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Human placental GLUT1 glucose transporter expression and the fetal insulin-like growth factor axis in pregnancies complicated by diabetes☆

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ABSTRACT

We have previously described regulation of syncytial GLUT1 glucose transporters by IGF-I. Despite this, it is not clear what signal regulates transplacental glucose transport. In this report we asked whether changes in GLUT1 expression and glucose transport activity in diabetic pregnancies were associated with alterations in the fetal IGF axis. Cord blood samples and paired syncytial microvillous and basal membranes were isolated from normal term pregnancies and pregnancies characterized by gestational diabetes type A2 (GDM A2) and pre-existing insulin-dependent diabetes mellitus (IDDM). Circulating IGF-I, basal membrane GLUT1 expression and glucose transporter activity were correlated with birth weight, but only in control, not diabetic groups. Basal membrane GLUT1 and transporter activity were correlated with IGF-I concentrations in control, but not diabetic groups. IGF binding protein (IGFBP) binding capacity showed a ≥50% reduction in the diabetic groups compared to control; both showed a higher level of free IGF-I. The absence of a correlation between birth weight and factors such as fetal IGF-I or GLUT1 expression in the diabetic groups suggests that IGF-I-stimulated effects may have reached a limiting threshold, such that further increases in IGF-I (or GLUT1) are without effect. These data support that fetal IGF-I acts as a fetal nutritional signal, modulating placental GLUT1 expression and birth weight via altered levels of fetal circulating IGFBPs. Diabetes appears to exert its effects on fetal and placental factors prior to the third trimester and, despite good glycemic control immediately prior to, and in the third trimester, these effects persist to term.

1. Introduction

In humans, there is no significant fetoplacental gluconeogenesis, therefore transfer of sufficient glucose from mother to fetus is essential for normal fetal growth. Glucose transfer takes place via glucose transporters embedded in the microvillous and basal membranes of the placental syncytiotrophoblast layer [1,2], thus regulation of syncytial transporter expression is important for fetal growth and energy needs.

In vitro, trophoblast GLUT1 protein expression is controlled by a number of regulatory elements. Previous work in this laboratory has shown that the reduction in growth that occurs as a result of chronic (altitude-induced) hypoxia is associated with a decrease in the expression of GLUT1 protein on the basal membrane of the syncytiotrophoblast [3] and with decreased placental delivery of glucose to the fetus [4]. We, and others, have previously shown an increase in basal membrane GLUT1 expression as a result of diabetes in pregnancy [5–7], a condition frequently associated with increased growth (macrosomia). These results suggest that the expression of basal membrane GLUT1 may be controlled, in part, by factors that respond to the nutritional or metabolic status of the fetus. As the placenta is the conduit for fetal nutrient delivery, it has first claim on the nutrient flow passing to the fetus. Despite this, placental growth is well correlated with fetal

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growth, suggesting that the placental growth trajectory may be modulated by fetal nutritional/growth signals so as to match fetal growth [2].

We have demonstrated that in vitro, placental GLUT1 is positively modulated by insulin-like growth factor I via the type 1 IGF receptor in the basal and microvillous membranes of the syncytiotrophoblast [8]. We have now extended these studies to in vivo measurements of GLUT1 where there may be significant changes in both GLUT1 and circulating fetal growth factors, including IGF-I. The primary aim of this study was to test the hypothesis that under the differing nutritional/metabolic conditions of diabetic pregnancy, a fetal signal, transmitted via circulating fetal IGF-I, modulates the expression of placental GLUT1.

2. Methods and materials

2.1. Subject selection

Placental tissue from control and diabetic pregnancies was obtained with written informed consent under procedures approved by the Institutional Review Boards of New Jersey Medical School (Newark, NJ) and MetroHealth Medical Center (Cleveland, OH). Primary criteria for inclusion in the diabetic groups were diagnosis of (1) Type 1, class B diabetes (onset at age 20 or older or with duration of < 10 years, diet and insulin-controlled) referred to hereafter as IDDM, or (2) class A2 gestational diabetes (diet and insulin-controlled), referred to hereafter as GDM A2. Secondary criteria required an absence of medical or obstetric complications other than diabetes, maternal age between 18 and 40 yr, term delivery (37+ weeks gestation) and singleton pregnancy. Exclusion criteria included the existence of nephropathy, hypertension (essential or pregnancy-related), and conditions that might indicate altered uteroplacental blood flow or substrate delivery. Normal, gestational age-matched control placental tissue was obtained and processed in a manner identical to the diabetic tissue. The inclusion and exclusion criteria for the normal, control subjects were as described for the diabetic groups, but in the absence of diabetes.

2.2. Placental syncytial membrane preparation

Placental syncytial microvillous and basal membranes were prepared as described previously [9], using Mg^{2+} precipitation and density gradient centrifugation. Membranes were stored at −80 °C until use. Enrichment, as assessed by alkaline phosphatase activity, was ≥20-fold in the microvillous samples, as seen previously, and did not differ between the control and diabetic groups [5]. Basal membranes showed minimal cross contamination by the microvillous fraction.

2.3. Cord serum

Venous blood was obtained at delivery from the umbilical cord of the placentae from which syncytial membranes were later prepared. Serum prepared from the cord blood was aliquoted into tubes containing aprotinin and frozen at −80 °C until assay.

2.4. Immunoblotting

In these investigations slot immunoblotting was used to measure the expression of the GLUT1 glucose transporter and the type 1 insulin-like growth factor receptor (IGF-IR). Slot blotting was used because it enables quantitation of these proteins in a manner that assures that the densitometric measurement of the immunoreactive band is in the linear region of the detection curve, as described previously [5]. Slot-blots permit comparison of multiple subjects on the same blot, quantification of the GLUT1 protein and preclude saturation effects. In preliminary investigations we showed that both antibodies produce a single immunoreactive band on Western blot, as also shown for GLUT1 in our previous reports [1,5] and from the manufacturer's data. When carrying out the slot blot, an increasing series of seven standards (1–32 μg membrane protein) were run in duplicate on each blot to assess linearity, and unknown sample concentrations were adjusted to generate band densities in the mid-range of these standards. The reference standards were composed of a mixture of equal quantities of all the samples and the same standards were used on each slot blot. After dilution to the appropriate protein concentration, syncytial membrane fractions were solubilized in 1% SDS. Following solubilization, samples were diluted 50-fold in Tris-buffered saline, pH 7.4 (TBS), reducing the SDS levels to ensure protein binding to the nitrocellulose. After sample loading, the quantity of protein loaded on to the membranes was determined using Sypro Ruby fluorescent stain (Invitrogen, Carlsbad, CA) to enable correction for differences in protein loading. The fluorescent signal was measured on a Typhoon 9410 imager (GE Healthcare BioScience Corp., Piscataway, NJ). Membranes were then blocked with SuperBlock (Pierce Biotechnology, Rockford, IL) for 60 min and incubated with rabbit polyclonal anti-GLUT1 or anti-IGF-IR β-subunit antibodies (1:20,000; EMD Millipore, Cat# 07-1401, 04-298) in TBS containing 3% BSA for 120 min. Specificity was assured by absence of a signal following omission of the primary antibody. After washing with TBS containing 0.05% Tween 20 (TBST; 2 × 10 min, 2 × 5 min), a secondary antibody was applied (HRP-coupled goat anti-rabbit IgG, 1:40,000) for 60 min. After washing with TBST, immunoreactive bands were visualized on film by chemiluminescence (SuperSignal West Pico, Pierce Biotechnology) and digitized. All the preceding steps were performed at room temperature. Slot density was quantitated by densitometry (ImageJ v1.46, NIH) and the intensity of the signal obtained on immunoblot was normalized using the protein loading values obtained from the Sypro Ruby measurements.

2.5. Western ligand blotting

Western ligand blotting (WLB) was performed on umbilical cord serum samples to measure IGFBP binding using a Western-Ligand Blotting Kit (IBT Gmbh, Binzwangen, Germany). Serum samples were diluted 1:4 with a non-reducing Laemmi sample buffer and subjected to SDS-PAGE using 8–16% gradient gels (Criterion, Bio-Rad). After protein transfer to nitrocellulose, membranes were blocked and incubated with biotinylated IGF-II (1:500 in TBS containing 3% BSA; 40 ng/mL) overnight at 4 °C. After washing with TBST, a detection agent was applied (HRP-streptavidin. 1:20,000; Thermo-Fisher) for 60 min at room temperature, followed by a further wash, and chemiluminescent detection as described above. A series of four pooled samples was run on each gel and the average, summed intensity of the bands was used for inter-gel normalization. Total intensity per lane was used as a measure of total binding capacity. Isoform identity was determined from molecular weight measurements, employing a series of low-molecular weight reference proteins (6.5–66 K; Sigma-Aldrich, Cat# M3913). Intensity in the individual bands in each lane was used as a measure of the binding capacity of individual IGFBP isoforms.

2.6. Growth factor assays

Growth factor concentrations were measured in umbilical cord serum using ELISA kits obtained from Diagnostic Systems Laboratories (Webster, TX). Concentrations of insulin, total IGF-I, total IGF-II and free IGF-I were measured according to the manufacturer's instructions.

2.7. Glucose transport assays

Transport of glucose across microvillous and basal membranes was measured using a light-scattering method as described previously [10]. Membrane vesicle samples (MVM or BM), loaded with 100 mM raffinose, 10 mM Hepes/Tris, pH 7.4 after overnight incubation at 4 °C, were mixed 1:1 in a Hi-Tech stopped-flow spectrophotometer (SF50, Hi-Tech, Salisbury, UK) with 20 mM raffinose, 80 mM glucose, 10 mM

Hepes/Tris, pH 7.4 to create an inwardly directed glucose gradient. The stopped-flow apparatus had a mixing time < 2 msec and used a 50 W quartz halogen lamp in series with a single grating monochromator as a light source (excitation 500 nm). Scattered light was measured in a flow cell at a 90° angle to the incident beam using a photomultiplier and the current thus generated was digitized to produce 2500 data points over the selected time interval and recorded for later analysis. All experiments were performed at room temperature and six or more curves were obtained for each condition. Transport experiments were analyzed by fitting the light scattering data to a double exponential function, the two exponentials representing transporter-mediated and diffusional components of glucose transport. The exponential time constants and amplitudes obtained for the fast (transporter-mediated) component were used to calculate the transporter-mediated glucose flux, J_{glc} ($nmol \cdot s^{-1} \cdot mg^{-1}$).

2.8. Statistical methods

The data quoted here are given as the mean ± standard error of the mean. Group comparisons were made by Student's *t*-test, ANOVA using Dunnett's test, or Kruskal-Wallis with Dunn's multiple comparisons test, as appropriate. Linear regression analysis was used to examine the interdependency of data variables. In all analyses, a value of $p \leq 0.05$ was taken to indicate significance.

2.9. Other

Fetal % body fat was measured as described previously [11]. Protein concentrations were measured using the Bio-Rad Quick Start Assay (Hercules, CA).

2.10. Materials

The primary antibodies against the GLUT1glucose transporter (rabbit polyclonal) and the type 1 IGF receptor β subunit (rabbit polyclonal) were obtained from EMD-Millipore (Burlington, MA). HRP-coupled secondary antibodies and protease inhibitor mix were obtained from Sigma Chemical Co. (St. Louis, MO). Biotin-labeled IGF-II was obtained from CellSciences (Newburyport, MA). Streptavidin-HRP was obtained from Thermo-Fisher (Grand Island, NY) Nitrocellulose for immunoblotting was obtained from Bio-Rad (Hercules, CA), Sypro Ruby protein blot stain from Invitrogen (Carlsbad, CA) and SuperSignal West Pico Chemiluminescence detection kits from Pierce Biotechnology (Rockford, IL). ELISA kits for the assay of insulin, IGF-I, IGF-II and free IGF-I were obtained from Diagnostic Systems Laboratories (Webster, TX).

3. Results

3.1. Subject demographics

The groups in this study consisted of 28 normal (control) pregnancies, 39 pregnancies diagnosed with gestational diabetes type A2 (GDM A2) and 18 pregnancies with preexisting, type 1 insulin-dependent diabetes (< 10 years duration; IDDM). Pre-pregnancy weight and BMI were higher in both diabetic groups but other maternal characteristics were similar (Table 1A).

Neonatal characteristics are shown in Table 1B. There was no difference in the mean birth weight between control and diabetic neonates however placental weight was increased in both the GDM A2 and IDDM groups (463 ± 16 g vs. 545 ± 18 g or 566 ± 26 g; $p < 0.005$). As a result, the corresponding birth weight to placental weight ratio was decreased. Neonatal birth length, ponderal index, flank skinfold thickness, % body fat and 1 and 5 min Apgar scores did not differ between the groups.

Table 1A
Maternal characteristics.

	Control (28)	GDM A2 (39)	IDDM (18)
Age (yr)	27.7 ± 1.1	29.4 ± 1.0	30.4 ± 0.8
Pre-pregnancy weight (kg)	64.6 ± 2.5	94.9 ± 5.5*	88.9 ± 4.3*
Weight gain (kg)	16.4 ± 1.4	12.9 ± 1.8	11.8 ± 1.8
BMI	24.7 ± 0.9	34.6 ± 1.9*	34.3 ± 1.5*

* > Control, $p < 0.05$ (ANOVA).

Table 1B
Neonatal characteristics.

	Control (28)	GDM A2 (39)	IDDM (18)
Gestational age at delivery (weeks)	39.2 ± 0.2	38.5 ± 0.2	38.1 ± 0.2
Birth weight (g)	3417 ± 84	3388 ± 94	3416 ± 78
Placental weight (g)	463 ± 16	557 ± 22*	566 ± 26*
Birth/placental weight ratio	7.55 ± 0.26	6.22 ± 0.19**	6.21 ± 0.26**
Ponderal index	2.74 ± 0.05	2.74 ± 0.06	2.78 ± 0.05
% body fat	12.5 ± 0.7	12.1 ± 0.8	13.5 ± 0.7
Flank skinfold thickness (mm)	3.7 ± 0.2	3.8 ± 0.2	4.5 ± 0.3
Apgar (1 min) (n = 19,14,10)	8.3 ± 0.5	8.3 ± 0.3	7.9 ± 0.5
Apgar (5 min) (n = 19,14,10)	8.7 ± 0.3	8.8 ± 0.2	8.9 ± 0.1

* > Control, $p < 0.05$ (ANOVA).
** < Control, $p < 0.05$ (ANOVA).

Table 2
Glycemic status.

	Control	GDM A2	IDDM
	n = 25	n = 27	
1 h Glucose tolerance test (mmol/L)	6.1 ± 0.3	9.7 ± 0.4*	ND
	n = 11	n = 25	n = 13
Gestational age at sampling	31.3 ± 1.0	28.6 ± 0.9	26.2 ± 1.2
HbA_1c	5.6 ± 0.2	6.0 ± 0.2	6.0 ± 0.2
	n = 20	n = 30	n = 18
Gestational age at sampling	39.2 ± 0.3	38.5 ± 0.2	38.2 ± 0.3
HbA_1c	5.5 ± 0.1	6.1 ± 0.1**	6.0 ± 0.1**

ND: Not done.
* > Reference cut-off of 7.8 mmol/L.
** > Control, $p < 0.05$.

3.2. Glycemic characteristics

An initial 1 h glucose tolerance test (GTT) was administered to those in the control and GDM A2 groups, prior to GDM diagnosis. The data in Table 2 shows that those who were later classified as GDM, showed a significantly higher blood glucose concentration in the 1 h test. Those failing the 1 h test were assessed by a 3 h 100 g OGTT. Those classified as GDM A2 had a fasting blood glucose concentration of 5.56 ± 0.22 mmol/L and concentrations of 11.56 ± 0.33, 9.78 ± 0.39 and 6.78 ± 0.50 mmol/L at 1, 2 and 3 h following glucose administration (n = 27). In addition, HbA_1c was measured in all three groups in the early third trimester and at delivery (Table 2). These data show that while both diabetic groups had higher values than the control, both were within the normal range (4–7%, 3rd trimester, [12,13]). More importantly perhaps, the values in control, GDM A2 and IDDM groups were stable across the third trimester, implying good glycemic control immediately prior to and during this period.

3.3. Fetal and placental growth

We examined the maternal factors that might influence birth weight and placental weight in normal vs. diabetic pregnancies. Gestational age at delivery, pre-pregnancy weight, pre-pregnancy BMI and gestational weight gain were not correlated with either birth weight or

Table 3
Fetal circulating growth factors.

Growth factor	Control (n = 19)	GDM A2 (n = 26)	IDDM (n = 18)
Insulin (pmol/L)	114 ± 15	136 ± 30	179 ± 41
IGF-I (nmol/L)	8.51 ± 0.65	9.67 ± 0.64	9.73 ± 0.54
Free IGF-I (pmol/L)	32.7 ± 5.2	105.9 ± 20.9*	146.4 ± 24.8*
IGF-II (nmol/L)	71.3 ± 4.4	81.8 ± 5.1	69.9 ± 3.5

* > Control, $p < 0.05$.

placental weight.

3.4. Fetal circulating growth factors

Insulin, IGF-I, IGF-II and free IGF-I were measured in fetal cord serum obtained from control and diabetic pregnancies. Growth factor concentrations were determined by ELISA and the results are shown in Table 3. Most notably, there was no difference in total IGF-I among the groups. There were also no differences among control, GDM A2 and IDDM groups for insulin and IGF-II. However, free IGF-I was increased in both the GDM A2 (105.9 ± 20.9 pmol/L) and IDDM groups (146.4 ± 24.8 pmol/L) compared to control (32.7 ± 9.2 pmol/L; n = 26, 16, 19; $p < 0.05$).

3.5. Circulating growth factors and fetoplacental growth

We examined the relationship between birth weight, placental weight and the fetal circulating growth factors. Fetal circulating IGF-I and birth weight correlated positively in the control group ($r^2 = 0.54$, $p = 0.0003$; Fig. 1A). This relationship was not found in either of the diabetic groups. Similarly, a positive relationship was found between IGF-I and placental weight for the control group ($r^2 = 0.37$, $p = 0.006$; Fig. 1B) but not for either diabetic group. There was no significant relationship between birth weight or placental weight and any of the other circulating growth factors.

Supporting the relationship between IGF-I and placental and birthweight is the data concerning the percentage of body fat (%BF) in the neonates from these pregnancies. While %BF is correlated with birthweight in all groups (Fig. 2A, $r^2 = 0.71$, n = 70; $p < 0.0001$), % BF was correlated with IGF-I only in the control group (Fig. 2B, $r^2 = 0.60$, n = 19; $p < 0.0001$).

3.6. Syncytial GLUT1 protein expression

Slot immunoblotting for the GLUT1 glucose transporter was performed on paired microvillous and basal membranes isolated from control and diabetic placenta. The GLUT1 data was normalized to protein loading using measurements of Sypro Ruby fluorescence for the same slots. The results for the normalized GLUT1 expression measurements are shown in Fig. 3A and B. Fig. 3A shows that there is no difference in microvillous membrane GLUT1 expression between control vs. either GDM A2 or IDDM (0.15 ± 0.01 vs. 0.16 ± 0.01 or 0.16 ± 0.01; n = 22, 35, 17; NS). However, the basal membrane data (Fig. 3B) shows a significant increase in GLUT1 expression in membranes from the GDM A2 and IDDM diabetic groups (0.076 ± 0.007 vs. 0.127 ± 0.003 or 0.117 ± 0.006; n = 19, 24, 14; $p < 0.05$).

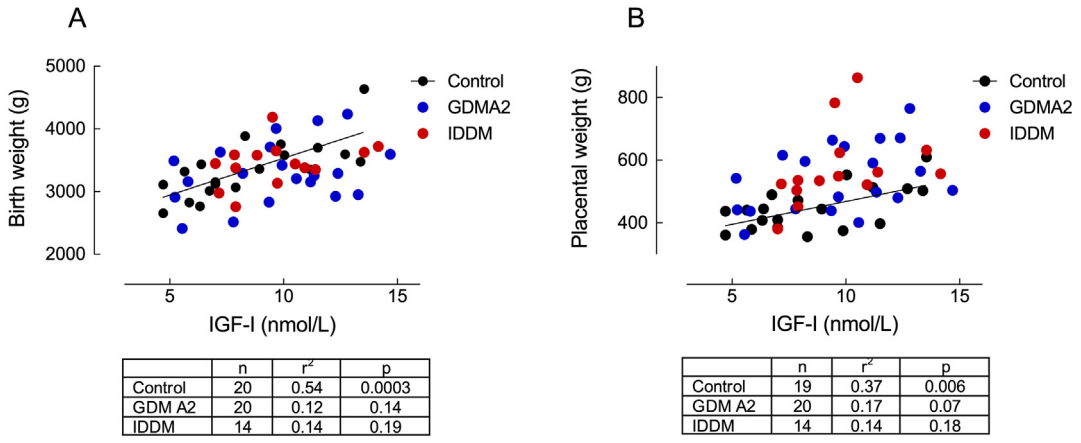
3.7. Microvillous and basal membrane glucose transport

Glucose transport was determined using a light-scattering technique developed in this laboratory [10], measuring the zero-*trans* uptake of D-glucose using a 40 mM glucose gradient. Rapid, mediated transport (J_{glc}) was measured for a subset of the microvillous and basal membrane samples used to measure GLUT1 expression. Glucose uptake into microvillous membrane vesicles did not differ between the control and both the GDM A2 and IDDM groups (Fig. 4A; 25.3 ± 1.2 vs. 27.0 ± 1.5 or 21.6 ± 1.0 $nmol.s^{-1}.mg^{-1}$, n = 10, 10, 10; NS). In contrast, basal membrane vesicle glucose uptake (Fig. 4B) was almost doubled in diabetics, from 31.4 ± 6.7 $nmol.s^{-1}.mg^{-1}$ in the control to 61.5 ± 5.0 or 60.7 ± 6.1 $nmol.s^{-1}.mg^{-1}$ in the GDM A2 and IDDM groups (n = 10, 10, 10; $p < 0.05$).

The light-scattering technique also provided a measure of transmembrane diffusional transport (J_D). Diffusional (non-protein mediated) glucose transport rates were approximately 10-fold lower than the transporter-mediated rates (3.4 ± 0.1 and 2.6 ± 0.2 $nmol.s^{-1}.mg^{-1}$; control microvillous and basal membranes, n = 9, 10) and did not differ between the groups (Fig. 4C).

3.8. GLUT 1 expression, glucose transport and birth/placental weight

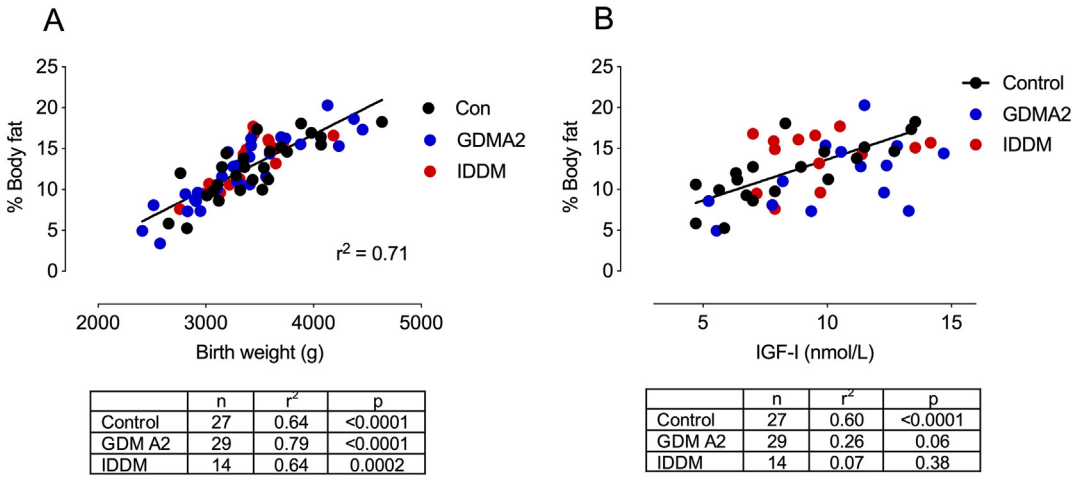
There was no correlation found between microvillous membrane GLUT1 expression and either birth or placental weight. Similarly, there was no significant relationship observed between microvillous glucose transport rates and either birth or placental weight. Basal membrane GLUT1 expression however, was positively correlated with birth weight for the control group (Fig. 5A; $r^2 = 0.26$, $p = 0.030$, n = 18) but not for either the GDM A2 or IDDM groups. Basal membrane glucose transport was also correlated with birth weight in the control group (Fig. 5B; $r^2 = 0.45$, $p = 0.035$, n = 10) but not in the GDM A2 or IDDM groups. Thus, basal membrane GLUT1 expression, and glucose transport appear



	n	r^2	p
Control	20	0.54	0.0003
GDM A2	20	0.12	0.14
IDDM	14	0.14	0.19

	n	r^2	p
Control	19	0.37	0.006
GDM A2	20	0.17	0.07
IDDM	14	0.14	0.18

Fig. 1. IGF-I and birth and placental weights: A. Birth weights (g) are plotted against IGF-I (nmol/L) for control, GDM A2 and IDDM groups. The regression line is plotted for the control group; no correlation was observed for the diabetic groups. B. Placental weights (g) are plotted against IGF-I (nmol/L) for control, GDM A2 and IDDM groups. The regression line is plotted for the control group; no correlation was observed for the diabetic groups.



	n	r^2	p
Control	27	0.64	<0.0001
GDM A2	29	0.79	<0.0001
IDDM	14	0.64	0.0002

	n	r^2	p
Control	27	0.60	<0.0001
GDM A2	29	0.26	0.06
IDDM	14	0.07	0.38

Fig. 2. Relationship between neonatal % body fat and (A) birth weight or (B) IGF-I.
A. Birthweights for control, GDMA2 and IDDM groups are plotted against neonatal % body fat. The regression line and the r^2 value are for all the groups combined. B. Neonatal % body fat is plotted against IGF-I (nmol/L) for control, GDM A2 and IDDM groups. The regression line is plotted for the control group only; no correlation was observed for the diabetic groups.

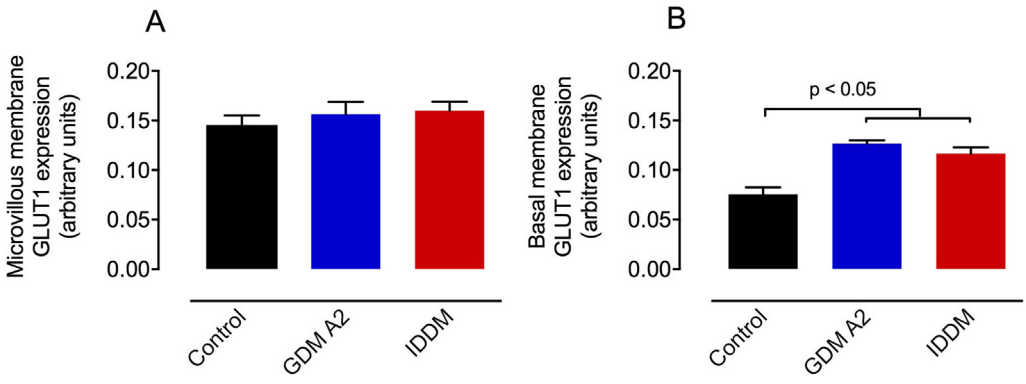


Fig. 3. GLUT1 expression on syncytial microvillous and basal membranes. A. Expression of GLUT1 protein, determined by slot-blotting, on microvillous membranes from control, GDM A2 and IDDM pregnancies (NS, ANOVA; n = 22, 38, 17). B. Expression of GLUT1 protein on basal membranes from control, GDM A2 and IDDM pregnancies ($p < 0.05$, ANOVA; n = 19, 24, 14).

to have reached a maximal level in the diabetic groups such that further increases in either have no effect on birthweight, resulting in loss of the correlation seen in the control group. There was no relationship between basal membrane GLUT1 or glucose transport and placental weight.

Despite the positive associations between birthweight and both IGF-I and basal membrane GLUT1 expression in the control group, there was no correlation between fetal circulating IGF-I and basal membrane GLUT1 expression in the diabetic groups. Nor was there any relationship between GLUT1 expression or glucose transport and any of the other circulating growth factors in the control or in the diabetic groups.

3.9. Fetal circulating IGF binding proteins

In view of the increased free IGF-I observed in the GDM A2 and IDDM groups compared to control, we examined another component of the fetoplacental IGF axis, fetal circulating IGF binding proteins (IGFBPs). Rather than measure the expression of the many individual IGFBPs, we chose to determine a functional measure, IGFBP binding capacity, for comparison between the control and diabetic groups. The same serum samples used for measurement of circulating growth factors were utilized to measure IGF binding proteins in a Western ligand blot assay using biotinylated IGF-II. A series of four pooled reference samples were run on each gel and used to normalize blot densities. The ligand blot revealed the presence of multiple bands with molecular weights of 37.6 ± 0.7, 33.6 ± 0.7, 31.0 ± 0.8 and 29.5 ± 0.8 (Fig. 6A; n = 4 for each, obtained from the pooled, reference samples). These blots displayed a pattern seen previously in human pregnancy [14,15] and were consistent with IGFBP 3, 2, 1, and 4 respectively. By summing the total quantity of bound IGF-II, we calculated the total binding capacity in each sample. The comparison between the control and diabetics groups is shown in Fig. 6B, demonstrating a drop in total binding capacity of > 50% in both the GDM A2 and IDDM groups compared to controls ($p < 0.0001$; n = 13, 10, 9). When broken down by isoform, the major changes in binding capacity are attributable to decreases in IGFBP2 and IGFBP3 for both GDM A2 and IDDM groups (Fig. 6C; $p < 0.05$, n = 10, 9). There were no changes in IGFBP1 or IGFBP4. The reduction in IGFBP total binding capacity in the GDM A2 and IDDM groups is consistent with the increased levels of free IGF-I seen in both.

3.10. Expression of type 1 insulin-like growth factor receptor protein

As our previous in vitro results demonstrated up-regulation of basal membrane GLUT1 by IGF-I [8], we decided to investigate whether changes in the expression of the placental type 1 insulin-like growth factor receptor (IGF-IR) might be contributory to the relationship between IGF-I and birth or placental weight. Expression of the type 1 insulin-like growth factor receptor (IGF-IR) in microvillous and basal membranes was measured using a polyclonal antibody to the β (transmembrane) subunit of the receptor. IGF-IR expression was determined by slot blotting since preliminary assays demonstrated a single immunoreactive band by Western blotting (data not shown). Expression on the basal membrane of the control group was similar to that on the microvillous membrane (1.44 ± 0.15 vs. 1.07 ± 0.23, n = 7, 7; N.S.). There were increases in both microvillous and basal

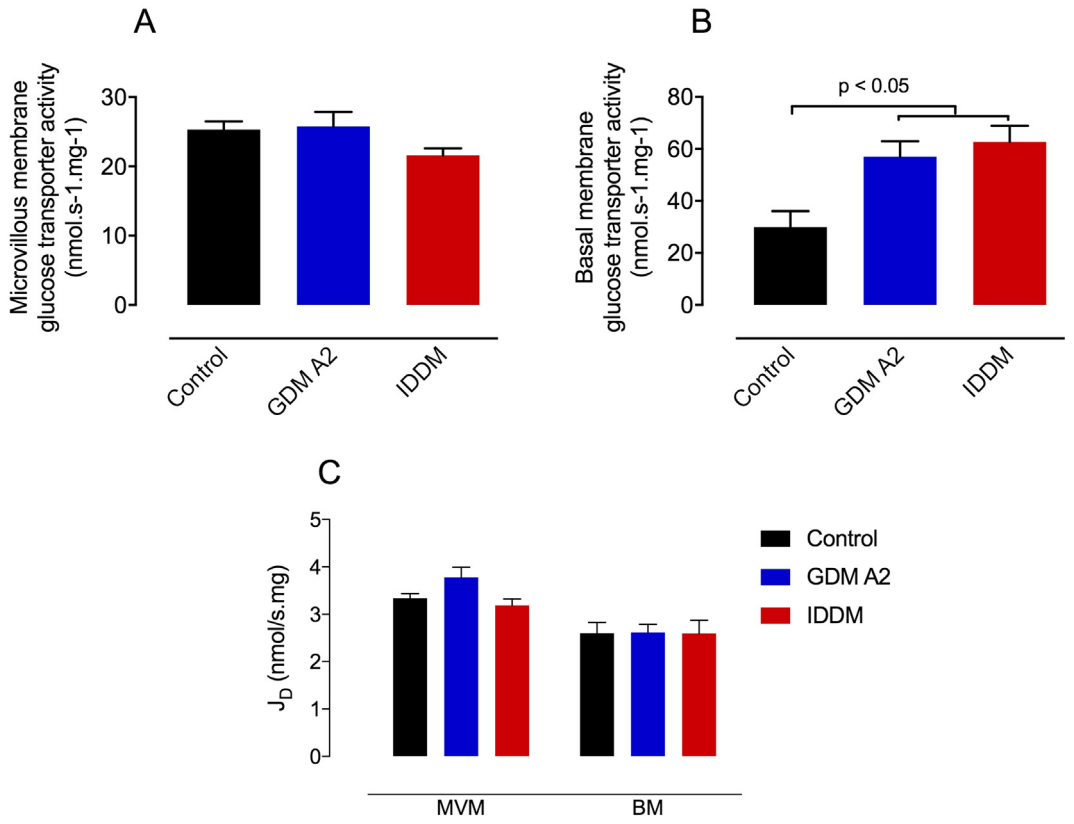
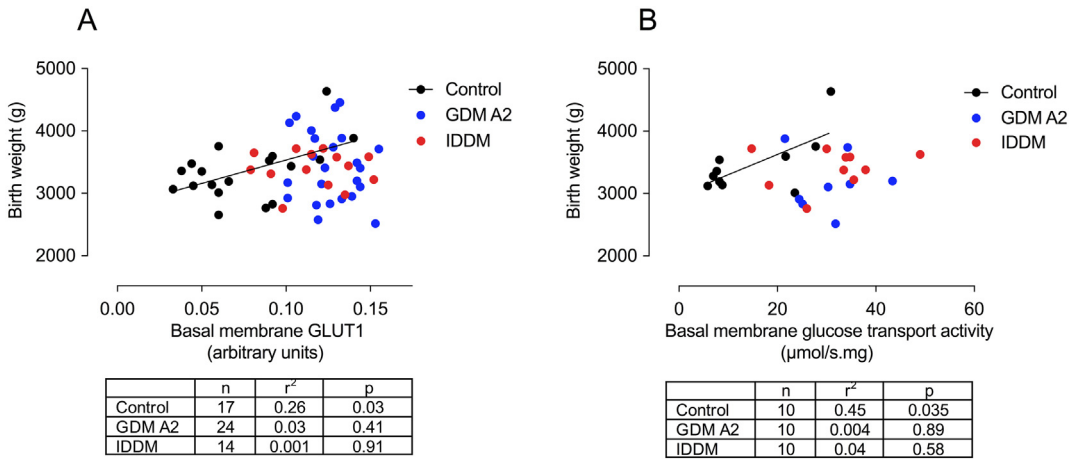


Fig. 4. Glucose transport activity of syncytial microvillous and basal membranes. A. Glucose transporter activity, determined by stopped-flow, of microvillous membranes from control, GDM A2 and IDDM pregnancies (NS, ANOVA; n = 9, 10, 10). B. Glucose transporter activity of basal membranes from control, GDM A2 and IDDM pregnancies ($p < 0.05$, ANOVA; n = 10, 8, 10). C. Non-protein mediated diffusional transport activity of microvillous and basal membranes from control, GDM A2 and IDDM pregnancies (numbers as for Fig. 4A).

membrane IGF-IR in the GDM A2 group, compared to control (Fig. 7). On the basal membrane, IGF-IR expression in the GDM A2 group was also greater than that in the IDDM, whereas on the microvillous membrane, expression in the IDDM group was not significantly different from either control or GDM A2 groups.

3.11. GLUT1 glucose transporter and the type 1 IGF receptor

In view of the increased expression in the GDM A2 group of the IGF-IR on the basal membrane of the syncytiotrophoblast, we tested whether, rather than IGF-I, the factor connecting changes in GLUT1 and the IGF axis might be the type 1 IGF receptor (IGF-IR). We examined the relationship between the expression of GLUT1 and IGF-IR on the microvillous and basal membranes from control, GDMA2 and IDDM placentas. Microvillous membrane GLUT1 showed no correlation with microvillous or basal membrane IGF-IR, for control, GDM A2 or IDDM pregnancies. Basal membrane GLUT1 expression, by contrast, was correlated with microvillous IGF-IR (Fig. 8), but only in control



	n	r^2	p
Control	17	0.26	0.03
GDM A2	24	0.03	0.41
IDDM	14	0.001	0.91

	n	r^2	p
Control	10	0.45	0.035
GDM A2	10	0.004	0.89
IDDM	10	0.04	0.58

Fig. 5. Relationship between birth weight and basal membrane GLUT1 expression and glucose transporter activity. A. Birth weight plotted against GLUT1 expression in basal membranes from control, GDM A2 and IDDM pregnancies. The regression line is plotted for the control group only; no correlation was observed for the diabetic groups. B. Birth weight plotted against glucose transport activity for basal membranes from control, GDM A2 and IDDM pregnancies. The regression line is plotted for the control group; no correlation was observed for the diabetic groups.

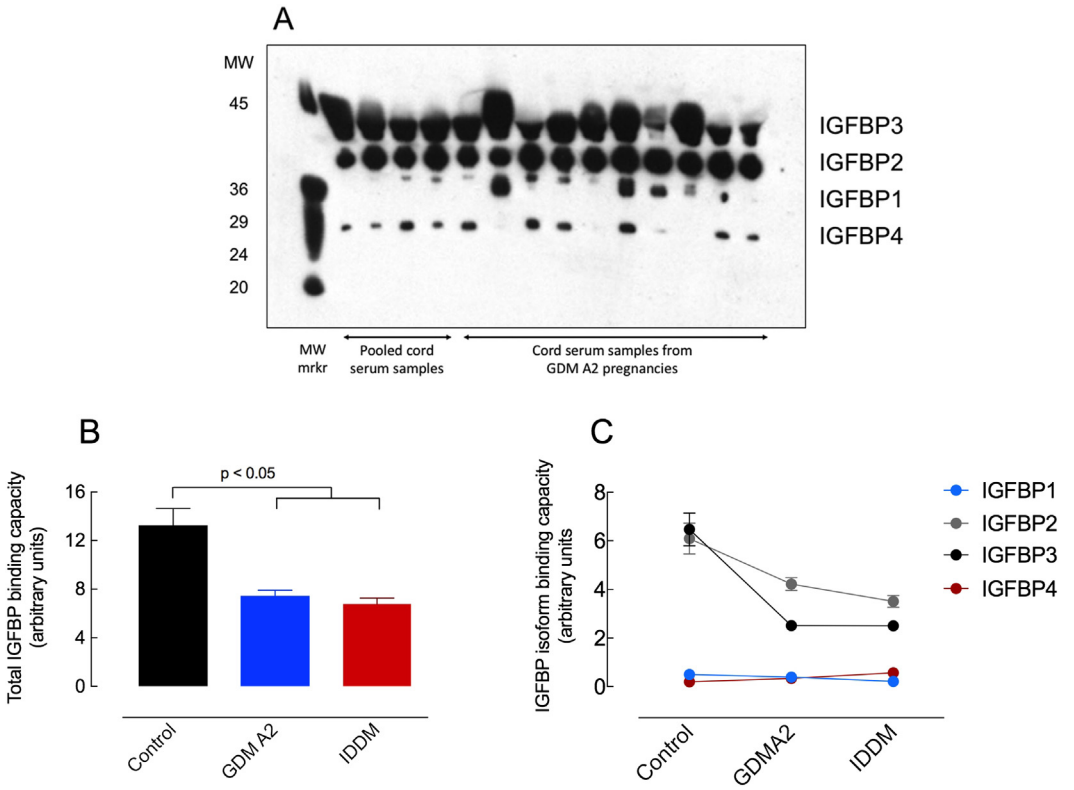


Fig. 6. IGF binding proteins in control, GDM A2 and IDDM pregnancies. A. An example of a Western ligand blot of serum from pooled normalization samples (lanes 2–5, from left) and from GDM A2 pregnancies (lanes 6–15). Ligand binding performed with biotinylated IGF-II. B. Quantification of Western ligand binding, measuring total binding capacity for control, GDM A2 and IDDM pregnancies ($p < 0.05$; n = 13, 10, 9). C. Binding capacity for IGFBP1–4 for serum samples from control, GDM A2 and IDDM pregnancies (n = 13, 10, 9).

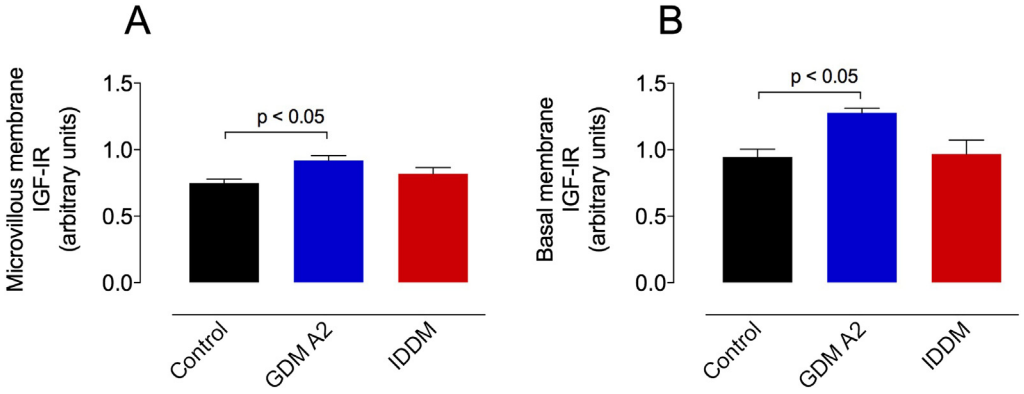
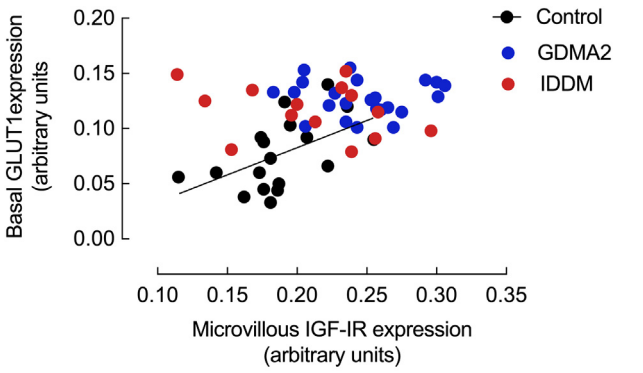


Fig. 7. Type 1 IGF receptor in control and diabetic pregnancies. A. Expression of type 1 IGF receptor β subunit in syncytial microvillous membranes from control, GDM A2 and IDDM pregnancies ($p < 0.05$, ANOVA; n = 22, 35, 17). B. Expression of type 1 IGF receptor β subunit in syncytial basal membranes from control, GDM A2 and IDDM pregnancies ($p < 0.05$, ANOVA; n = 21, 23, 16).

pregnancies.

4. Discussion

Although a link between fetal growth and placental nutrient transport has long been apparent, a description of the mechanism has been elusive. IGF-I has been suggested as a fetal nutritional signal by many investigators, however the evidence has been confusing and contradictory [16,17]. Our previous studies demonstrated a positive relationship between placental GLUT1 protein expression and IGF-I in vitro [8]. In this study we sought to determine whether and how this translates into alterations in GLUT1 under the abnormal metabolic conditions generated in pregnancies complicated by diabetes. We compared the protein expression of GLUT1 in syncytial microvillous and basal membranes with the concentrations of circulating fetal growth factors and IGF binding proteins as well as the syncytial type 1 IGF receptor from term control pregnancies and gestational (GDM A2) and pre-gestational (IDDM) diabetics. The most striking observation was the clear correlation, in the control group, between growth variables such as birth weight and factors such as fetal circulating IGF-I or GLUT1 expression, a correlation which was not present in either of the diabetic groups. This suggests that above a certain threshold of circulating IGF-I, further increases in IGF-I have no effect, due possibly to receptor saturation or decreased sensitivity. Similarly, beyond a particular level of basal membrane GLUT1 expression, further increases in GLUT1 (and transplacental glucose transport), have no further effect; beyond this threshold level, glucose transport no longer affects fetal and/or placental growth. It is notable that both diabetic groups showed a higher level of free IGF-I, likely due to a decrease in fetal circulating IGFBP binding capacity. We conclude that this data supports the hypothesis that fetal IGF-I acts as a fetal nutritional signal, contributing to the regulation of placental GLUT1 expression via the altered levels of



	n	r^2	p
Control	19	0.29	0.02
GDM A2	23	0.0002	0.95
IDDM	14	0.09	0.30

Fig. 8. Basal membrane GLUT1 and type 1 IGF receptors. Dependence of basal membrane GLUT1 expression on microvillous membrane expression of the type 1 IGF receptor β subunit for control, GDM A2 and IDDM pregnancies. The regression line is for the control group only; no correlation was observed for the diabetic groups.

fetal circulating IGFBPs.

We previously reported up-regulation of basal membrane GLUT1 expression and glucose transport, in both the GDM A2 and IDDM groups [5]. Our data differs from that of Jansson et al., who showed changes only in placentas from pregnant women whose diabetes was present before pregnancy, not in gestationally-induced diabetes. Their subsequent report confirmed the findings with pregestational diabetics [7], however their analyses combined gestational diabetics controlled by diet alone with those requiring insulin for glycemic control. Another study, examining GLUT expression by quantitative morphometric analysis, showed an increase in the expression of both GLUT4 and GLUT9 in insulin-dependent diabetic pregnancies (pregestational and gestational) as compared to control [18]. However, an increase in GLUT1 expression was observed only in pregestational diabetics, similar to the studies of Jansson et al. [6,7]. There are still questions regarding the pregestational and gestational diabetics. In data similar to that reported here, Tarrico et al. showed a clear increase in placental weight in their large study of gestational diabetic pregnancies [19], whereas those in the GLUT1 studies described above [7] were not increased. Another study, which differentiated between diet- and diet plus insulin-controlled gestational diabetics, was consistent with ours in showing increased GLUT1 in the latter group [20]. Finally, there is the question of what mechanism might account for the effects of pregestational diabetes on GLUT expression. As the pregestational diabetics are being treated for diabetes before and, presumably during early pregnancy, it is not clear why these cases should show similar changes in syncytial basal membrane GLUT1 to the gestational diabetics. One might anticipate a greater degree of maternal hyperglycemia and/or excessive insulin resistance in the gestational diabetics compared to control, prior to diagnosis. Perhaps, in the changing metabolic environment of early pregnancy, maternal hyperinsulinemia stimulates glucose transporter expression directly in the pregestational diabetics, leading to fetal hyperglycemia and the subsequent placental response.

Data for the various circulating growth factors is similar to that described previously in multiple reports for insulin, IGF-I and IGF-II [21]. Many of these reports also show the positive correlation between IGF-I and birth or placental weight seen in our data. The levels of free IGF-I have been less commonly reported but those we report here are similar to previously published values [22,23]. Unlike the decrease reported in type 1 gestational diabetics by Higgins et al. [24], we observed significant increases in free IGF-I in both diabetic groups. A less common aspect of this report is the quantitation of IGFBPs in cord serum. Other investigators have performed Western ligand blots previously on maternal and fetal sera [14,15,25], showing a similar distribution of IGFBP isoforms, however most reports on IGFBPs have quantified the changes in specific isoforms by Western blot [15,25–27]. In view of the multiple, post-translational modifications known to be possible for the IGFBPs, including phosphorylation, glycosylation and proteolysis [28], many of which modify IGF binding, we felt that the functional determination of binding capacity was a better measure of the changes taking place in diabetic pregnancy. We chose to determine IGFBP binding capacity directly using a Western ligand blot of the fetal serum samples, using biotinylated IGF-II as the ligand. Our Western ligand blots look similar to those reported by Giudice et al. for cord serum [25], to those given by Miell et al. for coelomic and amniotic fluid [15] and those determined by Sakai et al. for maternal serum [14]. A decrease in total binding capacity occurs in both diabetic groups, consistent with the concomitant increases in free IGF-I. The changes appear to be in the binding capacities of IGFBP2 and IGFBP3 in both diabetic types; IGFBP1 and IGFBP4 are unaffected. These measurements do not however provide any detail on the changes in other factors which might alter binding capacity.

The quantitative data on the protein expression of the type 1 IGF receptor (IGF-IR) is novel and shows a similar distribution, in term syncytiotrophoblast, to both the microvillous and basal membranes. This in contrast to the observations of Hiden et al. [29] who described IGF-IR as being confined to the basal membrane or to the findings of Fang et al. [30] who suggested that IGF-IR was located primarily on the microvillous surface of the syncytium. However, our results are in agreement with other reports [31–33] showing that the receptor is distributed between both membrane surfaces. Moreover, our data show equivalent quantities on both membranes. Quantitation of the receptor shows that expression in the gestational diabetics is increased on both microvillous and basal membranes of GDM A2 group compared to both the control and pre-gestational diabetics. Nevertheless, when basal membrane GLUT1 expression is plotted against either microvillous or basal membrane IGF-IR, the pattern is similar as for other parameters examined in this report; only the control group shows a correlation, whereas there is none for either diabetic group. It appears that GLUT1 expression in the diabetic groups may have been increased to some limiting value, such that increasing IGF-IR expression no longer has an effect.

The combination of data reported here leads us to the following conclusions. Under normal circumstances, birth and placental weight are positively regulated by fetal circulating IGF-I, at least in part through the increased expression and activity of basal membrane GLUT1 glucose transporters. In pregnancies complicated by diabetes however, there is a decrease in circulating IGFBP2 and IGFBP3, leading to an increase in fetal circulating free IGF-I. As a result of the elevated free IGF-I, there is increased expression of basal membrane GLUT1 (and associated transport activity) to a limiting, threshold level, beyond which factors operating to increase GLUT1 no longer have any effect. It is not clear what limits the maximal expression of basal membrane GLUT1. GLUT1 is inserted into both of the opposing faces of the epithelial syncytial layer, a relatively rare phenomenon which is also found in the human blood-brain barrier [34,35]. While GLUT3, also expressed on the microvillous surface of the syncytium [36], contains an apical targeting domain [37], GLUT1 seems to lack a targeting domain. It is possible that the microvillous and basolateral membrane distribution follows some type of hierarchical mechanism, whereby GLUT1 is targeted for apical transport until some crucial threshold or saturation limit is reached, at which point GLUT1 protein is directed to its secondary target, the basolateral membrane. This mechanism is very similar to the apical saturation effect postulated by Marmorstein et al. [38], in which target proteins interact with an apical sorting receptor structure prior to incorporation into lipid rafts [39] for transport and membrane insertion. The logical extension of this is that increased GLUT1 expression may also eventually saturate the basal membrane of

the syncytium, reaching its maximal, threshold level, consistent with the data obtained in the diabetic groups.

There is no immediately apparent mechanism for the down-regulation of IGFBPs in the gestational and pre-gestational diabetic groups. It is notable however, that the changes measured in the diabetic groups, such as the reduced IGFBPs and the increased free-IGF-I, as well as the increased placental weight, were determined at term. This follows 8–10 weeks of relative euglycemia in these pregnancies, eliminating many of the potential stimulatory agents which might be perturbed by chronic hyperglycemia. This observation raises the possibility of a more permanent form of stimulatory effect occurring via epigenetic alteration. IGFBP promoter methylation (and hence reduced IGFBP expression) has been shown to be associated with impaired glucose tolerance [40] and type 2 diabetes [41]. Further studies will be necessary to explore this possibility. What does seem likely from the aggregate data in the literature is that conditions earlier in pregnancy, prior to the accepted early third-trimester point for diagnosis of gestational diabetes, may have a lasting influence on parameters such as GLUT1 expression and placental weight in the third trimester, despite tight glycemic control in the last 8–10 weeks of pregnancy.

Transparency document

The Transparency document associated with this article can be found, in online version.

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