Glucose transporter protein responses to selective hyperglycemia or hyperinsulinemia in fetal sheep

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Anderson, Marianne S., Judy Flowers-Ziegler, Utpala G. Das, William W. Hay, Jr., and Sherin U. Devaskar. Glucose transporter protein responses to selective hyperglycemia or hyperinsulinemia in fetal sheep. Am J Physiol Regulatory Integrative Comp Physiol 281: R1545–R1552, 2001.—The acute effect of selective hyperglycemia or hyperinsulinemia on late gestation fetal ovine glucose transporter protein (GLUT-1, GLUT-3, and GLUT-4) concentrations was examined in insulin-insensitive (brain and liver) and insulin-sensitive (myocardium and fat) tissues at 1, 2.5, and 24 h. Hyperglycemia with euinsulinemia caused a two- to threefold increase in brain GLUT-3, liver GLUT-1, and myocardial GLUT-1 concentrations only at 1 h. There was no change in GLUT-4 protein amounts at any time during the selective hyperglycemia. In contrast, selective hyperinsulinemia with euinsulinemia led to an immediate and persistent twofold increase in liver GLUT-1, which lasted from 1 until 24 h with a concomitant decline in myocardial tissue GLUT-4 amounts, reaching statistical significance at 24 h. No other significant change in response to hyperinsulinemia was noted in any of the other isoforms in any of the other tissues. Simultaneous assessment of total fetal glucose utilization rate (GURf) during selective hyperglycemia demonstrated a transient 40% increase at 1 and 2.5 h, corresponding temporally with a transient increase in brain GLUT-3 and liver and myocardial GLUT-1 protein amounts. In contrast, selective hyperinsulinemia led to a sustained increase in GURf, corresponding temporally with the persistent increase in hepatic GLUT-1 concentrations. We conclude that excess substrate acutely increases GURf associated with an increase in various tissues of the transporter isoforms GLUT-1 and GLUT-3 that mediate fetal basal glucose transport without an effect on the GLUT-4 isoform that mediates insulin action. This contrasts with the tissue-specific effects of selective hyperinsulinemia with a sustained increase in GURf associated with a sustained increase in hepatic basal glucose transporter (GLUT-1) amounts and a myocardial-specific emergence of mild insulin resistance associated with a downregulation of GLUT-4.

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tions of ovine fetal glucose transporter proteins GLUT-1, GLUT-3, and GLUT-4 in insulin-insensitive (brain and liver) and insulin-sensitive (myocardium and fat) tissues. Simultaneously, the total fetal glucose uptake/utilization rate was calculated using transplacental steady-state diffusion technique with tritiated water tracer and application of the Fick principle (12, 14).

MATERIALS AND METHODS

Animal Preparation

Columbia-Rambouillet mixed-breed pregnant ewes, each carrying a single fetus, were obtained from Nebeker Ranch (Santa Monica, CA). All studies, animal surgery, and animal care procedures were in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the University of Colorado Health Sciences Center Institutional Animal Care and Use Committee. The animals were cared for and were studied in the University of Colorado Health Sciences Center Perinatal Research Facility, which is accredited by the NIH, the U.S. Department of Agriculture, and the Association for the Assessment and Accreditation of Laboratory Animal Care.

Studies were performed at 132 days (SE ± 0.7) of a 147-day gestation. This timing allowed selective measurement of the effects of hyperglycemia or hyperinsulinemia on fetal glucose transporter proteins in late gestation (90% term) but before potentially confounding increases in fetal hormones or catecholamines seen after 135 days of ovine gestation (5, 19). At 120 days of gestation, surgery was performed to place polyvinyl infusion and blood sampling catheters in the ewe and fetus using standard procedures that have been previously described (1). Fetal catheters for infusion were placed in the distal fetal inferior vena cava via hindlimb pedal veins. Fetal sampling catheters were placed in the distal fetal aorta via hindlimb pedal arteries and into the common umbilical vein by a direct approach at the base of the cord. A maternal femoral arterial sampling catheter and femoral venous infusion catheters were placed via a single groin incision. The catheters were tunneled subcutaneously to exit through a flank incision and were kept in a plastic pouch secured to the ewe’s flank. The catheters were flushed every other day with heparinized (100 U heparin/ml) 0.9% wt/vol sodium chloride in water. For infection prophylaxis, the ewe was given intramuscular injections of gentamicin (American Pharmaceutical Partners, Los Angeles, CA), 80 mg, and procaine penicillin G (Vedco, St. Joseph, MO), 600,000 U, before surgery. The fetus was given intra-amniotic ampicillin (Apothecon, Bristol-Meyers Squibb, New York, NY), 500 mg, at the time of surgery. A priming bolus infusion of D50W [−330 mg (18.3 mM) dextrose/kg maternal weight] followed by a variable infusion of D50W, beginning with an infusion rate of 20 ml/h that provided 3.7 mg (0.21 mM) dextrose·min−1·kg−1 maternal weight. Fetal arterial plasma glucose concentration was measured every 10 min, and the maternal glucose infusion rate was adjusted until fetal plasma glucose was stable at the target concentration. Hyperglycemic clamp was maintained for the duration of the study by small changes in the rate of glucose infusion into the ewe made intermittently in response to measured fetal arterial plasma glucose concentrations.

A Student's t-test was performed on all relevant data. Statistical analysis demonstrated a significant difference (p < 0.05) from baseline.

Study Design

Animals were equally divided into two study groups. One group of fetuses was made hyperglycemic while normal plasma insulin concentrations were maintained, and the other was made hyperinsulinemic while normal plasma glucose concentrations were maintained (13). These two study groups were subdivided into three experimental groups according to the duration of hyperglycemia or hyperinsulinemia (1, 2.5, or 24 h).

In both study groups, tritiated water was infused into the fetus to measure umbilical blood flow by the transplacental steady-state diffusion technique and to allow calculation of umbilical glucose uptake/utilization rate by the fetus using Fick principle methodology, as previously described (1, 12, 14). Control period blood samples for fetal glucose, insulin, and tritiated water concentrations were obtained at four times, each 10 min apart, after 90 min of tritiated water infusion and prior to starting study infusions. Basal glucose concentration, or fetal euglycemia, was defined as the mean fetal arterial plasma glucose concentration measured during the four control draws.

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maternal arterial plasma glucose and insulin concentrations. Fetal arterial oxygen saturation was measured at the beginning and at the end of the control and experimental blood drawing periods to verify fetal well-being. Study infusions were maintained to autopsy, which occurred as soon as possible after study. Certain animals were euthanized after the control period of the study and served as the “zero time” control group.

Tissue Collection

The ewes and fetuses were injected with lethal intravenous injections of pentobarbital (Sleepaway pentobarbital sodium, Fort Dodge Laboratories, Fort Dodge, IA). Fetal weight was measured. Samples of fetal brain, liver, perirenal fat, and myocardium were obtained within 5 min of induction of anesthesia. Samples were immediately snap-frozen in liquid nitrogen and then stored at −70°C until analysis.

Biochemical Assays

Plasma glucose concentrations were measured in duplicate using a YSI model 2700 analyzer (Yellow Springs Instrument, Yellow Springs, OH). To measure plasma insulin concentrations, blood samples were immediately centrifuged at 4°C for 3 min and the plasma was stored at −70°C until analysis with a Linco (St. Charles, MO) rat insulin RIA kit using ovine insulin standards (Eli Lilly, Indianapolis, IN). Blood oxygen saturation and hemoglobin concentration were measured using a radiometer OSM3 hemoximeter (Copenhagen, Denmark).

Glucose Transporter Protein Assays

Ovine tissues, which included fetal brain (cerebral cortex), liver, myocardium (ventricle), or white adipose tissue (perirenal), were thorously washed and then homogenized using a Tekmar Tissuemizer (Cincinnati, OH). The tissue samples were then sonicated (60 sonic, Dismembrator; Fisher Scientific, Pittsburgh, PA) using two 10-s cycles of 5–7 W to ensure adequate homogenization of tissue. Protein content was assessed by the Bio-Rad dye-binding assay (Bio-Rad, Richmond, CA). Fifty micrograms of protein in the ovine tissue homogenates were subjected to discontinuous 10% SDS-PAGE followed by electroblot transfer to nitrocellulose (Trans-Blot transfer medium; Bio-Rad Laboratories, Hercules, CA), which were subjected to Western blotting as previously described (1, 6). The primary antibodies consisted of an affinity purified rabbit anti-rat antibody that was generated against the hemocyanin-limpet linked rat GLUT-1 (1:2,000 dilution), rat GLUT-4 (1:500 dilution) COOH-terminal 16 amino acids (6), or the ovine GLUT-3 (1:20 dilution) COOH-terminal 10 amino acids (26), which were synthesized as oligopeptides. These antibodies have been previously characterized and the isoform specificity confirmed (6). In contrast to GLUT-3, which was not detected with an anti-mouse GLUT-3 antibody (26), rat and ovine GLUT-1 and GLUT-4 proteins were readily detected with the anti-rat glucose transporter antibodies (6). After being washed in PBS-0.1% Tween 20, the membranes treated with the GLUT-1, GLUT-3, or the GLUT-4 antibodies were incubated with a peroxidase-linked goat anti-rabbit IgG (1:2,500 dilution) for 1 h at room temperature and subsequently exposed to a chemiluminescence reagent (Amersham Pharmacia Biotech, Little Chalfont, UK). The chemiluminescence was captured by autoradiography over an optimal period of time (1–5 min; Ref. 6). Glucose transporter protein concentrations were assessed by densitometry once the presence of linearity between the time of autoradiographic exposure and the optical density was established. The results were expressed as a percentage of the mean of the corresponding basal period control values.

Calculations

Umbilical blood flow was calculated by the transplacental steady-state diffusion technique using tritiated water (3H₂O) as the tracer (29). Net umbilical (fetal) glucose uptake rate (UGU_f) was calculated by application of the Fick principle as

\[ \text{UGU}_f(\text{mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}) = \text{ubilical blood flow (ml/min)} \times \text{Gv} - \text{Ga (mg/dl)} \]

where Gv and Ga are the umbilical venous and the fetal arterial plasma glucose concentrations. Net umbilical (fetal) glucose uptake rate was considered equal to fetal glucose utilization rate, as there is no net fetal glucose production when the fetus receives normal or above normal rates of glucose supply from the placenta and has normal to high plasma glucose and/or insulin concentrations (12, 14).

Data Analysis

All results are means ± SE. When two groups were compared, the Student’s t-test was used. Differences when comparing more than two time points were determined by the Kruskal-Wallis test followed by a post-hoc t-test, given the small sample size limitation in sheep investigations.

RESULTS

Plasma Glucose and Insulin Concentrations

Table 1 depicts the maternal and fetal plasma glucose and insulin concentrations measured at control and during the experimental period. The hyperglycemic, euinsulinemic clamp experiments achieved a doubling of maternal and fetal plasma glucose concentrations in the presence of maternal hyperinsulinemia (2.7-fold increase). Euinsulinemia was maintained in

<table>
<thead>
<tr>
<th>Group</th>
<th>Control period</th>
<th>Hyperglycemic, Euglycemic Group</th>
<th>Intergroup P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal glucose, mM</td>
<td>3.9 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td>P = NS</td>
</tr>
<tr>
<td>Fetal glucose, mM</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>P = NS</td>
</tr>
<tr>
<td>Maternal insulin, µU/ml</td>
<td>19 ± 4</td>
<td>27 ± 6</td>
<td>P = NS</td>
</tr>
<tr>
<td>Fetal insulin, µU/ml</td>
<td>13 ± 2</td>
<td>16 ± 2</td>
<td>P = NS</td>
</tr>
<tr>
<td>Clamp period</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal glucose, mM</td>
<td>6.4 ± 0.4*</td>
<td>4.9 ± 0.4</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Fetal glucose, mM</td>
<td>2.1 ± 0.2*</td>
<td>1.3 ± 0.4</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Maternal insulin, µU/ml</td>
<td>52 ± 11*</td>
<td>41 ± 11</td>
<td>P = NS</td>
</tr>
<tr>
<td>Fetal insulin, µU/ml</td>
<td>15 ± 4</td>
<td>73 ± 15*</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>

Values are means ± SE. NS, Not significant. *P < 0.01 or more, when the mean clamp values were compared with the respective control value.
the fetus. The hyperinsulenic, euglycemic clamp experiments achieved fetal hyperinsulinemia (4.6-fold) with no change in maternal and fetal glucose concentrations.

Effects of Selective Hyperglycemia on Tissue Glucose Transporter Concentration

**Insulin-insensitive tissues (brain and liver).** Hyperglycemia with euinsulinemia caused no significant change in brain GLUT-1 concentrations at 1, 2.5, or 24 h but caused a twofold increase in GLUT-3 concentrations at 1 h after the infusion (Fig. 1). A similar acute threefold increase in fetal liver GLUT-1 concentrations was noted at 1 h as well (Fig. 2). Although the change in GLUT-1 protein concentration did not show continued statistically significant elevation at 2.5 and 24 h, it remained well above control at those time points.

**Insulin-sensitive tissue (myocardium and adipose tissue).** Hyperglycemia with euinsulinemia led to a 1.8-fold increase in myocardial GLUT-1 concentrations at 1 h but no change in myocardial GLUT-4 amounts at any time examined (Fig. 3). No change was observed in adipose tissue GLUT-1 or GLUT-4 concentrations (Fig. 4).

Effects of Selective Hyperinsulinemia on Tissue Glucose Transporter Concentration

**Insulin-insensitive tissues (brain and liver).** Selective hyperinsulinemia led to no change in brain GLUT-1 or GLUT-3 concentrations (Fig. 5). Liver GLUT-1 concentrations, however, were significantly increased at all time points with insulin stimulation (Fig. 6).

**Insulin-sensitive tissue (myocardium and adipose tissue).** Selective hyperinsulinemia led to no increase in myocardial GLUT-1 and GLUT-4 concentrations, but a 40% decline in GLUT-4 concentrations was measured at 24 h (P < 0.01; Fig. 7). Akin to the myocardial GLUT-1 concentrations.
dium, no change in adipose tissue GLUT-1 or GLUT-4 concentrations was measured (Fig. 8).

**Fetal Glucose Utilization Rate**

The mean control net fetal glucose utilization/uptake rate for the hyperglycemic study was $7.1 \pm 0.5$ and $6.4 \pm 0.6 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ for the hyperinsulinemic study (not significant). During the hyperglycemic clamp (Fig. 9), fetal umbilical glucose uptake rate was $10.8 \pm 0.9 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ at 1 h ($P < 0.05$ vs. control), $8.9 \pm 0.7 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ at 2.5 h ($P < 0.05$ vs. control), and $7.5 \pm 0.7 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ at 24 h (not significant vs. control). During the hyperinsulinemic clamp (Fig. 10), fetal glucose utilization rate at 1 h was $11.4 \pm 1.0 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, $10.2 \pm 1.0 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ at 2.5 h, and $10.2 \pm 0.5 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ at 24 h ($P < 0.05$ at all three time points vs. control).

**DISCUSSION**

Our present observations support the concept of independent and separate effects of acute changes in plasma glucose and insulin concentrations on fetal tissue glucose transporter protein concentrations. These effects were time dependent and tissue and isoform specific. Although findings of time dependency and tissue and isoform specificity were similar to our previous observations in investigations involving chronic hyperglycemia (6), the actual quantitative effects were dissimilar. Chronic hyperglycemia (10–20 days) in a previous study led to a universal decline in fetal ovine tissue GLUT-1 concentrations in both insulin-sensitive and -insensitive tissues except for the brain (6). In contrast, our present investigations reveal that within 1 h, hyperglycemia in the absence of any perturbation in circulating insulin levels caused significant increases in liver and myocardial GLUT-1 concentrations and in brain neuronal GLUT-3 levels (16). A similar acute increase in GLUT-1 transporter concentration was measured in insulin-responsive skele-
GLUT-4 expression in adult cells. tive to GLUT-1, which contrasts with the higher concentrations in the adult rat. These adult findings suggest that the fetal cells adapt to a high substrate load by initially increasing the portal of basal glucose entry into most cells, including the neurons (16). This change was evanescent, however, disappearing by 2.5 h after the initiation of selective hyperglycemia. Paralleling these changes in fetal tissue GLUT-1 concentrations is the observation of a transient increase in total fetal glucose utilization. This self-limited increase in GLUT-1 perhaps serves a protective function against ongoing enhanced intracellular glucose entry, glycolysis, and lactic acid accumulation, which could prove detrimental to the developing cells (6).

Excess availability of glucose alone did not alter GLUT-4 concentrations in fetal ovine myocardial or adipose tissue, suggesting no acute change in tissue insulin sensitivity. In contrast, in the adult rat, high glucose containing perfusate circulating into the skeletal muscle led to a 20% decline in GLUT-4 concentrations in the plasma membrane, whereas low glucose perfusate caused a 30% increase in plasma membrane GLUT-4 concentrations (21). These observations in the adult rat indicated that perturbations in glucose alone led to a change in translocation of GLUT-4, which in turn either suppressed or augmented the insulin sensitivity of the adult tissue to subsequent hyperglycemic or hypoglycemic insults (21). These changes occurred independent of alterations in insulin or catecholamine concentrations (21). Of note was the fact that these changes were seen at 2 h subsequent to the perturbation. In contrast, hyperglycemia had no effect on GLUT-1 concentrations in the adult rat. These adult observations, although setting a precedent for acute and selective changes in GLUT-4 distribution independent of alterations in insulin concentrations, were dissimilar to our current fetal results. In contrast to the adult, the fetus reacted more quickly to high circulating glucose concentrations, within 1 h, and the changes were in GLUT-1 but not in GLUT-4. This difference may stem from unique developmental characteristics, as ovine fetal tissues express very little GLUT-4 relative to GLUT-1, which contrasts with the higher GLUT-4 expression in adult cells.

Further, because we assessed the total glucose transporter protein concentrations in tissue homogenates, the fetal changes were not due to translocation alone but related to changes in total cellular GLUT-1 and GLUT-3 amounts. Unlike GLUT-4, GLUT-1 resides mainly in the plasma membrane of fetal tissues (hepatocytes, hematopoietic cells, and myocardium; Refs. 25, 26). Thus translocation plays a minor role in further augmenting its function in the fetus. The increases of fetal GLUT-1 and GLUT-3 seen at 1 h are not compatible with synthesis of nascent GLUT-1 or GLUT-3 but perhaps are related to increased stabilization of the endogenous GLUT-1 or GLUT-3 proteins causing an increase in the protein half-life brought about by elevated glucose concentrations (17). Alternatively, similar to previous reports with the adult rat GLUT-4 (27), fetal hyperglycemia might have unmasked the COOH terminus of either GLUT-1 or GLUT-3 in a tissuespecific manner, thereby affecting their detectability with the antibodies that we used that are directed to the COOH terminus.

The development-related isoform-specific effects may be dependent on the predominant isoform that plays a key role in mediating glucose transport, namely, GLUT-1 in the fetus (24, 28) and GLUT-4 in the adult (21). Whereas high glucose suppressed adult GLUT-4 (21), hyperglycemia in the fetus resulted in an increase in GLUT-1 in both the insulin-insensitive (liver) and insulin-sensitive (myocardium) tissues. Thus whereas the acute hyperglycemia-induced decrease in GLUT-4 in the insulin-responsive adult serves an immediate and appropriate role to decrease cell glucose uptake (21), the hyperglycemia-induced acute increase in GLUT-1 (basal) and GLUT-3 (basal) in the fetus appears inappropriate by encouraging excess entry of glucose into cells and potentially resulting in an adverse intracellular environment. Fortunately, following this teleological reasoning, the fetal GLUT-1 and GLUT-3 changes were short-lived. In contrast, no changes in fetal GLUT-4 were observed in the insulin-responsive tissues examined, suggesting no acute change in fetal insulin action secondary to hyperglycemia alone.
Except in liver tissue, selective hyperinsulinemia did not alter tissue GLUT-1 or neuronal GLUT-3 concentrations, indicating no acute effect on the mechanisms underlying basal glucose entry into fetal tissues. The sustained increase in fetal liver GLUT-1 concentrations was paralleled by a persistent increase in the fetal glucose utilization rate. Although the liver is not considered an insulin-sensitive tissue in the classical sense, it is insulin responsive. This insulin responsivity may stem from an insulin-induced increase in GLUT-1 concentrations. GLUT-1 in the fetal mammalian liver is found on the plasma membranes of hepatocytes and hematopoietic cells and is responsible for the unidirectional entry of glucose into cells.

Although hyperinsulinemia is known to alter synthesis of adult GLUT-4 over time, within the acute time frame that we examined, no effect was noted on the total GLUT-4 content in myocardial or adipose tissue. This observation supports the absence of a change in fetal insulin sensitivity within 24 h, except for myocardial GLUT-4 levels, which began declining. This decrease in myocardial GLUT-4 was also observed with 15–20 days of fetal hyperglycemia as previously reported (6). This indicates that while a prolonged exposure to excess glucose was required for the development of insulin resistance as heralded by a decline in GLUT-4 (6), 24 h of hyperinsulinemia alone was sufficient to set the stage for the early emergence of fetal myocardial insulin resistance. An absence of a similar change in adipose tissue GLUT-4 is perhaps related to the need for a longer period of substrate-driven ovine fetal adipose tissue accumulation to herald the onset of insulin resistance. A separate study (11) did show increased GLUT-4 in another insulin-responsive tissue, skeletal muscle, but only at 2.5 h of insulin stimulation, similar to findings in the adult rat. Investigations in the 5-day-old neonatal lamb revealed no effect of hyperinsulinemia with euglycemia on skeletal muscle total GLUT-4 amounts, despite undertaking the study over 5 h with a 100-fold higher insulin dose than we employed in our fetal study (11). Our present studies do not rule out the possibility of an insulin-induced redistribution of GLUT-4 in fetal insulin-responsive tissues akin to the adult (18, 20). Studies utilizing subfractionation procedures (9) and immunolocalization with quantitative microscopic analysis (23) are needed to address the question of GLUT-4 translocation in the fetus.

Perspectives

These observations support an immediate but transient cellular adaptation of augmented glucose uptake associated with a transient increase in GLUT-1 concentration in response to an acute hyperglycemic perturbation in the in utero metabolic milieu. In contrast, alterations in the hormonal milieu (insulin) did not demonstrate any acute effects on the mechanisms underlying insulin-induced cellular glucose transport, except for a sustained increase in hepatic GLUT-1 concentrations and an increase in skeletal muscle GLUT-4 concentrations (1). Whether chronic hyperinsulinemia in the presence of hyperglycemia or euglycemia leads to adaptations of fetal ovine glucose transporter proteins toward enhancing or limiting intracellular substrate delivery remains to be investigated, as do the mechanisms, such as GLUT gene transcription or translation and posttranslational protein stability, that underlie both acute and chronic adaptation to changes in circulating glucose and insulin concentrations.

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