Overexpression of GLUT3 Placental Glucose Transporter in Diabetic Rats

Pascal Boileau, Caroline Mrejen, Jean Girard, and Sylvie Hauguel-de Mouzon
Centre de Recherche sur l'Endocrinologie, Moléculaire et le Développement, Centre National de la Recherche Scientifique, 92 190 Meudon-Bellevue, France

Abstract

The localization of the two major placental glucose transporter isoforms, GLUT1 and GLUT3 was studied in 20-d pregnant rats. Immunocytochemical studies revealed that GLUT1 protein is expressed ubiquitously in the junctional zone (maternal side) and the labyrinthine zone (fetal side) of the placenta. In contrast, expression of GLUT3 protein is restricted to the labyrinthine zone, specialized in nutrient transfer. After 19-d maternal insulinopenic diabetes (streptozotocin), placental GLUT3 mRNA and protein levels were increased four-to-fivefold compared to nondiabetic rats, whereas GLUT1 mRNA and protein levels remained unmodified. Placental 2-deoxyglucose uptake and glycogen concentration were also increased fivefold in diabetic rats. These data suggest that GLUT3 plays a major role in placental glucose uptake and metabolism.

The role of hyperglycemia in the regulation of GLUT3 expression was assessed by lowering the glycemia of diabetic pregnant rats. After a 5-d phlorizin infusion to pregnant diabetic rats, placental GLUT3 mRNA and protein levels returned to levels similar to those observed in nondiabetic rats. Furthermore, a short-term hyperglycemia (12 h), achieved by performing hyperglycemic clamps induced a fourfold increase in placental GLUT3 mRNA and protein with no concomitant change in GLUT1 expression.

This study provides the first evidence that placental GLUT3 mRNA and protein expression can be stimulated in vivo under hyperglycemic conditions. Thus, GLUT3 transporter isoform appears to be highly sensitive to ambient glucose levels and may play a pivotal role in the severe alterations of placental function observed in diabetic pregnancies. (*J. Clin. Invest.* 1995. 96:309–317.) Key words: diabetes • pregnancy • trophoblast • glucose transport • immunocytochemistry

Introduction

Glucose is the major fuel for growth and energy metabolism of feto-placental tissues (reviewed in reference 1, 2). One of the major functions of the placenta is to ensure adequate transfer of glucose from maternal to fetal circulation. Glucose transport across the cell membrane occurs through a stereospecific, saturable, and facilitative diffusion process dependent on glucose transporter proteins. At least five different facilitative glucose transporters encoded by separate genes (GLUT1–5) have been characterized in mammalian tissues (reviewed in reference 3, 4). Among these transporters, GLUT1 and GLUT3 proteins are predominantly expressed in human and rat placenta (5–8). The mRNAs of these two major placental glucose transporter isoforms have been recently localized in the rat by in situ hybridization. GLUT1 mRNA has been found in regions of high glucose metabolism, whereas GLUT3 mRNA is expressed in regions devoted to nutrient transfer processes (9). However, the localization and the regulation of placental GLUT1 and GLUT3 proteins have not been determined.

In general, glucose transporter mRNA and protein expression are controlled in vivo by the metabolic and hormonal status. In the rat, long-term insulinopenic diabetes results in increased liver GLUT2 mRNA (10) and decreased GLUT4 mRNA levels in skeletal muscle (11) and adipose tissue (12). In vitro, glucose deprivation increases GLUT1 mRNA and protein levels in adipose and muscle cell lines (13, 14). Thus, changes in glucose and insulin levels appear to be important modulators of the expression of several facilitative glucose transporters.

Although the placenta contains a large number of insulin receptors located on trophoblast cells (15), placental glucose transport is not likely to be regulated by insulin (16–18). This is strengthened by the observation that the insulin-responsive glucose transporter isoform, GLUT4 is expressed at very low levels in the placenta (6, 9). By contrast, placental glucose transport is closely related to maternal plasma glucose concentration (1, 19), suggesting that placental glucose transporter(s) expression might be regulated by glucose. This is supported by the findings that glucose transport from the mother to the fetus is enhanced in diabetic rats (20). Furthermore, diabetes in human pregnancy is associated with abnormalities in feto-placental growth, and it has been hypothesized that maternal hyperglycemia could lead to fetal growth alterations, i.e., feto-placental macrosomia and congenital malformations (21–23). This also favors a major role for maternal glucose homeostasis in feto-placental development.

The aims of the present study were: (*a*) to localize rat placental GLUT1 and GLUT3 proteins and to assess their respective role in the transfer of glucose from maternal to fetal circulation; (*b*) to study the expression of GLUT1 and GLUT3 transporter isoforms in placentas of insulinopenic diabetic rats; and (*c*) to determine the effect of hyperglycemia on the regulation of placental GLUT3 mRNA and protein expression.

Methods

Animals. 9-wk-old female Wistar rats (IFFA Credo, l'Arbresle, France) were housed at 21°C with light from 7:00 a.m. to 7:00 p.m. They were

Address correspondence to Sylvie Hauguel-de Mouzon, Centre de Recherche sur l'Endocrinologie, Moléculaire et le Développement, Centre National de la Recherche Scientifique, 9, rue Jules Hetzel, 92 190 Meudon-Bellevue, France. Phone: 33-1-45-07-52-18; FAX: 33-1-45-07-58-90.

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fed ad lib. a standard laboratory chow (62% carbohydrate, 26% protein, and 12% fat in caloric percentage). The day of mating was considered as day 1 of gestation. Pregnancy was confirmed by abdominal palpation on day 14 of gestation. Diabetes was induced on day 1 of gestation by an intraperitoneal injection (65 mg/kg body weight) of streptozotocin (STZ)[1] (Sigma Chemical Co., St Louis, MO). Diabetes was diagnosed on day 3 after STZ injection by measuring plasma glucose levels on blood sampled from the tail vein. A group of diabetic rats received a subcutaneous infusion of phlorizin (Sigma Chemical Co.) from day 15 to day 20 of gestation, to ensure a constant inhibition of renal tubular glucose reabsorption. Phlorizin dissolved in 40% (wt/vol) propylene glycol was delivered at a rate of 0.4 mg/kg body weight per day via an implantable mini-osmotic pump (Alzet; Charles River, St. Aubin les Elbeuf, France) implanted in the intrascapular region under light ether anaesthesia. Placentas were sampled after maternal laparotomy on day 19–20 of gestation. In some experiments, 9-wk-old male rats were used for sampling testis.

Euglycemic hyperinsulinemic and hyperglycemic clamp studies. 3 d before the experiments, undwelling catheters were inserted in the left jugular vein and the right carotid artery under light ether anaesthesia so that the rats could be further handled under awake state (24). Euglycemic hyperinsulinemic clamps were performed as previously described (25). Briefly, human insulin (Actrapid Novo, Copenhagen, Denmark) was infused at a constant rate of 20 nmol/h per kg through the jugular vein. Blood glucose was maintained at normoglycemic levels by a variable infusion of glucose (30% wt/vol). Glucose infusion was started 1 min after the beginning of insulin infusion, and was set at 1.8 mmol/min per kg on the basis of previous experiments. Then, glucose infusion was periodically adjusted to maintain euglycemia. Blood samples (10 μl) were withdrawn from the carotid artery every 5 min during the first hour, and then every 30 min until the end of the experiments. Blood glucose concentration was determined extemporaneously with a glucose analyzer (model YSI 23A; Yellow Springs Instrument Co., Yellow Springs, OH). Additional samples (100 μl) were taken hourly for plasma insulin and glucose determinations. The samples were immediately centrifuged at 4°C, and the plasma was frozen at −20°C. At the end of the experiments the rats were killed by an overdose of pentobarbital, the placentas were removed, quickly frozen in liquid nitrogen, and stored at −80°C. Hyperglycemic clamps were performed similarly except that insulin and glucose infusions were adjusted to maintain maternal glycemia at a level of 655±12 mg/dl.

Immunohistochemistry. Placentas were sampled on day 20 of gestation. Placental samples were fixed in 2.5% paraformaldehyde for 3 h, and washed three times in PBS at pH 7.4 containing 2 mM PMSF and 0.05% azide. Fixed material was soaked overnight at 4°C in 15% sucrose. Samples were then snap frozen in isopentane, chilled to −70°C with liquid nitrogen, and embedded in Tissue-Tek (Miles Inc., Naperville, IL). 8-μm thick serial sections of placenta were obtained with a cryostat (Reichert-Jung 2800 Frigocut E; Leica, Heidelberg, Germany) then laid on glass slides. They were incubated with PBS containing 2% BSA and 0.05% azide for 2 h, then incubated with anti-GLUT3 (1/200) or anti-GLUT1 antibodies (1/500) for 1 h at 23°C. The incubation with the primary antibody was followed by four 10-min PBS washes at 23°C. The incubation with the secondary antibody, FITC-labeled goat anti-rabbit IgG (Sanofi Diagnostics Pasteur, Marnes la Coquette, France) was carried out in the dark at 23°C for 1 h. Placental sections were stained with Hoescht 33258 (5 μg/ml; Sigma Chemical Co.) for 15 min at 23°C then washed as described above and mounted in Mowiol (Hoescht, Frankfurt, Germany) with propylgallate (Sigma Chemical Co.). Sections were examined with a fluorescence microscope equipped with filters selective for FITC.

Membrane preparations. Postnuclear placental membranes were obtained according to the following procedure. All steps were performed at 4°C. Intact placentas were obtained after maternal laparotomy on day 19–21 of gestation. Tissue samples (1.5 g wet weight) were homogenized in 5 ml Hepes-Sucrose buffer (Hepes 25 mM, sucrose 250 mM, dithiotreitol 2 mM, PMSF 1 mM, 1% aprotinine [10 mg/ml], pH 7.4) with 20 strokes of a dounce homogenizer. The homogenate was centrifuged at 1,000 *g* for 10 min and the supernatant, for 20 min at 10,000 *g*. The resulting supernatant was centrifuged at 100,000 *g* for 1 h and the crude membrane pellet was resuspended in 100 μl of Hepes buffer pH 7.4 then frozen at −80°C before use. Crude membranes from rat brain were prepared as previously described (26).

Immunoblotting. Equal amounts of membrane proteins (100 μg) were solubilized in Laemmli buffer without heating (27) and separated by SDS-PAGE using 10% slab gels. After electrophoresis, proteins were transferred electrically to a nitrocellulose membrane (BA 85; Schleicher & Schuell, Dassel, Germany) at 0.25 A for 35 min using a Miliblot apparatus (Millipore Corp., Guyancourt, France). Transfer was confirmed by Ponceau S staining of the membrane. Nonspecific binding sites were blocked by an overnight incubation of the nitrocellulose membrane in phosphate buffered saline with 0.1% Triton (PBST) containing 5% nonfat dried milk. After three 10-min washes in PBST, the nitrocellulose filter was incubated in 0.1% PBST containing 2% nonfat dried milk and primary antibodies (1/200 dilution for anti-GLUT3, 1/500 for anti-GLUT1) and allowed to incubate for 4 h at room temperature. Blots were then subjected to three 10 min washes in 0.1% PBST before addition of 2 μCi ^{125}I-protein A (Amersham International, Les Ulis, France) in 10 ml PBST containing 2% nonfat dried milk for 1 h at room temperature. After appropriate washes in PBST, the filters were exposed to autoradiography with two intensifying screens (Cronex, Dupont, Kolen & Delhumeau, Saint-Denis, France) at −80°C for 16–72 h to obtain images within the linear range of the x-ray film for subsequent quantitation. Apparent protein molecular weights were determined by comparison with rainbow markers (Amersham International). Immunoreactivity was quantitated by scanning densitometry of the autoradiograms (GS-300 scanning densitometer; Hoefer Scientific Instruments, San Francisco, CA). Linear responses were obtained with protein concentrations ranging from 10 to 50 μg for brain membranes and 50–100 μg for placental membranes. A peptide corresponding to the 13 COOH-terminal amino acids of the mouse GLUT3 (28) was used to establish the specificity of anti-GLUT3 antibody reactivity in competition studies. Prior addition to the nitrocellulose filter, anti-GLUT3 antibody (1/200) was preincubated for 2 h at 37°C in PBST in the presence of GLUT3 peptide (50 μg/ml). The incubation of the filter was then carried out in PBST containing 2% nonfat dried milk as described above.

Glycosidase digestion. Placental membranes (100 μg protein) were resuspended in 50 mM phosphate 20 mM EDTA buffer pH 6.1 (final vol 50 μl) containing 2% Triton X-100, 0.2% SDS, 1% β-mercaptoethanol, 0.4 U *N*-endoglycosidase F (Sigma Chemical Co.), and 5 μg neuraminidase (Sigma Chemical Co.). Incubation was carried out for 24 h at 37°C and proteins were precipitated with 5% trichloracetic acid. The resultant pellets were rinsed with acetone, dried, and resuspended in Laemmli sample buffer before electrophoresis and immunoblotting as described above.

RNA extraction and Northern blot analysis. Total RNA from placenta, brain, and testis was extracted according to Chirgwin's procedure (29). RNA concentration was determined by absorbance at 260 nm. All samples had a 260/280 absorbance ratio close to 2. For Northern blot analysis, total RNA (30 μg) was denatured in a solution containing 110 mM formaldehyde, 48% formamide, 1 × Mops, 5.3% glycerol, and 0.1% bromophenol blue by heating at 65°C for 15 min. RNA were electrophoresed on 1% agarose/0.6 M formaldehyde gels, transferred by capillarity to hybond-N membrane (Amersham International). The integrity of the RNA blotted was evaluated by visualization of the ethidium bromide stained 28S and 18S ribosomal RNA subunits by ultraviolet shadowing. Hybridizations were performed with α[^{32}P]dCTP labeled cDNA probes for 16 h at 42°C, in solutions containing 40% deionized formamide, 7.5% dextran sulfate, 8 × Denhardt's, 30 mM Tris/HCl pH 7.5, and 1% SDS. After overnight hybridization, the membranes were washed twice for 30 min with 2 × SSC/0.1% SDS at 42°C and once for 30 min with 0.1 × SSC/0.1% SDS at 55°C, then exposed

1. *Abbreviations used in this paper:* ^{3}H-2DG, 2-deoxy-1[^{3}H]glucose; PBST, PBS with 0.1% Triton; STZ, streptozotocin.

for 6–24 h at −80°C with intensifying screens. Quantification of the specific signals was performed by scanning densitometry. After stripping of the glucose transporter probes, the blots were rehybridized with an antisense synthetic oligonucleotide (24 mer) specific for the 18S ribosomal subunit to correct for the exact amount of RNA transferred to the filters (30).

In vivo measurement of placental 2-deoxyglucose uptake. The method used for measuring placental glucose uptake has been described previously (17). Briefly, 19-d pregnant rats were anesthetized with an intraperitoneal injection of pentobarbital (30 mg/kg body weight). 30 μCi of 2-deoxy-[1[^{3}H]-glucose (^{3}H-2DG), 20 Ci/mmol (Amersham International) were injected via the maternal saphenous vein. Maternal arterial blood was sampled from the carotid artery 1, 3, 5, 10, 15, 20, 30, 40, and 60 min after the injection. The measurement of the specific activity was determined on 50-μl deproteinized blood samples, counted using a scintillation counter (Betamatic 2; Kontron, St. Quentin en Yvelines, France). The integral of specific activity ^{3}H-2DG/glucose was calculated over 60 min. After the last blood sampling, rats were killed by an overdose of pentobarbital and placentas were immediately excised. The amount of ^{3}H-2DG-6-phosphate accumulated in the placenta was determined by differential precipitation in Somogyi reagent and in 6% $HClO_4$ on aliquots of tissue digested for 1 h at 60°C in 1 M NaOH and neutralized. An index of glucose accumulation rate was calculated as the amount of ^{3}H-2DG-6-phosphate taken up by the placenta divided by the integral of ^{3}H-2DG/glucose specific activity in the blood. Results are expressed in nmol of glucose accumulated per min and per mg placenta.

Measurement of placental glycogen concentration. Placentas sampled from control or diabetic rats were snap frozen in liquid nitrogen and kept at −80°C until the measurements. Determination of glycogen content was performed according to the method described by Chan and Exton (31) on placental homogenates prepared by pooling two placentas from each rat.

Biochemical determinations. Protein concentrations were determined by the Bio-Rad assay (Bio-Rad Laboratories, Munich, Germany) using BSA as standard. Glucose concentration was assessed on plasma samples by spectrophotometry using the glucose oxidase method (Peridochrom; Boehringer-Mannheim, Mannheim, Germany). Plasma immunoreactive insulin was determined by radioimmunoassay (Kit Oris Industrie, Gif-sur-Yvette, France) using rat insulin as standard.

Antibodies and cDNA probes. The antibodies used were rabbit polyclonal antisera to the intracellular COOH terminus of rat brain GLUT1 (amino acids 479–492; East Acres Biologicals, Southbridge, MA) and to the COOH-terminal 13 amino acid sequence of mouse GLUT3 (amino acids 481–493), generously provided by Dr. Gwyn Gould (University of Glasgow, Scotland, United Kingdom). In some experiments, GLUT3 antibody produced by East Acres Biologicals with the same mouse peptide and the same immunization procedure than those utilized by Dr. G. W. Gould was used for comparison. It gave essentially the same results. GLUT3 and GLUT1 cDNA probes were prepared from a mouse GLUT3 cDNA fragment coding for amino acids 207–394 subcloned into pGEM4Z (provided by Pr. G. I. Bell, University of Chicago, Chicago, IL) and from a 1.75-kb human GLUT1 cDNA fragment subcloned into pUC19 (provided by Dr. M. J. Birnbaum, Harvard Medical School, Boston, MA). Probes were labeled with α[^{32}P]dCTP using the multiprime labeling system kit (Amersham International). The peptide corresponding to the COOH-terminal 13 amino acids of the mouse GLUT3, sequence: NSMQPVKETPGNA (28), was purchased from Neosystem (Strasbourg, France).

Statistical analysis. Results are expressed as means±SE. Statistical analysis was performed by Student's *t* test for unpaired data (Statworks Software, Calabasas, CA).

Results

Expression of GLUT3 glucose transporters in rat tissues. To assess the relative amount of GLUT3 in rat tissues, we have compared GLUT3 mRNA levels in placenta, brain, and testis, three tissues reported to specifically express this glucose transporter isoform. The highest levels of GLUT3 mRNA were observed in testis and placenta whereas lower levels were found in brain. This might be due to the fact that neuronal cells only are a major site for GLUT3 expression (32). GLUT1 mRNA levels were determined in the same tissues. Highest expression was observed in placenta (Fig. 1).

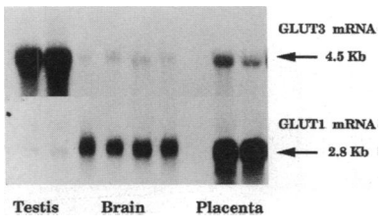

Figure 1. Expression of GLUT3 and GLUT1 mRNA in rat tissues. Brain and placental tissues were obtained from pregnant rats on day 19 of gestation. Testis were from 12-wk-old rats. Total RNA from frozen tissue samples was extracted as described in Methods, and Northern blots were performed with 30 μg total RNA per lane. Autoradiograms were exposed at −80°C for 6 h and each one is representative of two separate experiments.

Localization of placental GLUT1 and GLUT3 glucose transporters by immunofluorescent staining. The localization of GLUT1 and GLUT3 proteins was studied on parasagittal sections of placentas obtained from three pregnant rats (day 20 of gestation). The junctional and labyrinthine zones of the rat placenta were identified by the size of their nuclei. The cytotrophoblast cells of the junctional zone have larger nuclei than those of the labyrinthine cells (14–18 μm and 4–6 μm, respectively). The size of the nuclei was determined on tissue sections stained with Hoescht dye (Fig. 2 *C*, and Fig. 3, *B* and *D*). After incubation of serial semithin placental sections with anti-GLUT1 and anti-GLUT3 antibodies, a unipolar distribution of GLUT3 protein (Fig. 2 *B*) was first detected at a low magnification (×100); it was distinct from the homogeneous distribution of GLUT1 protein (Fig. 2 *A*). At a higher magnification (×400) GLUT1 protein was found in both the junctional zone which corresponds to the interface between maternal and fetal tissues and the labyrinthine zone (Fig. 3 *A*) specialized for materno–fetal exchanges. GLUT3 protein was found exclusively in the labyrinthine zone (Fig. 3 *C*). However it was impossible to determine the precise location of GLUT3 and GLUT1 proteins within a specific layer of the placental barrier by light microscopy.

Characterization of placental GLUT3 glucose transporter. GLUT3 glucose transporter was detected in postnuclear placental and brain membranes prepared from 19-d pregnant rats. Specificity of the detection was determined by competition with an homologous mouse GLUT3 peptide. In the absence of GLUT3 peptide, a broad diffuse signal composed of several bands migrating between 46 and 90 kD was detected in the placenta, whereas in the brain, a major band was observed at 45–55 kD (Fig. 4 *A*). Homologous mouse GLUT3 peptide eliminated detection of the major bands in brain and placenta, leaving a noncompetable signal at 45 kD in the placenta and a nonspecific signal at 80 kD in the brain. The noncompetable signal at 45 kD should not be mistaken for placental glucose transporter. The pattern of migration of placental GLUT3 is characteristic of a heavily glycosylated protein. Indeed, digestion of solubilized placental membranes with endoglycosidase F and neuraminidase produced a decrease in the apparent GLUT3

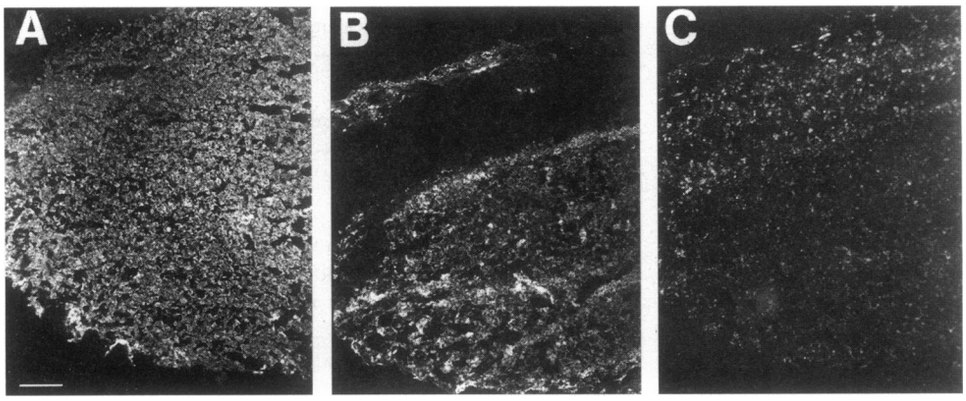

Figure 2. Structural localization of GLUT1 and GLUT3 glucose transporters in rat placenta on day 20 of gestation. Photographs of serial parasagittal sections showing the junctional and the labyrinthine zones localized on Hoescht-stained sections according to the morphological characteristics of their nuclei (*C*). GLUT1 protein staining was ubiquitous (*A*), whereas GLUT3 protein staining was restricted to the labyrinthine zone (*B*). ×100. Bar, 1.4 mm.

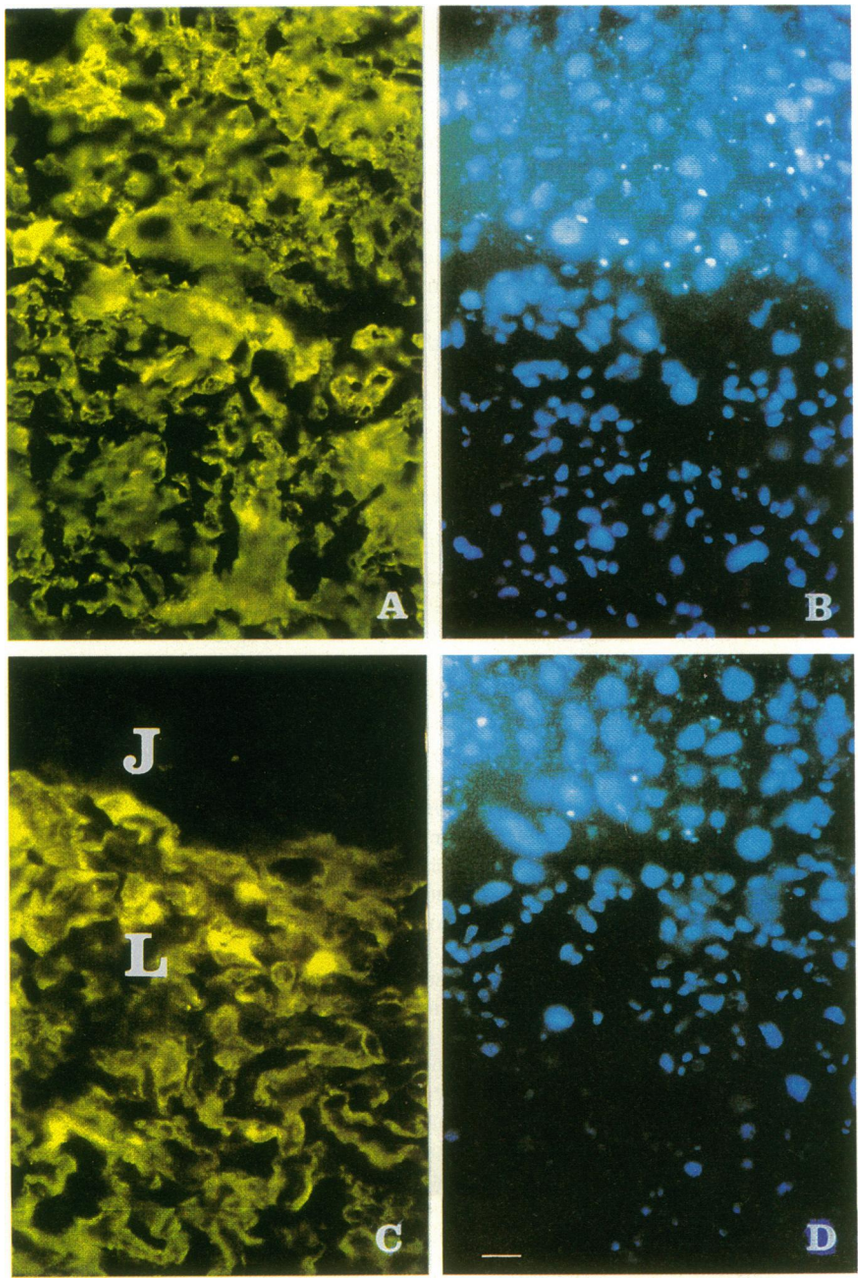

Figure 3. Immunohistochemical detection of placental GLUT1 and GLUT3 glucose transporter proteins. Semithin sections from day 20 rat placenta were incubated with (*A*) anti-GLUT1 antibody (1/500) and (*C*), anti-GLUT3 antibody (1/200). The same sections were also stained with Hoescht (*B* and *D*). Size of nuclei were used to locate the junctional zone (*J*) and the labyrinthine zone (*L*). ×400. Bar, 15 μm.

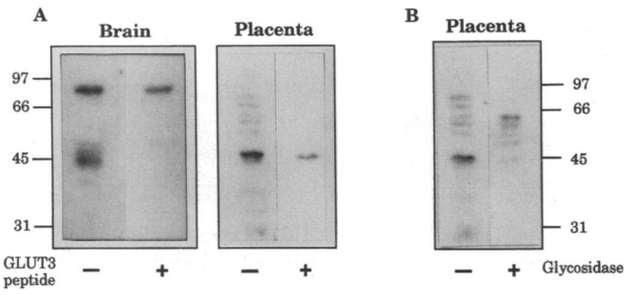

Figure 4. Immunological characterization of GLUT3 protein in rat placenta. (*A*) Effect of GLUT3 peptide competition. 50 μg brain and 100 μg placental membrane proteins were electrophoresed and transfered as described. The immunoblots were then incubated with anti-GLUT3 antibody in the absence or presence of 50 μg/ml GLUT3 peptide in the incubation buffer. (*B*) Effect of glycosidases on the electrophoretic mobility of GLUT3 protein. 100 μg placental membrane proteins were incubated for 24 h at 37°C in the presence of neuraminidase and endoglycosidase F as described in Methods. Samples containing equal amount of membrane proteins subjected (+) or not (−) to glycosidase treatment were dissolved in Laemmli buffer, electrophoresed on the same blot, and probed with anti-GLUT3 antibody as described. Position of the molecular weight markers (kD) is shown.

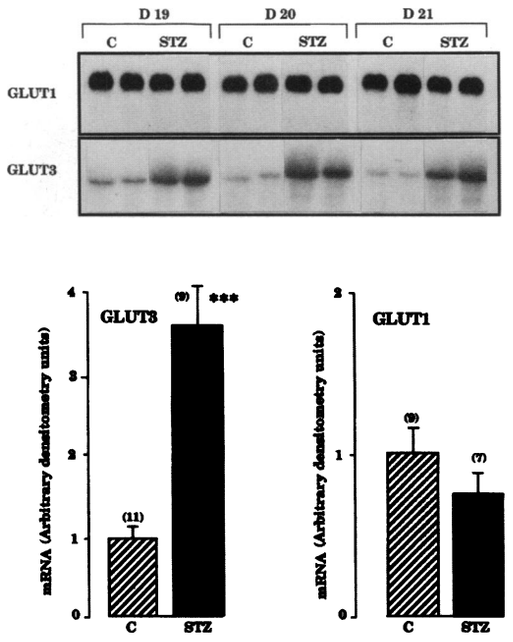

Figure 5. Placental glucose transporter mRNA levels in diabetic rats. (*Top*) Northern blot sequentially hybridized with GLUT1 and GLUT3 cDNA probes. 30 μg total RNA were blotted in each lane. (*Bottom*) Means±SE of scanning densitometry data obtained after autoradiography of three independent Northern blots. Number of animals in each group is represented in parentheses. One placenta sampled from each rat was extracted individually. *C*, RNA obtained from control pregnant rats, *STZ*, RNA obtained from STZ-induced diabetic pregnant rats. Difference statistically significant for *** $P < 0.001$ as compared to controls.

molecular weight to a sharper band of 46–60 kD (Fig. 4 *B*) consistent with the removal of carbohydrates from the polypeptide chain. Similar results were obtained with placental membranes from diabetic rats (result not shown).

Effect of diabetes on placental glucose transporter mRNA levels. STZ-diabetic pregnant rats were killed on day 19–21 of gestation. Age-matched nondiabetic pregnant rats were used as controls. Maternal, placental, and fetal weights as well as plasma glucose and insulin levels are reported in Table I. Diabetic pregnant rats were hyperglycemic (449±11 mg/dl vs 91±2 mg/dl, $P < 0.001$) and hypoinsulinemic (4±1 μU/ml vs 37±3 μU/ml, $P < 0.001$). Placental weights were higher in diabetic than in control rats ($P < 0.01$). By contrast, fetal weights were lower in diabetic rats ($P < 0.001$). The placental levels of GLUT1 and GLUT3 mRNAs were assessed by Northern blot analysis. Three Northern blots were performed independently using total RNA extracted from placentas of 9 STZ and 11 control rats. Quantification of the autoradiographic signals showed that GLUT1 mRNA levels were similar in placentas of diabetic and control rats whereas GLUT3 mRNA levels were increased fivefold ($P < 0.001$) in placentas of diabetic compared to those of control rats (Fig. 5).

Effect of diabetes on placental GLUT1 and GLUT3 protein levels. Placentas were obtained from four control and four diabetic rats on day 20 of pregnancy. Western blot analysis shows that GLUT1 and GLUT3 proteins appear as diffuse signals ranging from 46 to 90 and 46 to 110 kD, respectively (Fig. 6, *top*). The broadness of the bands is characteristic of heavily glycosylated proteins as has been previously shown in the human placenta (33). By contrast, the brain GLUT1 and GLUT3 isoforms were detected as narrow 45–50 kD bands (Fig. 6, *bottom*). GLUT1 protein level was similar in placentas of diabetic and control rats, whereas GLUT3 protein levels were increased threefold in placenta from diabetic rats. When quantitated by densitometry and expressed in arbitrary units, the mean values were 30±1.9 in placentas from diabetic rats compared to 7.6±0.8 in placentas from control rats ($P < 0.002$) (Fig. 6). Since diabetes did not induce any modifications in the amount of brain glucose transporters, these data clearly show that diabetes specifically induced an overexpression of GLUT3 in the placenta at both the mRNA and protein levels.

Regulation of GLUT3 expression in diabetic rats. To evaluate the role of hyperglycemia in the regulation of GLUT3 expression, six STZ-diabetic rats were treated with phlorizin from day 15 to 20 of gestation. Phlorizin infusion resulted in a significant decrease in maternal plasma glucose levels from 440±11 to 248±23 mg/dl, with no change in plasma insulin levels (Table II). Quantitation of specific signals by scanning densitometry showed that under these conditions, GLUT3 mRNA and protein levels were decreased 2.5-fold (10.7±4 vs

Table I. Physiological Parameters in STZ-Diabetic and Control Pregnant Rats on Day 20 of Gestation

	Maternal weight	Fetal weight	Placental weight	Maternal glycemia	Maternal insulinemia
	g	*g*	*g*	*mg/dl*	*μU/ml*
Controls (n = 6)	251±17	4.73±0.28	0.49±0.02	91±2	37±3
Diabetics (n = 6)	190±8*	2.20±0.05‡	0.58±0.02*	449±11‡	4±1‡

Values are means±SE. * $P < 0.01$ and ‡ $P < 0.001$ as compared to control rats. n, number of pregnant rats. Parameters from four fetuses and four placentas sampled from each pregnant rat were pooled and used as one individual value.

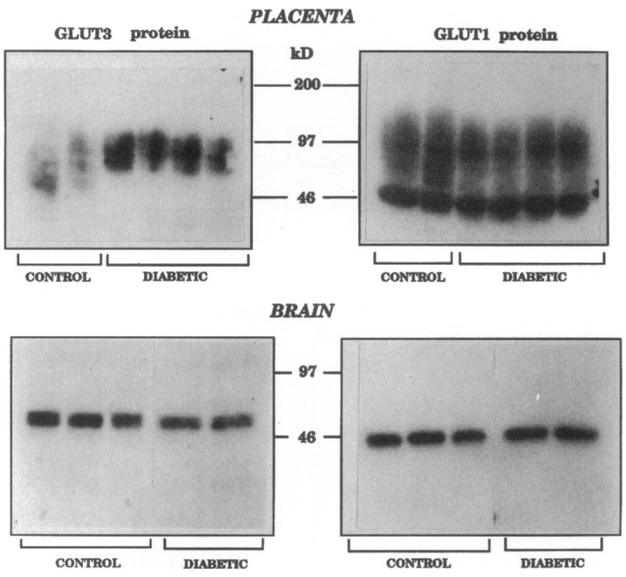

Figure 6. Effect of STZ-induced diabetes on GLUT1 and GLUT3 proteins. 100 μg crude placental and 50 μg brain membrane proteins from 20-d control and diabetic pregnant rats were solubilized in Laemmli buffer containing 2 mM DTT and electrophoresed on 10% SDS-PAGE. Proteins were transferred on nitrocellulose filters by electrotransfer. The nitrocellulose filters were hybridyzed with anti-GLUT1 or anti-GLUT3 antibodies diluted 1/500 and 1/200, respectively, and exposed to autoradiography for 6–18 h at −80°C. Molecular mass markers are indicated in kD. Western blots. (*Top*) placental membranes. (*Bottom*) Brain membranes. Each autoradiogram is representative of two independent Western blots performed with placental and brain membranes obtained from four control and four diabetic rats.

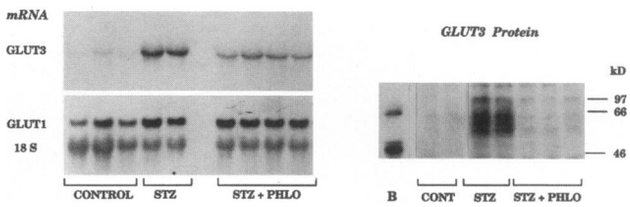

Figure 7. Regulation of placental GLUT3 expression in STZ-diabetic rats. Diabetic rats were infused with phlorizin from day 15 to 20 of gestation. Placentas were obtained on day 20 of gestation. Total RNA was extracted and analyzed by Northern blotting. Crude placental and brain membranes were prepared as described in Methods. (*Left*) Northern blot performed with 30 μg total RNA loaded in each lane and hybridized with a GLUT3 cDNA probe. The same blot has been hybridized with a GLUT1 cDNA probe and an oligonucleotide specific for the 18S RNA ribosomal subunit to visualize the exact amount of RNA blotted. (*Right*) Western blot performed with 100 μg placental membranes. *STZ,* tissue sampled from diabetic rats; STZ + PHLO, tissue sampled from diabetic rats treated with phlorizin; *B,* 70 μg brain membranes from control rats. Each autoradiogram is representative of three independent experiments performed with placental membranes prepared from seven control, nine diabetic, and six diabetic rats treated with phorizin.

4.5±0.5 arbitrary units) and 3-fold (30±1.9 vs 9.7±2.4 arbitrary units), respectively, compared to diabetic rats (Fig. 7).

Placental 2-deoxyglucose uptake and glycogen concentration in control and diabetic rats. Placental 2-deoxyglucose uptake and glycogen concentration were determined in 19-d control and diabetic pregnant rats. Both parameters were fivefold higher in placentas from diabetic compared to control rats (Table III). Since protein concentrations were unmodified in diabetic and control placentas, the results were similar when expressed per milligram tissue wet weight or milligram protein.

Table II. Plasma Glucose and Insulin Levels During Euglycemic and Hyperglycemic Clamps in 20-d-old Pregnant Rats

	Maternal glycemia	Maternal insulinemia
	mg/dl	*μU/ml*
Controls (10)	89±4	30±3
Euglycemic hyperinsulinemic clamps 12 h (5)	96±4	4820±204*
Hyperglycemic clamps 6 h (4)	655±12*	130±12*
Hyperglycemic clamps 12 h (6)	613±45*	450±30*
Diabetics (8)	440±11*	5±1*
Diabetics + phlorizin (6)	248±23‡	7±1*

Values are means±SE. * $P < 0.001$ as compared to control and as compared with diabetic rats. Number of pregnant rats in each group represented in parentheses.

This indicates an increase in the overall placental glucose utilization and storage ability in placentas of diabetic animals.

Effects of maternal hyperglycemia and hyperinsulinemia on placental GLUT1 and GLUT3 mRNA levels. Hyperglycemic clamps were performed for 6 or 12 h in awake, unrestrained 20-d pregnant rats to determine the short-term effect of hyperglycemia on GLUT1 and GLUT3 mRNAs. Maternal glucose concentrations were raised and clamped at 655±12 mg/dl and 613±45 mg/dl, respectively, resulting in a maternal insulinemia of 130±12 μU/ml after 6 h and 450±30 μU/ml after 12 h (Table II). Placental GLUT1 and GLUT3 mRNA levels were not altered after 6 h whereas GLUT3 mRNA concentration was increased three- to fourfold after the 12 h hyperglycemia (Fig. 8). Euglycemic–hyperinsulinemic clamps were also performed to evaluate a possible effect of changes in maternal insulinemia. Maternal plasma glucose concentration was thus clamped at 96±4 mg/dl whereas plasma insulin was raised to 4,820±205 μU/ml during 12 h (Table II). Under these conditions, placental GLUT1 and GLUT3 mRNA levels were not modified (Fig. 8).

Discussion

The rat placenta is composed of two main regions, the junctional and the labyrinthine zones. The junctional zone (maternal side)

Table III. Glucose Uptake and Glycogen Concentration in Control and Diabetic Rat Placenta

	Control	Diabetic
[^{3}H]2-deoxyglucose uptake (nmol/min per g)	50±7.8 (6)	284±65 (7)*
Glycogen (mg/g tissue)	2.1±0.2 (8)	10.6±1.1 (9)‡
Protein (mg/g tissue)	77.9±3.4	77.7±3.6

Results are presented as mean values±SE for the number of animals indicated which are represented in parentheses. * $P < 0.002$ and ‡ $P < 0.001$ vs controls. Four placentas were sampled from each animal and analyzed individually for the different parameters. Plasma glucose levels were 82±9 and 415±33 mg/dl in control and diabetic rats, respectively.

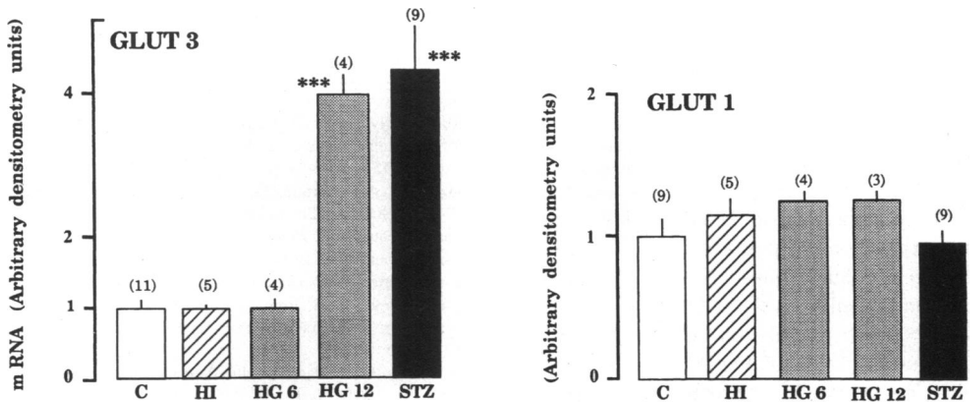

Figure 8. Effect of maternal hyperglycemia and hyperinsulinemia on placental GLUT1 and GLUT3 mRNA levels. Hyperglycemic and euglycemic hyperinsulinemic clamp studies were performed in 20-d pregnant rats. At the end of the experiment, placentas were sampled for RNA analysis. Shown are scanning densitometry data obtained from two individual Northern blots (30 μg total RNA per lane). Results are expressed as means±SE. The number of placentas sampled from each rat are represented in parentheses. *C,* control; *HI,* 12-h euglycemic hyperinsulinemic clamps; *HG6,* 6-h hyperglycemic clamps; *HG12,* 12-h hyperglycemic clamps; *STZ,* streptozotocin-induced diabetic rats. Differences statistically significant for *** $P < 0.002$ as compared to controls.

corresponds to the interface between the maternal and fetal tissues, and is the site of trophoblast proliferation. The labyrinthine syncytiotrophoblast layer (fetal side) is specialized in materno–fetal exchanges, and is also referred to as the placental barrier (34). Using light microscopic immunohistochemistry, we found that GLUT3 protein was exclusively localized in the labyrinthine zone whereas GLUT1 protein was detected both in the labyrinthine and junctional zones (Figs. 2 and 3). These observations are consistent with data obtained by in situ hybridization in the rat placenta showing an homogeneous distribution of GLUT1 and GLUT3 mRNAs in the labyrinthine zone (9), and by immunohistochemistry in the mouse placenta showing an homogeneous distribution of GLUT1 and GLUT3 proteins in the same region (35). Although the precise localization of GLUT1 and GLUT3 proteins within the trilaminar placental barrier requires electron microscopic studies, the presence of GLUT3 protein in the region of materno–fetal exchanges suggests that this isoform could be specifically involved in the transfer of glucose from mother to the feto-placental unit. In the rat brain, GLUT1 protein is detected in the microvessels of the blood–brain barrier (28, 36, 37) whereas GLUT3 is localized in neuronal cells (32, 37, 38). The localization of GLUT1 and GLUT3 proteins in specific brain areas have led to the hypothesis of a preferential transfer of glucose from blood to neuronal cells (28). Based on the observation that the low K_m GLUT3 transporter is expressed at highest levels in tissues with an hematotissular barrier, i.e. brain, testis, and placenta (28, 39, and Fig. 1), it is tempting to speculate that this transporter isoform would serve preferentially to ensure a vectorial transport of glucose from the maternal to the fetal circulation, as proposed by Zhou and Bondy (9). Thus, mechanisms similar to those described in the brain could also prevail in the placenta to deliver maternal glucose to meet specific metabolic adaptations within tissues of the feto-placental unit.

GLUT1 and GLUT3 proteins were also readily detected in the placenta by immunoblotting. Both glucose transporter isoforms appear heavily glycosylated as shown by the broad range of migration on SDS-PAGE (Figs. 4 and 6). The major immunoreactive bands detected with the anti-GLUT3 antibody are effectively competed by an homologous GLUT3 peptide, indicating that this heavily glycosylated protein represents GLUT3 glucose transporter isoform (Fig. 4 *A*). Glycosidase digestion before electrophoresis and immunoblotting confirms that placental GLUT3 protein contains N-linked oligosaccharides (Fig. 4 *B*) as previously reported for most glucose transporter isoforms, including GLUT3 (7, 26, 37). A high level of glycosylation seems a characteristic of placental tissue since only lower molecular weight isoforms of GLUT3 are detected in brain and testis (26, 37, 39), two other tissues expressing high levels of GLUT3. By contrast the heavy glycosylation pattern of placental GLUT1 protein appears as a more constant feature of GLUT1 isoform as observed in several tissues and cells including erythrocytes, liver, and adipocytes (26, 40).

The fivefold increase in placental glucose uptake and glycogen concentration found in diabetic rats (Table III) confirms previous reports showing an increase in placental glucose transfer and utilization in STZ-diabetic pregnant rats (20, 41) as well as in placental glycogen concentration in diabetic human (42), mouse (35), and rat (43).

Since GLUT1 mRNA and protein levels remained unchanged in the placenta of diabetic rats (Figs. 5 and 6), this glucose transporter isoform cannot be implicated in the placental metabolic changes observed in diabetes. By contrast, the four- to fivefold increase in GLUT3 mRNA and protein expression in placentas of diabetic rats suggests that GLUT3 could be responsible for the enhanced glucose uptake and glycogen accumulation. An increase in placental GLUT3 mRNA levels has also been recently reported in diabetic pregnant mice (35). The 30% increase in the placental weight of diabetic rats (20, 33, and Table I) is unlikely to account for the fourfold increase in GLUT3 expression and the fivefold increase in glucose uptake and glycogen accumulation. These adaptations favor the idea that the placenta cannot be regarded as a simple filter between the mother and the fetus. Under certain pathological conditions, it could also function as a reservoir to prevent an accelerated flux of maternal glucose toward the fetal circulation. The overexpression of placental GLUT3 could also contribute to maintain a positive materno–fetal glucose gradient to supply an adequate flux of glucose to the fetus. This would in turn limit fetal growth retardation since the diabetic fetus is clearly not capable of using glucose at a normal rate.

The potential role of maternal hyperglycemia in the stimulation of GLUT3 expression was evaluated by lowering maternal glycemia. Infusing phlorizin to the diabetic pregnant rats induced a two- to threefold decrease in maternal plasma glucose levels maintained for 5 d (without any concomitant change in

insulin levels). This resulted in a twofold decrease in GLUT3 mRNA and protein levels (Fig. 7) and suggests that maternal hyperglycemia plays a major role in the increase in GLUT3 mRNA and protein levels observed in diabetes. A different type of regulation is more likely to prevail in the brain since GLUT3 protein expression was neither increased by insulinopenic diabetes in adult rats (Fig. 6) and mice (44) nor in rat fetuses (45, 46). To establish the respective role of glycemia and insulinemia in the modifications of GLUT3 gene expression, we performed euglycemic hyperinsulinemic, and hyperglycemic clamps in 20-d pregnant rats. Supraphysiologic plasma insulin levels maintained during 12 h in pregnant rats (> 4,000 μU/ml) did not increase placental GLUT3 gene expression (Table II, Fig. 8), suggesting that maternal insulinemia is not involved in the regulation of GLUT3 mRNA levels. By contrast, placental GLUT3 mRNA levels were enhanced threefold after 12 h hyperglycemic clamps (Fig. 8). The similar increase in GLUT3 mRNA levels observed under short-term hyperglycemia and long-term diabetes supports the idea that hyperglycemia per se could be responsible for the regulation of placental GLUT3 gene expression.

The mechanisms involved in the regulation of placental GLUT3 expression by glucose have not been defined in the present study. However, the expression of an increasing number of genes implicated in glucose metabolism have been shown to be regulated by one or several metabolites (glucose-6-phosphate or a metabolite of the hexosamine pathway) possibly via a glucose responsive sequence located in the 5′ flanking region of these genes (47–50). Whether or not the regulation of placental GLUT3 expression involves similar mechanisms remains to be determined. In this respect, studies of transcriptional steps will contribute to further understand the modifications of placental GLUT3 gene expression induced by maternal hyperglycemia.

In summary, this study provides the first evidence that GLUT3 mRNA and protein expression can be stimulated in vivo under various hyperglycemic conditions. These findings might be of major importance to establish the specific contribution of GLUT3 glucose transporter to the overall regulation of placental glucose transport and utilization. Further characterization of placental GLUT3 gene expression will help to shed light on severe pregnancy-associated disorders such as those encountered in diabetes (macrosomia, malformations) and intrauterine growth retardation (hypoglycemia, low birth weight).

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