Protein expression of PI3-K p85 α and PI3-K p110 in adipose tissue (b) and skeletal muscle (d) collected from the non-obese pregnant cohort ($n=8$ per group). Expression is measured via absorbance reading at 450 nm. Student's t -test, * $P<0.05$ NGT (non-obese) versus NGT (obese).

(Stump *et al.* 2006). The action of insulin to increase glucose uptake is controlled initially by the transport of glucose across the cell membrane, which takes place by facilitated diffusion of GLUT-4 (Scheck *et al.* 1991). Studies in T2DM models have demonstrated impaired glucose transport via IRS-1/PI3-K-dependent signalling (Scheck *et al.* 1991, Rondinone *et al.* 1997).

In this study, GLUT-1 and -4 transporters were investigated. In response to GDM, GLUT-4 mRNA expression was lower in both skeletal muscle and adipose tissue in both non-obese and obese GDM cohorts and NGT obese women. Skeletal muscle protein expression was similar to mRNA expression, while adipose tissue demonstrated no changes in GLUT-4 transporter with the exception of higher GLUT-4 in adipose tissue from obese GDM women compared to controls. GLUT-1 mRNA expression was lower in both adipose tissue and skeletal muscle from non-obese GDM women and NGT obese women compared to non-obese NGT controls. No changes in skeletal muscle protein expression were noted, but adipose tissue demonstrated lower GLUT-1 expression in both obese GDM women and obese NGT women compared to controls.

Overall decreased GLUT expression in both tissues was noted in individuals with GDM and/or obesity. Others have shown that GLUT-4 and subsequent glucose uptake is lower in adipose tissue and skeletal muscle from women with GDM (Garvey *et al.* 1992, 1993). This may be caused by defective insulin-signalling upstream as discussed above. Consequently, blood glucose levels remain high in the maternal bloodstream as there is a decreased capacity, due to lower GLUT transporter expression, to uptake glucose into the cell. Our data demonstrate that women with obesity and/or GDM have decreased adipose tissue and skeletal muscle GLUT-4 transport consequently increasing free glucose within maternal blood circulation and availability to the placenta. In keeping with this, our previous studies have shown increased GLUT-1 expression in the GDM placenta (Colomiere *et al.* 2009). Furthermore, studies have shown increased maternal-foetal glucose transport in placenta from women with insulin-controlled GDM (Osmond *et al.* 2001). The combination of these factors suggests that defective glucose transport prior to birth may be involved in pre-programming the foetus for increased risk of diabetes and obesity in adulthood.

PEPCK, a cytosolic decarboxylase enzyme involved in gluconeogenesis, is regulated by hormones that are involved in the maintenance of glucose homeostasis (Holoak *et al.* 2006). A secondary role for PEPCK is glyceroneogenesis, or the production of glycerol. This is essential for the synthesis of triglycerides and the release of free fatty acids (FFA) into the blood stream (Beale *et al.* 2004). Studies have shown glyceroneogenesis to occur in skeletal muscle (Nye *et al.* 2008). In addition, overexpression of cytosolic PEPCK in the skeletal muscle of the mouse resulted in a marked increase in intramyocellular triglyceride levels (Hakimi *et al.* 2007). In this study, skeletal muscle PEPCK mRNA expression was higher in obese NGT women compared to non-obese NGT controls. This increase could reflect the amount of triglycerides within these skeletal tissues, due to maternal obesity. Further investigation is warranted to elucidate the precise role of PEPCK overexpression and/or gluconeogenesis and reciprocal glyceroneogenesis in maternal obesity.

GSK-3 α and -3 β are serine/threonine protein kinases that are involved in the storage of glucose into glycogen. Defects in GS activity and GSK-3 expression are early events in the development of insulin resistance where glycogen synthesis is impaired in T2DM (Eldar-Finkelman *et al.* 1999). Studies have shown that inhibition of GSK-3 in Zucker diabetic fatty rats leads to an improvement in both insulin action and glucose uptake (Cline *et al.* 2002). Our study demonstrates higher adipose tissue GSK-3 (α and β) mRNA expression in GDM women from both non-obese and obese cohorts compared to BMI-matched NGT controls. Previous studies performed in CHO/IR/IRS-1 cells overexpressing GSK-3 demonstrate decreased tyrosine phosphorylation of IRS-1 and IR- β (Eldar-Finkelman & Krebs 1997). Therefore, GSK-3 overexpression in adipose tissue may negatively impact on the insulin-signalling pathway, particularly at IR and its IRS-1, and thus may play a central role in the regulation of insulin action and glucose metabolism.

Previous studies have demonstrated overexpression of GSK-3 isoforms in skeletal muscle samples from insulin resistance states (Ciaraldi *et al.* 2006). In contrast to this, our study demonstrated lower skeletal muscle GSK-3 (α and β) mRNA expression in GDM women. Additionally, skeletal muscle GSK-3 β mRNA expression was decreased in obese NGT women. Others have demonstrated that insulin resistance in muscle cells reduces glucose uptake and local storage of glucose as glycogen (Patel *et al.* 2008).

The role for GSK-3 in skeletal muscle from diabetic pregnancies is currently unknown and the mechanisms behind GSK-3 require further investigations. Measuring glycogen synthase activity may aid to determine whether decreases in GSK-3 mRNA

expression disrupt glycogen synthesis and furthermore establish the role of tissue-specific alterations in GSK-3 in women with GDM.

The development of insulin resistance during late pregnancy shifts maternal energy metabolism from carbohydrate to lipid oxidation and thus, spares glucose for the growing foetus (Sivan *et al.* 1998). In the third trimester, postprandial FFA levels increase and insulin sensitivity worsens by 40–60% compared with pre-pregnancy (Catalano *et al.* 1993, 1999). Insulin's ability to suppress whole-body lipolysis is reduced during late pregnancy (Sivan *et al.* 1999) and to a greater degree in women with GDM (Xiang *et al.* 1999), consequently contributing to increased postprandial FFA levels. An intracellular mechanism responsible for insulin suppression of lipolysis involves activation of the insulin-signalling pathway. Defects in the transduction of the insulin-signalling pathway can therefore increase adipocytes lipolysis and the release of FFA into the mother's bloodstream, where it may have an effect on skeletal muscle glucose metabolism and placental transport of nutrients between mother and foetus.

In conclusion, adipose tissue and skeletal muscle collected from women with maternal obesity or GDM (independent of obesity) display impaired insulin signalling, which appears to be regulated through IRS-1/Akt/PDK-1-dependent pathway. This study suggests that impaired insulin-signalling transduction disrupts glucose transport, glycogen synthesis and possibly disrupts lipid metabolism in adipose tissue. Furthermore, GSK-3 and enzymes involved in gluconeogenesis may be involved in both obesity and diabetes during pregnancy. Post-partum longitudinal studies, involving repeating this study on maternal tissues 1 year post-pregnancy, could confirm long-term effects of GDM and obesity during pregnancy. This would allow a more in-depth understanding into the aetiology of GDM and maternal obesity and the future risk of T2DM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M Colomiere carried out all experimental studies, did all the data/statistical analysis and wrote the manuscript; M Lappas conceived the study. M Permezel assisted in patient analysis. M Lappas drafted the manuscript and assisted in data analysis.

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