Human Reproduction Vol.20, No.2 pp. 521–530, 2005 doi:10.1093/humrep/deh596
Advance Access publication November 3, 2004.

Glucose transporter isoform 4 is expressed in the syncytiotrophoblast of first trimester human placenta

A.Ericsson[1,3], B.Hamark[2], T.L.Powell[1] and T.Jansson[1]

[1]Department of Physiology and Pharmacology and [2]Department of Obstetrics and Gynecology, Perinatal Center, Göteborg University, 405 30 Göteborg, Sweden

[3]To whom correspondence should be addressed. E-mail: anette.ericsson@fysiologi.gu.se

BACKGROUND: Placental glucose transport mechanisms in early pregnancy are poorly understood. The aims of this study were to investigate the expression of glucose transporter (GLUT) isoforms 1, 3 and 4 in first trimester villous tissue, to assess the effects of insulin on glucose uptake and compare them with term. METHODS: The expression of GLUT isoforms was investigated using immunohistochemistry, Western blot and reverse transcription (RT)–PCR in trophoblast tissue from terminations at 6–13 weeks gestation and term. The effects of insulin (300 ng/ml, 1 h) on glucose uptake were studied in villous fragments. RESULTS: In the first trimester, GLUT1 and GLUT3 were present in the microvillous membrane and the cytotrophoblast, and GLUT4 in perinuclear membranes in the cytosol of the syncytiotrophoblast (ST). GLUT4 protein (48 kDa) and mRNA were identified in trophoblast homogenates. Whereas GLUT1 was expressed abundantly in term placenta, the expression of GLUT3 and 4 was markedly lower at term compared with first trimester. Insulin increased glucose uptake by 182% ($n = 6$, $P < 0.05$) in first trimester fragments, but not in term fragments. CONCLUSIONS: The insulin-regulatable GLUT4 is expressed in the cytosol of first trimester ST compatible with a role for GLUT4 in placental glucose transport in early pregnancy. The placental expression pattern of GLUT isoforms in early pregnancy is distinct from that later in pregnancy.

Key words: diabetes/fetal growth/glucose transporter/insulin/term

Introduction

Glucose is the primary source of energy for the fetus and the placenta. The placenta consumes at least one-third of the glucose taken up from the maternal circulation (Ginsburg and Jeacock, 1964; Sakurai *et al.*, 1969). Since fetal gluconeogenesis is small, the fetus is critically dependent on the net transport of glucose across the syncytiotrophoblast (ST), the transporting epithelium of the placenta. Transplacental glucose transport is a facilitated carrier-mediated diffusion process (Schneider *et al.*, 1981), which in the human term placenta primarily involves glucose transporter (GLUT) isoform 1. The total fetal glucose consumption increases rapidly toward term due to the almost 20-fold increase in fetal weight during the second half of pregnancy. The increased requirements for glucose transport are met by an increase in surface area (Teasdale, 1980) and the maintenance of a high density of GLUT1 protein in the placenta. The GLUT1 protein expression and mediated glucose transport per unit of protein in the fetal-facing plasma membrane of the ST have been found to increase in late second trimester and to remain unchanged thereafter (Jansson *et al.*, 1993). In contrast, GLUT1 mRNA expression has been demonstrated to be 5-fold higher in first trimester than in term placenta (Hauguel-de Mouzon *et al.*, 1994). The novel glucose transporter isoform GLUT12 shows homology to GLUT4 and exhibits a similar pattern of distribution to GLUT4 since it is expressed in skeletal and cardiac muscle as well as in adipose tissue (Rogers *et al.*, 2002). Recently mRNA transcripts of GLUT12 were identified in the human placenta (Rogers *et al.*, 2002). Protein expression of GLUT12 has been shown further to be expressed predominantly in the ST in first trimester, whereas GLUT12 was expressed primarily in villous arterial smooth muscle cells and stromal cells at term (Gude *et al.*, 2003). Moreover, GLUT3 has been found along the surface of cytotrophoblasts (CTs) and to some extent in the ST of first trimester human placenta (Ogura *et al.*, 2000). GLUT3 mRNA expression has been shown to be highest in early gestation, with decreasing levels in mid- and full-term placenta (Sakata *et al.*, 1995).

The insulin-sensitive isoform GLUT4 is expressed almost exclusively in tissues that are insulin targets. These tissues exhibit an acutely regulated glucose transport system that responds within minutes to insulin (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). Insulin receptors (IRs) have been detected in the ST microvillous plasma membrane (MVM) of first trimester and term human placenta; however, receptor density was found to decrease markedly towards term (Desoye *et al.*, 1994). Several studies have

demonstrated that short-term stimulation with insulin was ineffective in altering glucose transport in placenta at term (Challier *et al.*, 1986; Urbach *et al.*, 1989; Boileau *et al.*, 2001). Many studies have failed to detect significant expression levels of the insulin-responsive GLUT4 isoform in placenta (Takata *et al.*, 1990; Hauguel-de Mouzon *et al.*, 1994; Barros *et al.*, 1995; Kainulainen *et al.*, 1997). However, Xing *et al.*, (1998) demonstrated a GLUT4 signal in intravillous stromal cells of human term placenta that co-localizes with IRs, which may suggest that placental glucose metabolism could be stimulated by fetal insulin. With respect to pregnancies complicated by insulin-dependent diabetes (IDDM), late first and early second trimester often represent a period of gestation that is characterized by a suboptimal glucose control even in patients with successful management during the second half of pregnancy. In some studies (Rey *et al.*, 1999), first trimester HbA_{1C} values in diabetic pregnancies have been identified as one factor that correlates to birth weight. This suggests that placental glucose transporters may be subjected to regulation in first trimester and that the rate of fetal growth may be determined in part already early in pregnancy.

First trimester glucose transport mechanisms and regulation remain poorly understood. The objective of this study was to characterize glucose transporter isoform expression in first trimester human placenta and to address the question of whether insulin regulates trophoblast glucose uptake in early pregnancy. Using immunohistochemistry, the cellular distribution of GLUT1, 3 and 4 isoforms was investigated and protein expression of GLUT1 and 4 was studied further by western blots. In addition, expression of GLUT4 mRNA was assessed using reverse transcription (RT)–PCR. The response to 1 h of insulin stimulation on the mediated uptake of isotope-labelled methyl-D-glucose was then studied in fresh villous fragments isolated from first trimester trophoblast (6–8 weeks). For comparison, GLUT isoform expression and effects of insulin on glucose uptake were also studied in term villous tissue.

Materials and methods

Tissue collection

First trimester trophoblast tissue (6–13 weeks of gestation) was collected from 27 women who underwent terminations by means of vacuum extraction at Sahlgren's University Hospital, Göteborg. All patients were healthy and none had evidence of complications during the index pregnancy or had had complications in previous pregnancies. Trophoblast tissue was identified from the aborted material and rinsed. Term placentas were obtained immediately after vaginal delivery or Caesarean section from women with normal uncomplicated pregnancies ($n = 18$). All term pregnant women attended the obstetric antenatal clinic at Sahlgren's University Hospital, Göteborg. The amniotic sac, chorionic plate and decidua were removed and trophoblast tissue was collected from different areas of the placenta and rinsed in physiological saline. Villous tissue to be used in glucose uptake studies was maintained at room temperature; all other samples were placed on ice. All protocols were approved by the Committee for Research Ethics at Göteborg University and informed consent was obtained from patients prior to tissue collection. Gestational age was determined from the first day of the last menstrual period and for term pregnancies confirmed by ultrasound at 16–18 weeks of gestation.

Immunohistochemistry

The immunohistochemical study was performed as described previously (Johansson *et al.*, 2000). In brief, tissue samples were rinsed in cold physiological saline and placed in a fixation solution containing zinc salts at 4°C (Beckstead, 1994, 1995) for 4–6 days. After fixation, tissue samples were dehydrated through a graded series of ethanol to xylene, paraffin embedded and cut into sections (4 μm) that were mounted onto slides. The slides were then heated to 60°C for 20 min and cooled at room temperature. The paraffin was removed in xylene, and the sections were rehydrated in ethanol and placed in 0.1 mol/l phosphate-buffered saline (PBS). The slides were then boiled for 10 min in 10 mmo/l citrate buffer, pH 6.0 and thereafter cooled down for 30 min at room temperature. Tissue was blocked in normal horse serum (NHS) and 2.5% non-fat dry milk in PBS (NHS-blotto) for 45 min at room temperature. Sections were incubated overnight at 4°C in a humidified chamber in antibodies diluted in NHS-blotto. The antibodies used were monoclonal anti-GLUT4 1F8 raised in mouse (1:150, Biogenesis, UK), polyclonal anti-GLUT1 raised in goat (1:100–1:500, Santa Cruz Biotechnology, CA) or anti-GLUT3 raised in goat (1:100–1:500, Santa Cruz Biotechnology) against peptides mapping within the C-terminus. NHS-blotto was used as control of non-specific staining and tissue from rat muscle as positive control of GLUT4 staining. Specific staining of the primary antibodies for GLUT1 and 3 was tested by pre-incubating the antibodies with blocking peptides (1:20, Santa Cruz Biotechnology) for 24 h at +4°C. Next, the sections were incubated in biotinylated anti-mouse (1:300) or anti-goat (1:300) IgG diluted in 1.5% NHS in PBS, for 30 min at room temperature. Endogenous peroxidases were inhibited by placing slides in 0.6% H_2O_2 in methanol for 10 min. A Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used to detect secondary antibodies. Slides were incubated with ABC reagent for 30 min at room temperature. In order to visualize the antigen stain, the slides were incubated with 3,5-diaminobenzidine according to the glucose oxidase method (Shu *et al.*, 1988) until a black reaction product appeared (~1–6 min). Slides were then placed in PBS, dehydrated in ethanol, cleared in xylene and finally mounted with no counterstain.

Isolation of microvillous plasma membranes

The MVM of the ST was isolated from first trimester and term placenta as described previously (Illsley *et al.*, 1990; Johansson *et al.*, 2000) with some modifications. All steps were carried out at 4°C. In brief, villous tissue was dissected from term placenta and from aborted material. Placental tissue, ~2–4 g for first trimester and 100 g for term, was rinsed in saline and placed in buffer D (250 mmol/l sucrose, 10 mmol/l HEPES-Tris, the protease inhibitors 0.8 μmol/l antipain, 0.7 μmol/l pepstatin, 80 μmol/l aprotinin, and 2.5 ml/g of tissue EDTA). Villous tissue was homogenized and centrifuged for 15 min at 10 000 *g*. The supernatant was collected and the pellet resuspended in buffer D and again homogenized and centrifuged for 10 min (10 000 *g*). Centrifugation of the combined supernatant was then carried out at 125 000 *g* for 30 min. The resulting post-nuclear fraction (pellet) was resuspended in buffer D, and $MgCl_2$ (12 mmol/l) added. The mixture was stirred slowly for 20 min and then centrifuged at 2500 *g* for 10 min. The supernatant, containing the MVM vesicles, was collected and centrifuged at 125 000 *g* for 30 min and the pelleted MVM vesicles were homogenized in buffer D. The MVM suspensions were then centrifuged

finally at 125 000 *g* for 30 min and the pellets were homogenized in buffer D and snap-frozen in liquid nitrogen and stored at −80°C until use. The protein concentration of MVM fractions was determined using the Bradford assay (Bradford, 1976). In order to assess MVM enrichment, the activity of alkaline phosphatase, an MVM marker, was measured in MVM and homogenates (Bowers and McComb, 1966).

Western blot analysis

Homogenates and isolated MVM were used in western blots. Homogenates were prepared on ice by homogenizing first trimester villous tissue in cold buffer D, snap-frozen in liquid nitrogen and stored at −80°C. Proteins from homogenates and MVM were separated by SDS–PAGE as previously described by Johansson *et al.*, (2000), with minor changes. Homogenate and vesicle preparations were thawed on ice and diluted with buffer D and sample buffer to a final concentration of 1.0 μg/μl. Electrophoresis was carried out at 200 V with a 10% SDS–polyacrylamide gel onto which 10 μg of protein/lane was loaded. Pre-stained SDS marker proteins with a molecular weight range of 27 000–180 000 Da (Sigma Chemical Co., St Louis, MO) were used as a ladder. The gels were equilibrated in transfer buffer after electrophoresis, mounted with a nitrocellulose transfer membrane and blotted overnight at 30 V. The membranes were then blocked in 5% blotto buffer [5% dry milk (w/v) in PBS/0.1% Tween-20 (PBST)] overnight at 4°C. For detection of GLUT4, one membrane was incubated with anti-GLUT4 1F8 (1:200) diluted in PBST, and as control a second membrane was incubated in PBST only. The secondary peroxidase-labelled antibody (anti-mouse, 1:10 000; Vector Laboratories) was used in conjunction with enhanced chemiluminescence to visualize the GLUT4 signal on autoradiographic film. The nitrocellulose membrane with proteins from the homogenate was stripped in stripping solution (2% SDS, 62.5 mmol/l Tris–HCl, 100 mmol/l β-mercaptoethanol, pH 6.7) for 30 min at 55°C. The membrane was washed repeatedly in PBST buffer, blocked again in blotto and reprobed with monoclonal anti-pan cytokeratin (1:7000; Sigma) or polyclonal anti-GLUT1 antibody (1:5000; Chemicon, CA). Quantification of specific GLUT or cytokeratin signal was carried out by Image Gauge software (version 3.45; Fuji film). The mean density of the signal for the positive control human fat homogenate was arbitrarily assigned a value of 1 and the densities of the individual samples from first trimester and term were calculated relative to the density of the positive control. Tissue from rat liver was used as negative control for GLUT4 protein expression.

RNA extraction and RT–PCR for GLUT4

Total RNA was isolated from placental villous homogenates using the RNA STAT-60 protocol (Tel-Test Inc., TX) with a few modifications. A 1 g aliquot of villous tissue was homogenized with a Polytron (T25 basic, IKA Labortechnik, Germany) in 5 ml of RNA STAT-60 solution and nucleoprotein complexes allowed to dissociate for a few minutes at room temperature. Chloroform was added (1 ml) to each sample vial which was then shaken vigorously for 15 s and kept at room temperature for 3 min. The homogenate was centrifuged at 7000 *g* for 25 min at 4°C and the aqueous phase transferred to a new vial. RNA precipitation was carried out by addition of 2.5 ml of isopropanol, after which the vials were stored at room temperature for 10 min and then centrifuged at 12 000 *g* for 10 min at 4°C. The RNA pellet was washed twice in 75% ethanol and centrifuged at 7500 *g* for 5 min at 4°C. The pellet was then dissolved in RNase-free dH_2O. The RNA concentration and purity were determined spectrophotometrically by absorbance measurements at 260 and 280 nm. First strand cDNA synthesis was carried out with a Superscript RNase H^- reverse transcriptase kit (Invitrogen, San Diego, CA), random hexamer primers and deoxy NTPs (dATP, dCTP, dTTP and dGTP; Roche Diagnostics GmbH, Mannheim, Germany), as previously described (Blomgren *et al.*, 1999). Each amplification reaction (25 μl) was performed using the PCR reagents 0.2 mmol/l dNTP, 2.5 μl 10× PCR buffer (250 mmol/l Tris–HCl, pH 8.3, 375 mmol/l KCl, 15 mmol/l $MgCl_2$; Sigma), 1 U of *Taq* DNA polymerase (Sigma), 1 μmol/l upstream (U) and downstream (D) primers and 1/25 of the cDNA synthesis reaction. Oligonucleotide primers were synthesized by Cybergene AB (Huddinge, Sweden). Fragments of cDNA for GLUT4 were amplified using the primers GLUT4 U 5′-CTTCGAGACAGCAGGGGTAG-3′ and GLUT4 D 5′-AGGAGCAGAGCCACAGTCAT-3′. The annealing temperature was 58°C for GLUT4. The cycle number (46 cycles) was chosen such that the PCR product would be in the linear phase of amplification. The expected product size of GLUT4 cDNA was 175 bp in length. Samples of the RT–PCRs were separated on a 1.5% agarose/0.5× Tris borate EDTA-containing ethidium bromide gel for ~35 min. In order to verify the size of the PCR products, a 100 bp ladder was used (Roche Diagnostics GmbH). The identity of the PCR product was confirmed by sequencing which was performed by CyberGene AB using primer elongation techniques. The gels were exposed in a LAS 1000-cooled CCD camera (Fujifilm). RNA isolated from human breast fat was used as positive control and water as negative control.

Insulin stimulation of glucose uptake in fresh villous fragments

Mediated glucose uptake into fresh villous fragments was carried out according to a previously described method for amino acid transport by system A (Jansson *et al.*, 2003) with modifications. In brief, villous tissue was isolated from terminations at 6–8 weeks gestation ($n = 6$). Tissue was dissected into fragments of ~2 mm in diameter and secured to one end of silk suture, which in turn was attached to three specially designed hooks. Fragments were then placed in fresh medium [Dulbecco's modified Eagle's medium (Sigma) diluted 1:3 in Tyrode's buffer (135 mmo/l NaCl, 5 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 10 mmol/l HEPES and 5.6 mmol/l D-glucose) pH 7.4] at room temperature. Villous fragments were incubated in medium only (control) or in medium containing insulin (300 ng/ml) for 1 h at 37'C. Following incubation, the fragments were washed in glucose-free Tyrode's buffer ± phloretin (100 μmol/l), the inhibitor of facilitated glucose transporters, for 2 min at 23°C with continuous shaking. In order to measure the mediated glucose uptake, villous fragments were incubated in 3-*O*-(methyl-[^{14}C])-D-glucose ± phloretin (250 μmol/l) for 70 s (first trimester) or 20 s (term) at 23°C. An initial time course for first trimester ($n = 4–8$) and term ($n = 7$) villous fragments, respectively, was registered in order to demonstrate linear glucose uptake. Fragments were first incubated in DMEM/Tyrode's for 30 min at 37°C, washed as described above and then incubated in 3-*O*-(methyl-[^{14}C])-D-glucose medium ± phloretin for 30, 60, 90, 120 and 180 s in first trimester and 10, 20, 30 and 60 s in term at 23°C. Subsequently, fragments were washed three times for 20 s in glucose-free Tyrode's buffer with 100 μmol/l phloretin on ice to terminate uptake. The fragments were then lysed in dH_2O overnight in order to release ^{14}C-labelled glucose taken up by cells, and fragments subsequently were transferred to a new vial and treated with 0.3 mol/l NaOH overnight. Scintillation fluid (aquasafe 300 plus, Zinsser Analytic, Germany) was added to the vials containing dH_2O and released ^{14}C-labelled glucose, mixed thoroughly and counted in a β-counter. Protein concentration was measured after denaturation in NaOH using the Bradford assay (Bradford 1976). Subsequently, the 3-*O*-(methyl-[^{14}C])-D-glucose uptake could be calculated as

pmol/mg protein/min. The mediated glucose uptake was calculated by subtracting the glucose uptake in phloretin-containing medium from uptake in phloretin-free medium.

Data analysis

The number of experiments (*n*) represents the number of placentas studied. In studies of glucose uptake, experiments from one placenta were performed in triplicate and averaged. Results are given as mean ± SEM. Differences between groups in glucose uptake were evaluated statistically using ANOVA, followed by Student–Newman–Keuls test. Linear regression and correlation was applied on time courses for glucose uptake in first trimester and term villous tissue. Western blot densitometric analysis of GLUT1 and GLUT4 protein expression in first trimester and term homogenate were evaluated using the *t*-test. A P-value <0.05 was considered significant.

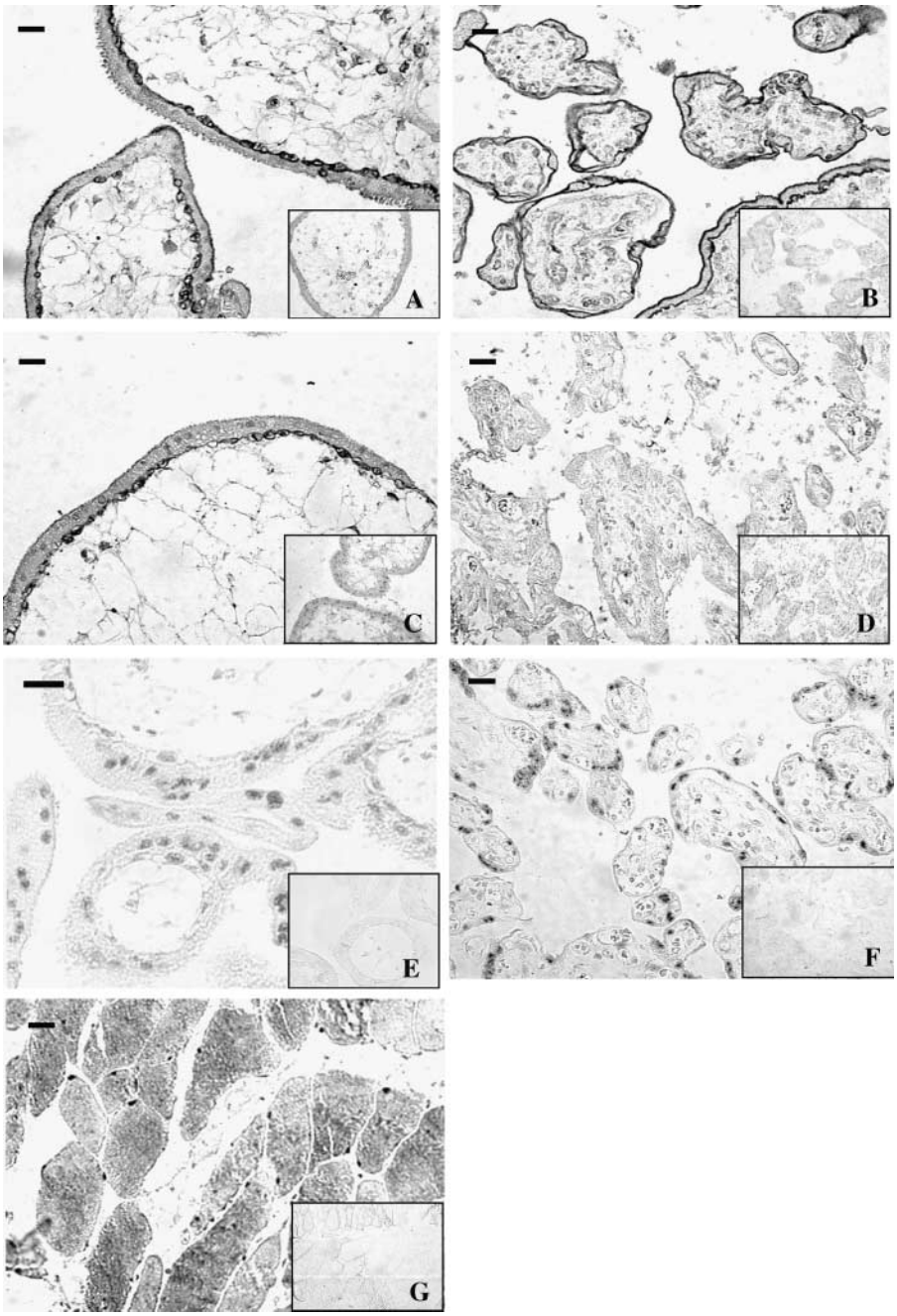

Figure 1. Sections of zinc-fixed first trimester [9 (A and C) and 6 (E) weeks of gestation] and term human placenta were stained with antibodies against GLUT1 (1:200), GLUT3 (1:200) or GLUT4 (1:150). Tissue of rat muscle (G) was used as a positive control of GLUT4 staining. GLUT 1 was detected in the microvillous membrane (MVM) of the syncytiotrophoblast (ST) and plasma membrane of underlying cytotrophoblast (CT) of first trimester placenta (**A**) and in the MVM and basal membrane (BM) at term (**B**). GLUT3 staining was found in MVM and CT at first trimester (**C**) but not at term (**D**). GLUT4 signal was observed in perinuclear membranes of the ST cytoplasm of first trimester (**E**), and also to some extent in the sections at term (**F**). GLUT4 signal was also demonstrated in rat muscle (**G**). Control sections showed no background staining (inserts). Magnification ×400 (A–D, F and G) and ×600 (E). The scale bar is 20 μm.

Results

Immunohistochemistry

GLUT1 expression was detected in both first trimester and full-term placentas. In first trimester placenta, at weeks 6, 9, 11 and 13, the maternal-facing MVM of the ST and the plasma membrane of the underlying villous CTs were densely stained at the optimal antibody concentration of 1:200 (Figure 1A). In full-term placenta, GLUT1 was clearly detected in the MVM and the basal membrane of the ST (Figure 1B). Optimal GLUT3 expression was detected at an antibody concentration of 1:200 in the MVM of the ST and plasma membrane of the CT in first trimester only (Figure 1C and D). The staining of primary GLUT1 and 3 antibodies was shown to be specific by pre-incubation with blocking peptides (data not shown). GLUT4 expression was detected in the ST of first trimester trophoblast tissue, whereas GLUT4 was localized to the cytoplasm primarily in perinuclear membranes and possibly a minor fraction in the MVM of the ST (Figure 1E). In term sections, less or no GLUT4 protein signal was detected in the cytosol of the ST. GLUT4 staining was clearly detected in rat muscle tissue (Figure 1G). No staining was detectable in any of the control sections (Figure 1A–G, inserts).

Western blot

Immunoblotting using homogenates and GLUT1 antibodies showed expression between 7 and 12 weeks of gestation (Figure 2A), where GLUT1 protein expression in first trimester homogenate was lower ($P < 0.05$) compared with term homogenates (Figure 3A). Western blot analysis of homogenates from first trimester trophoblast tissue and term placenta showed the presence of GLUT4 at an approximate size of 48 kDa (Figure 2C). The protein expression of GLUT4 was markedly higher ($P < 0.05$) in first trimester homogenates compared with term (Figure 3B). In addition, a signal of the same size was observed in human fat. No GLUT4 signal was detected in rat liver. We proceeded by isolating microvillous plasma membranes from villous tissue obtained from three patients undergoing terminations at 10–12 weeks of gestation and from three term pregnancies. The activity of alkaline phosphatase, a marker for MVM, was assayed in homogenates and MVM fractions. Alkaline phosphatase activity was on average enriched 6-fold ($n = 3$) in first trimester MVM, a value consistent with substantial enrichment of the microvillous plasma membrane in the MVM, albeit not as high as 14-fold in term MVM, commonly achieved in term preparations (Johansson *et al.*, 2000; Persson *et al.*, 2002b). Using western blot and anti-GLUT4 antibodies, no or only a weak signal was detectable in first trimester MVM and no signal at all at term (data not shown). GLUT1 probing upon stripping of the same membrane clearly showed a signal in first trimester and term MVM where the GLUT1 expression was increased at term compared with first trimester (Figure 2B). The morphology of trophoblast villi

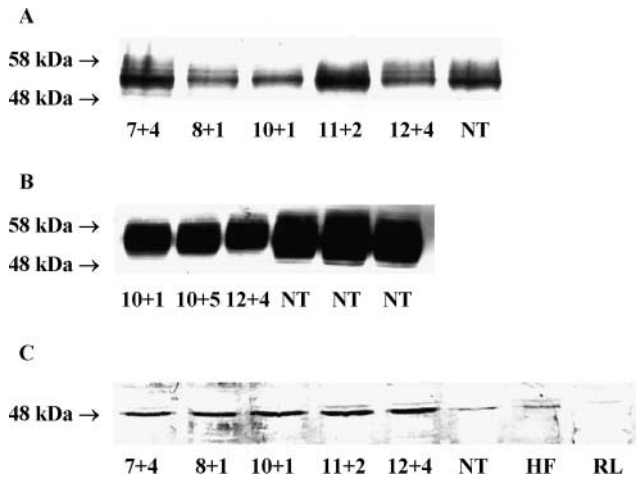

Figure 2. Western blot of GLUT1 (**A**) and GLUT4 (**C**) in homogenates from first trimester ($n = 5$) and term (NT, $n = 1$), and GLUT1 in MVM (**B**) from first trimester ($n = 3$) and term (NT, $n = 3$). Figures indicate the gestational age (weeks + days) of first trimester placentas. Homogenate of human fat (HF) was used as positive control and rat liver (RL) as negative control for GLUT4.

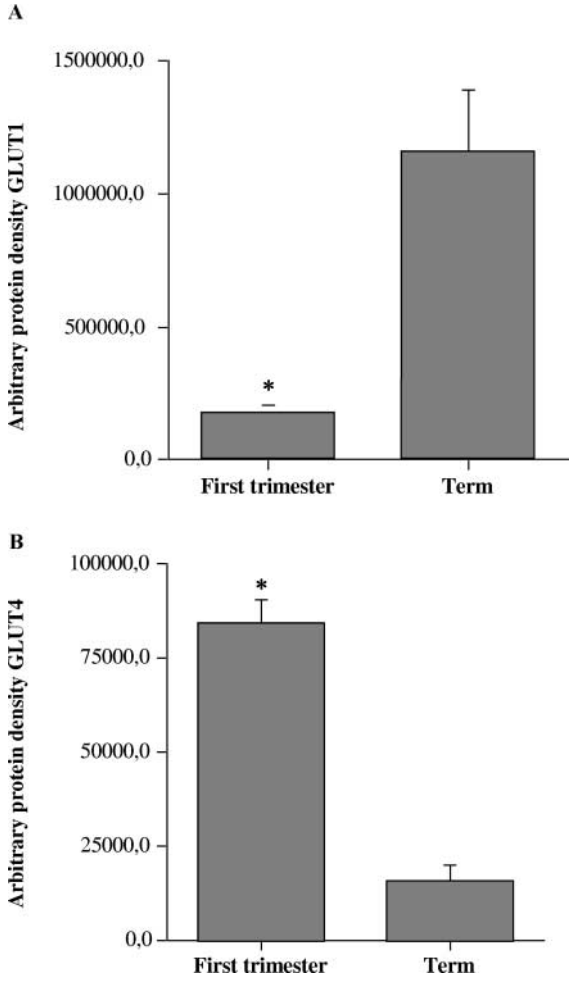

Figure 3. The mean density of GLUT1 (**A**) and GLUT4 (**B**) protein expression as measured by Western blot analysis in homogenates from first trimester ($n = 5$) and term ($n = 5$) trophoblast tissue. The protein expression of GLUT1 was significantly lower and GLUT4 was significantly higher ($P < 0.05$) in first trimester compared with term. The mean density of the positive control was assigned arbitrarily a value of 1 and the mean densities of first trimester and term calculated relative to the positive control. Values are given as means ± SEM.

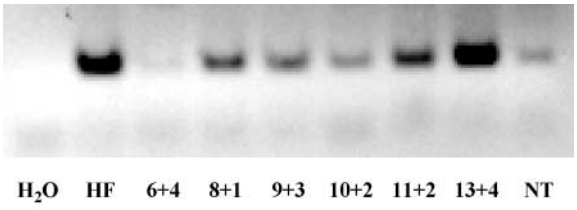

Figure 4. RT–PCR analysis of GLUT4 in first trimester ($n = 5$) and term placenta (NT, $n = 1$). The expected PCR product size was 175 bp. The figures indicate the gestational age of first trimester placentas (weeks + days). Water was used as negative control and human fat (HF) as positive control.

undergoes changes in late first trimester and between early and late pregnancy. In order to obtain a crude estimate of changes in the relative contribution of ST to homogenates of trophoblast villi between first trimester samples as well as between first trimester and term, western blots using an antibody against cytokeratin, an epithelial cell marker, were performed. No significant difference in cytokeratin protein expression was observed in these experiments (data not shown), suggesting that the relative contribution of ST cell mass to homogenates is similar in our samples.

mRNA expression of GLUT4

GLUT4 mRNA was detected and amplified in first trimester trophoblast tissue by RT–PCR (Figure 4). GLUT4 was observed in first trimester placenta at 6, 8, 9, 10, 11 and 13 weeks of gestation. A faint signal of GLUT4 product was detected in term placenta. Sequencing of the PCR product of 10+2 weeks confirmed a GLUT4-specific product of 175 bp.

Time dependence of glucose transport

Figure 5 demonstrates the linear uptake of 3-*O*-(methyl-[^{14}C])-D-glucose into villous fragments between 30 and 120 s in first trimester ($n = 4–8$) and between 0 and 30 s at term ($n = 7$, $P < 0.05$). In subsequent experiments, we used the time point 70 s for first trimester and 20 s for term to measure mediated glucose uptake.

Insulin regulation of glucose uptake

Villous fragments from first trimester placentas at 6–8 weeks of gestation were incubated in DMEM/Tyrode's buffer, with and without insulin (300 ng/ml) for 1 h at 37°C. Insulin concentrations used in these experiments are supraphysiological and have been shown to stimulate the activity of the amino acid transporter system A in term villous fragments *in vitro* (Jansson *et al.*, 2003). Insulin increased glucose uptake by 182% compared with control in first trimester fragments ($P < 0.05$, Figure 6). In contrast, no significant effect of insulin was observed on glucose uptake in term fragments.

Discussion

The novel aspect of the current study is the demonstration of the presence of the insulin-regulatable glucose transporter isoform GLUT4 in villous tissue of first trimester human placenta. GLUT4 was localized primarily to perinuclear

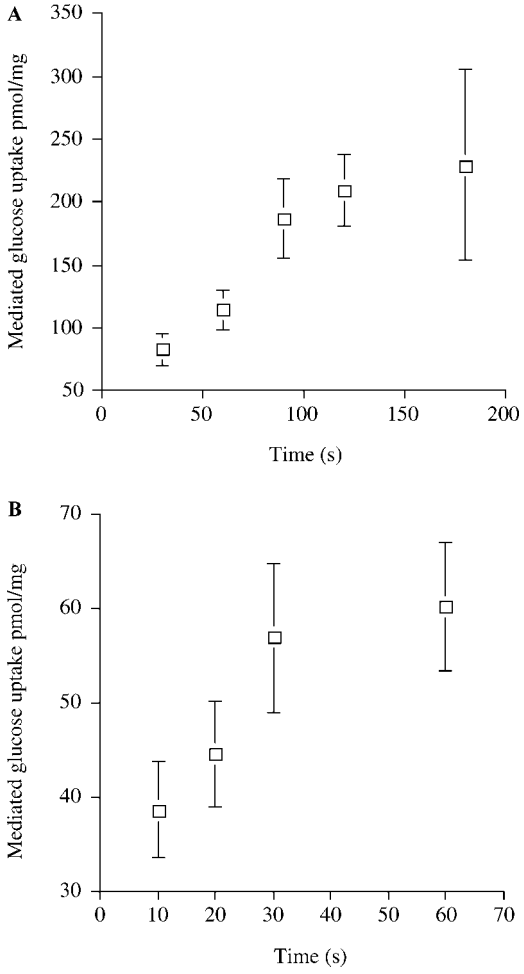

Figure 5. Time course of mediated glucose uptake in first trimester (**A**, $n = 4–8$) and term villous fragments (**B**, $n = 7$). Linear glucose uptake was observed between 30 and 120 s in first trimester and between 0 and 30 s at term ($P < 0.05$).

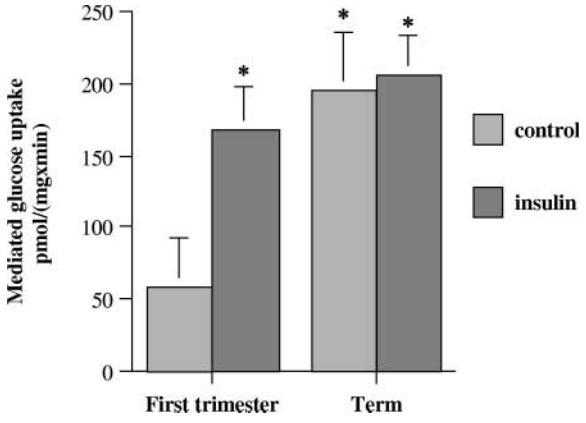

Figure 6. Isolated villous fragments from first trimester placental tissue at 6–8 weeks of gestation ($n = 6$) and fragments from term placentas ($n = 4$) were incubated for 1 h at 37°C with and without insulin (300 ng/ml). Basal glucose uptake was higher in term compared with first trimester ($P < 0.05$). Insulin stimulated glucose uptake in first trimester but not at term

membranes in the cytoplasm of the first trimester ST, the transporting epithelium of the human placenta. Furthermore, GLUT3 was found to be expressed in the MVM of the ST in first trimester. These findings are compatible with the possibility that GLUT4 and GLUT3 may play a role in placental glucose uptake in early pregnancy. In addition, we show that insulin stimulates mediated glucose uptake in villous fragments obtained from first trimester but not at term.

Previously, GLUT1 protein has been shown to be localized in ST plasma membranes and endothelial cells of human term placenta (Takata *et al.*, 1992; Jansson *et al.*, 1993; Hauguel-de Mouzon *et al.*, 1997), and the current study confirms this cellular distribution. The abundant expression of GLUT1 protein in the polarized plasma membranes of the term ST, together with the low expression or absence of other glucose transporter isoforms, such as GLUT3 (Jansson et al., 1993; Hauguel-de Mouzon et al., 1997) and GLUT4 (Xing et al., 1998), supports the view that GLUT1 is the primary GLUT isoform mediating transplacental transport in late gestation. Furthermore, the high level of expression of GLUT1 in the placental barrier in first trimester as shown in the current study and previously by Ogura et al. (2000) indicates an important role for this transporter in mediating placental transport of glucose also in early pregnancy. At the light microscopy level, it was difficult to distinguish GLUT1 staining of the basal plasma membrane of the ST due to the abundant GLUT1-positive CTs that are localized adjacent to the ST plasma membrane. In the current study, GLUT1 protein expression was higher in term compared with first trimester placental homogenates. This finding is probably not due to a marked difference in the contribution of ST cell mass to our samples between early pregnancy and term since cytokeratin protein expression did not differ between these samples.

In the human, GLUT3 is expressed predominantly in organs with high glucose requirements such as in the brain, testis and placenta (Gould *et al.*, 1991; Haber *et al.*, 1993). We have shown previously that GLUT3 protein is not expressed in significant amounts in ST plasma membranes isolated from term placenta (Jansson *et al.*, 1993). Subsequently, Hauguel-de Mouzon *et al.*, (1997) reported that trophoblast villous GLUT3 expression appears to be restricted to the fetal capillary endothelium at term. In first trimester placenta, the presence of GLUT3 protein has been identified along the surface of CTs and, to some extent, in the ST (Ogura *et al.*, 2000). Our results on GLUT3 protein expression in early pregnancy are in general agreement with the data of Ogura *et al.*, but we further add information on GLUT3 expression in the microvillous plasma membrane of the ST. However, in contrast to previous studies (Hauguel-de Mouzon *et al.*, 1997), we were unable to detect GLUT3 in vascular endothelium of term placenta. This discrepancy may be related to differences in fixation protocols or distinct sources for the GLUT3 antibodies.

GLUT4 is the major insulin-responsive glucose transporter isoform and is expressed primarily in striated muscle and adipose tissue (Birnbaum, 1989; Charron *et al.*, 1989; James *et al.*, 1989). In unstimulated adipocytes, most of the GLUT4 has been demonstrated to be present in perinuclear membranes (Guilherme *et al.*, 2000). The mechanisms by which insulin increases GLUT4-mediated glucose uptake are complex but involve both translocation of transporters from intracellular pools to the plasma membrane and an increase in the intrinsic activity of GLUT4 transporters already present at the cell surface (reviewed in Bryant *et al.*, 2002). Several investigators have failed to show expression of the GLUT4 isoform in human term placenta (Takata *et al.*, 1990; Barros *et al.*, 1995); however, Xing *et al.*, (1998) reported GLUT4 protein expression in intravillous stromal cells. To the best of our knowledge, we demonstrate for the first time GLUT4 protein expression primarily in the cytoplasm of first trimester STs, and less expression in the cytoplasm of term human placenta. GLUT4 was detected by immunohistochemistry in the syncytium throughout the second half of first trimester and at term. In addition, GLUT4 expression in late first trimester and term was confirmed by western blotting, showing GLUT4 to be present in placental homogenates and no or very low expression in isolated ST MVM at 12 weeks of gestation. The protein expression of GLUT4 was significantly higher in first trimester homogenates compared with term. At term, GLUT4 has been reported to be expressed in villous stromal cells (Xing *et al.*, 1998), and it has been suggested that GLUT4 may be important for glucose transport and conversion to glycogen in stromal cells in response to fetal insulin (Illsley, 2000). Although we found evidence for expression of GLUT4 by using western blot and immunohistochemistry, no distinct GLUT4 staining was found in stromal cells. Rather, the localization of the faint GLUT4 expression was found in perinuclear membranes of the ST cytoplasm. The reasons for this discrepancy may be due to those previously discussed for GLUT3 immunohistochemistry.

Insulin receptors are expressed in first trimester placenta, predominantly in MVM, the maternal-facing plasma membrane of the ST (Tavare and Holmes, 1989; Desoye *et al.*, 1994). Furthermore, these authors reported a distinct gestational pattern in insulin receptor expression since MVM of the ST at term only showed patches of weak insulin receptor signal, whereas the expression in fetal endothelium was higher compared with first trimester. According to our study, the same distinct gestational pattern does appear to apply to the expression of GLUT4 in the ST. The question of whether insulin regulates human placental glucose uptake and transport has been addressed quite extensively and remains controversial. Most studies (Challier *et al.*, 1986; Urbach *et al.*, 1989), but not all (Brunette *et al.*, 1990), show that insulin does not affect placental glucose transporters at term. In a first trimester trophoblast cell line, glucose transport activity was increased after 1 h of incubation with insulin (10 ng/ml), insulin-like growth factor (IGF)-I or IGF-II (Kniss *et al.*, 1994; Gordon *et al.*, 1995).

The finding of GLUT4 in the maternal–fetal interface in first trimester may suggest that the placental glucose transport in early pregnancy is sensitive to regulation by maternal insulin and plasma glucose levels. We proceeded by assessing the effect of insulin on the mediated uptake of methylglucose in individual villous fragments. The use of this

experimental system in transport studies has been thoroughly evaluated recently for measurements of system A uptake at term (Jansson *et al.*, 2003). The ultrastructural integrity of the fragments was confirmed in the previous study by using electron microscopy, showing an intact morphology up to 3 h of incubation. Furthermore, the production of the hormones human placental lactogen and 17β-estradiol was shown to be stable and the release of LDH very low, for up to 3 h (Jansson *et al.*, 2003). Other investigators have evaluated the use of another model of human placental explants from first trimester and term villous fragments and demonstrated stable conditions up to 4 h, when testing morphological, biochemical and physiological parameters (Sooranna *et al.*, 1999). The use of villous fragments in uptake studies assesses, in principal, the transport of glucose from the maternal circulation across the maternal-facing MVM into the ST cell and does not provide information of net transport of glucose to the fetus over the basal membrane. Our data show that insulin, at least in supraphysiological concentrations, stimulates glucose uptake in first trimester fragments studied *in vitro*. In contrast, term fragments did not respond to insulin. It is interesting to note that ST expression of GLUT4 (current study), GLUT12, a novel glucose transporter isoform that has been suggested to be insulin sensitive (Rogers *et al.*, 2002), and insulin receptors (Desoye *et al.*, 1994) decreases markedly from early to late gestation. Indeed, GLUT12 appears not to be expressed in term ST and we demonstrated only weak GLUT4 expression in the ST at term. It may be speculated that insulin stimulates villous glucose uptake by recruitment or activation of the GLUT4 and/or GLUT12 transporters in first trimester, and the lack of effect of insulin on villous glucose uptake at term might be related to the decrease/absence of GLUT4 and GLUT12 protein expression in ST at this stage of gestation. However, this hypothesis needs to be tested in further experiments. It appears less likely that the lower levels of expression of insulin receptors in MVM in late gestation can explain the lack of effect of insulin on glucose uptake since insulin, at the same concentration as used in the current study, increases system A activity in term villous fragments (Jansson *et al.*, 2003).

The data of the current and previous studies (Rogers *et al.*, 2002) clearly suggest that glucose transporter expression is strikingly different in first trimester ST compared with term. Indeed, high levels of ST expression of GLUT3, 4 and 12 appear to be unique to early pregnancy. Furthermore, an abundant expression of insulin receptors in MVM is a characteristic of early pregnancy (Desoye *et al.*, 1994). The physiological significance of the expression of insulin-regulated GLUT4 and GLUT12 in the cytosol of the syncytium in early pregnancy is unknown. It is possible that these transporters represent a mechanism by which maternal nutrition and metabolism, which alter insulin levels, influence placental glucose uptake and thereby possibly the growth trajectory of the placenta and fetus in the first part of pregnancy. Blood flow through the intervillous space cannot be demonstrated clearly until 10–12 weeks of gestation (Jaffe *et al.*, 1997), suggesting that the fetoplacental tissue prior to this time is more dependent on anaerobic metabolism and therefore has a relatively high glucose demand. It is therefore possible that the increased capacity for trophoblast glucose uptake provided by the recruitment of MVM insulin-sensitive glucose transporters is necessary to meet the relatively high glucose requirements in first trimester. Moreover, a relatively high glucose demand concomitant with a limited glucose supply from the maternal blood flow may result in low extracellular glucose concentrations in the vicinity of the maternal–fetal interface, providing a rationale for the presence of GLUT3, a high-affinity glucose transporter, in the maternal-facing plasma membrane of the ST early in pregnancy.

In pregnancies complicated by insulin-dependent diabetes mellitus, the occurrence of accelerated fetal growth remains high despite rigorous glucose control throughout the second half of pregnancy. Suboptimal glucose control in early pregnancy, as represented by significantly elevated HbA_{1C} values, is one predictor for fetal overgrowth in IDDM (Rey *et al.*, 1999), suggesting that metabolic disturbance during this critical period affects the fetal growth trajectory later in pregnancy. We have suggested previously that in the IDDM mothers with markedly disturbed glucose metabolism in the first trimester, altered maternal insulin and nutrient levels may affect placental transport capacity and metabolism for the remainder of the pregnancy (Jansson and Powell, 2000). This could result in increased nutrient transport and fetal growth even when the mother is normoglycaemic in the second half of pregnancy. Indeed, we have shown that GLUT1 protein expression and glucose transport activity (Jansson *et al.*, 1999) as well as the activity of system A (Jansson *et al.*, 2002) are increased in ST plasma membranes isolated from placentas obtained at term from IDDM pregnancies associated with accelerated fetal growth. However, intense insulin treatment of IDDM patients in order to achieve normoglycaemia in the first trimester has been shown to be inefficient in significantly decreasing the incidence of accelerated fetal growth (Persson and Hanson, 1996). In the light of the findings in the present study, it may be speculated that high levels of insulin achieved during attempts to normalize glucose levels in patients with IDDM in early pregnancy may increase placental glucose uptake by recruiting insulin-sensitive glucose transporters to the maternal-facing MVM. As a consequence, glucose transfer to the fetus increases and fetal growth is accelerated. However, whether changes in the activity and expression of insulin-regulatable glucose transporters in the first trimester placenta contribute to accelerated fetal growth in IDDM remains to be established.

Acknowledgements

We would like to thank Louise Svensson at the Department of Physiology and Pharmacology, Göteborg University for providing human fat for positive controls in this study. This study was supported by grants from the Swedish Research Council (10838), the Swedish Diabetes Association, The Novo Nordisk Foundation, Frimurare-Barnhus-direktionen, theÅhlens Foundation, theÅke Wiberg Foundation, the Sven Jerring Foundation, the Magnus Bergvall Foundation and the Willhelm and Martina Lundgrens Foundation.

References

Barros LF, Yudilevich DL, Jarvis SM, Beaumont N and Baldwin SA (1995) Quantitation and immunolocalization of glucose transporters in the human placenta. Placenta 16,623–633.

Beckstead JH (1994) A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. J Histochem Cytochem 42,1127–1134.

Beckstead JH (1995) A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues: addendum. J Histochem Cytochem 43,345.

Birnbaum MJ (1989) Identification of a novel gene encoding an insulin-responsive glucose transporter protein. Cell 57,305–315.

Blomgren K, Hallin U, Andersson AL, Puka-Sundvall M, Bahr BA, McRae A, Saido TC, Kawashima S and Hagberg H (1999) Calpastatin is up-regulated in response to hypoxia and is a suicide substrate to calpain after neonatal cerebral hypoxia-ischemia. J Biol Chem 274,14046–14052.

Boileau P, Cauzac M, Pereira MA, Girard J and Hauguel-De Mouzon S (2001) Dissociation between insulin-mediated signaling pathways and biological effects in placental cells: role of protein kinase, B and MAPK phosphorylation. Endocrinology 142,3974–3979.

Bowers GN Jr and McComb RB (1966) A continuous spectrophotometric method for measuring the activity of serum alkaline phosphatase. Clin Chem 12,70–89.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72,248–254.

Brunette MG, Lajeunesse D, Leclerc M and Lafond J (1990) Effect of insulin on D-glucose transport by human placental brush border membranes. Mol Cell Endocrinol 69,59–68.

Bryant NJ, Govers R and James DE (2002) Regulated transport of the glucose transporter GLUT4. Nat Rev Mol Cell Biol 3,267–277.

Challier JC, Hauguel S and Desmaizieres V (1986) Effect of insulin on glucose uptake and metabolism in the human placenta. J Clin Endocrinol Metab 62,803–807.

Charron MJ, Brosius FC, 3rd, Alper SL and Lodish HF (1989) A glucose transport protein expressed predominantly in insulin-responsive tissues. Proc Natl Acad Sci USA 86,2535–2539.

Cushman SW and Wardzala LJ (1980) Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. Apparent translocation of intracellular transport systems to the plasma membrane. J Biol Chem 255,4758–4762.

Desoye G, Hartmann M, Blaschitz A, Dohr G, Hahn T, Kohnen G and Kaufmann P (1994) Insulin receptors in syncytiotrophoblast and fetal endothelium of human placenta. Immunohistochemical evidence for developmental changes in distribution pattern. Histochemistry 101, 277–285.

Ginsburg J and Jeacock MK (1964) Pathways of glucose metabolism in human placental tissue. Biochim Biophys Acta 90,166–168.

Gordon MC, Zimmerman PD, Landon MB, Gabbe SG and Kniss DA (1995) Insulin and glucose modulate glucose transporter messenger ribonucleic acid expression and glucose uptake in trophoblasts isolated from first-trimester chorionic villi. 173,1089–1097.

Gould GW, Thomas HM, Jess TJ and Bell GI (1991) Expression of human glucose transporters in Xenopus oocytes: kinetic characterization and substrate specificities of the erythrocyte, liver, and brain isoforms. Biochemistry 30,5139–5145.

Gude NM, Stevenson JL, Rogers S, Best JD, Kalionis B, Huisman MA, Erwich JJ, Timmer A and King RG (2003) GLUT12 expression in human placenta in first trimester and term. Placenta 24,566–570.

Guilherme A, Emoto M, Buxton JM, Bose S, Sabini R, Theurkauf WE, Leszyk J and Czech MP (2000) Perinuclear localization and insulin responsiveness of GLUT4 requires cytoskeletal integrity in 3T3-L1 adipocytes. J Biol Chem 275,38151–38159.

Haber RS, Weinstein SP, O'Boyle E and Morgello S (1993) Tissue distribution of the human GLUT3 glucose transporter. Endocrinology 132, 2538–2543.

Hauguel-de Mouzon S, Leturque A, Alsat E, Loizeau M, Evain-Brion D and Girard J (1994) Developmental expression of Glut1 glucose transporter and c-fos genes in human placental cells. Placenta 15,35–46.

Hauguel-de Mouzon S, Challier JC, Kacemi A, Cauzac M, Malek A and Girard J (1997) The GLUT3 glucose transporter isoform is differentially expressed within human placental cell types. J Clin Endocrinol Metab 82,2689–2694.

Illsley NP (2000) Glucose transporters in the human placenta. Placenta 21,14–22.

Illsley NP, Wang ZQ, Gray A, Sellers MC and Jacobs MM (1990) Simultaneous preparation of paired, syncytial, microvillous and basal membranes from human placenta. Biochim Biophys Acta 1029,218–226.

Jaffe R, Jauniaux E and Hustin J (1997) Maternal circulation in the first-trimester human placenta—myth or reality? Am J Obstet Gynecol 176, 695–705.

James DE, Strube M and Mueckler M (1989) Molecular cloning and characterization of an insulin-regulatable glucose transporter. Nature 338,83–87.

Jansson N, Greenwood SL, Johansson BR, Powell TL and Jansson T (2003) Leptin stimulates the activity of the system A amino acid transporter in human placental villous fragments. J Clin Endocrinol Metab 88, 1205–1211.

Jansson T and Powell TL (2000) Placental nutrient transfer and fetal growth. Nutrition 16,500–502.

Jansson T, Wennergren M, Illsley NP and Clin J (1993) Glucose transporter protein expression in human placenta throughout gestation and in intrauterine growth retardation. J Clin Endocrinol Metab 77,1554–1562.

Jansson T, Wennergren M and Powell TL (1999) Placental glucose transport and GLUT 1 expression in insulin-dependent diabetes. Am J Obstet Gynecol 180,163–168.

Jansson T, Ekstrand Y, Bjorn C, Wennergren M and Powell TL (2002) Alterations in the activity of placental amino acid transporters in pregnancies complicated by diabetes. Diabetes 51,2214–2219.

Johansson M, Jansson T and Powell TL (2000) Na(+)-K(+)-ATPase is distributed to microvillous and basal membrane of the syncytiotrophoblast in human placenta. Am J Physiol Regul Integr Comp Physiol 279, R287–R294.

Kainulainen H, Jarvinen T and Heinonen PK (1997) Placental glucose transporters in fetal intrauterine growth retardation and macrosomia. Gynecol Obset Invest 44,89–92.

Kniss DA, Shubert PJ, Zimmerman PD, Landon MB and Gabbe SG (1994) Insulinlike growth factors. Their regulation of glucose and amino acid transport in placental trophoblasts isolated from first-trimester chorionic villi. J Reprod Med 39,249–256.

Ogura K, Sakata M, Okamoto Y, Yasui Y, Tadokoro C, Yoshimoto Y, Yamaguchi M, Kurachi H, Maeda T and Murata Y (2000) 8-Bromo-cyclicAMP stimulates glucose transporter-1 expression in a human choriocarcinoma cell line. J Endocrinol 164,171–178.

Persson A, Johansson M, Jansson T and Powell TL (2002b) Na(+)/K(+)-ATPase activity and expression in syncytiotrophoblast plasma membranes in pregnancies complicated by diabetes. Placenta 23,386–391.

Persson B and Hanson U (1996) Fetal size at birth in relation to quality of blood glucose control in pregnancies complicated by pregestational diabetes mellitus. Br J Obstet Gynaecol 103,427–433.

Rey E, Attie C and Bonin A (1999) The effects of first-trimester diabetes control on the incidence of macrosomia. Am J Obstet Gynecol 181,202–206.

Rogers S, Macheda ML, Docherty SE, Carty MD, Henderson MA, Soeller WC, Gibbs EM, James DE and Best JD (2002) Identification of a novel glucose transporter-like protein-GLUT-12. Am J Physiol Endocrinol Metab 282,E733–E738.

Sakata M, Kurachi H, Imai T, Tadokoro C, Yamaguchi M, Yoshimoto Y, Oka Y and Miyake A (1995) Increase in human placental glucose transporter-1 during pregnancy. Eur J Endocrinol 132,206–212.

Sakurai T, Takagi H and Hosoya N (1969) Metabolic pathways of glucose in human placenta. Changes with gestation and with added 17-beta-estradiol. Am J Obstet Gynecol 105,1044–1054.

Schneider NO, Calderon RO and de Fabro SP (1981) Isolation and characterization of cell membranes from human placenta. Acta Physiol Lat Am 31, 283–289.

Shu SY, Ju G and Fan LZ (1988) The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci Lett 85, 169–171.

Sooranna SR, Oteng-Ntim E, Meah R, Ryder TA and Bajoria R (1999) Characterization of human placental explants: morphological, biochemical and physiological studies using first and third trimester placenta. Hum Reprod 14,536–541.

Suzuki K and Kono T (1980) Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. Proc Natl Acad Sci USA 77,2542–2545.

Takata K, Kasahara T, Kasahara M, Ezaki O and Hirano H (1990) Erythrocyte/HepG2-type glucose transporter is concentrated in cells of blood-tissue barriers. Biochem Biophys Res Commun 173,67–73.

Takata K, Kasahara T, Kasahara M, Ezaki O and Hirano H (1992) Localization of erythrocyte/HepG2-type glucose transporter (GLUT1) in human placental villi. Cell Tissue Res 267,407–412.

Tavare JM and Holmes CH (1989) Differential expression of the receptors for epidermal growth factor and insulin in the developing human placenta. Cell Signal 1,55–64.

Teasdale F (1980) Gestational changes in the functional structure of the human placenta in relation to fetal growth: a morphometric study. Am J Obstet Gynecol 137,560–568.

Urbach J, Mor L, Ronen N and Brandes JM (1989) Does insulin affect placental glucose metabolism and transfer? Am J Obstet Gynecol 161, 953–959.

Xing AY, Challier JC, Lepercq J, Cauzac M, Charron MJ, Girard J and Hauguel-de Mouzon S (1998) Unexpected expression of glucose transporter 4 in villous stromal cells of human placenta. J Clin Endocrinol Metab 83,4097–4101.

Submitted on April 1, 2004; accepted on June 1, 2004