Effects of acute hyperinsulinemia on insulin signal transduction and glucose transporters in ovine fetal skeletal muscle

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Submitted 17 June 2004; accepted in final form 4 November 2004

Anderson, Marianne S., M. Thamotharan, Doris Kao, Sherin U. Devaskar, Liping Qiao, Jacob E. Friedman, and William W. Hay Jr. Effects of acute hyperinsulinemia on insulin signal transduction and glucose transporters in ovine fetal skeletal muscle. Am J Physiol Regul Integr Comp Physiol 288: R473–R481, 2005. First published November 11, 2004; doi:10.1152/ajpregu.00405.2004.—To test the effects of acute fetal hyperinsulinemia on the pattern and time course of insulin signaling in ovine fetal skeletal muscle, we measured selected signal transduction proteins in the mitogenic, protein synthetic, and metabolic pathways in the skeletal muscle of normally growing fetal sheep in utero. In experiment 1, 4-h hyperinsulinemic-euglycemic clamps were conducted in anesthetized twin fetuses to produce selective fetal hyperinsulinemia-euglycemia in one twin and euinsulinemia-euglycemia in the other. Serial skeletal muscle biopsies were taken from each fetus during the clamp and assayed by Western blot for selected insulin signal transduction proteins. Tyrosine phosphorylation of the insulin receptor, insulin receptor substrate-1, and the p85 subunit of phosphatidylinositol 3-kinase doubled at 30 min and gradually returned to control values by 240 min. Phosphorylation of extracellular signal-regulated kinase 1,2 was increased fivefold through 120 min of insulin infusion and decreased to control concentration by 240 min. Protein kinase B phosphorylation doubled at 30 min and remained elevated throughout the study. Phosphorylation of p70 S6K increased fourfold at 30, 60, and 120 min. In the second experiment, a separate group of nonanesthetized singleton fetuses was clamped to intermediate and high hyperinsulinemic-euglycemic conditions for 1 h. GLUT4 increased fourfold in the plasma membrane at 1 h, and hindlimb glucose uptake increased significantly at the higher insulin concentration. These data demonstrate that an acute increase in fetal plasma insulin concentration stimulates a unique pattern of insulin signal transduction proteins in intact skeletal muscle, thereby increasing pathways for mRNA translation, glucose transport, and cell growth.

IN ADULT ANIMALS and humans, insulin has primarily a metabolic role to regulate skeletal muscle glucose uptake with less effect on skeletal muscle protein synthesis than in rapidly growing tissues. Although increased serum insulin concentration in the fetal sheep has been shown to increase fetal skeletal muscle glucose uptake, especially at pharmacological doses (17, 18, 20, 21), insulin in the rapidly growing fetus seems to act largely as an anabolic hormone, promoting skeletal muscle protein synthesis and growth. In human fetuses, abnormal insulin concentrations contribute to variations in fetal growth; excessive insulin promotes fetal overgrowth, whereas insufficient insulin leads to fetal growth restriction. Animal studies have produced experimental deficiencies in insulin secretion in fetal lambs, resulting in fetal growth restriction and limited carcass protein accretion (27). Also, it has been shown in the neonatal pig that insulin action to promote skeletal muscle protein synthesis and growth decreases progressively with maturity and a decreasing rate of growth, unrelated to changes in serum insulin concentration (11, 24). The relative effect of insulin stimulation on intracellular insulin signaling pathways appears to change with maturation. These studies indicate that changes in the activity or amount of one or more intracellular insulin signaling proteins could account for preferential insulin signaling toward protein synthesis and growth in the fetus. To date, very little information exists regarding the ability of fetal hyperinsulinemia to activate steps in the insulin signaling cascade in the intact fetus.

To test the effects of acute fetal hyperinsulinemia on the pattern and time course of insulin signaling in ovine fetal skeletal muscle, we measured selected signal transduction proteins in the mitogenic, protein synthetic, and metabolic pathways in the skeletal muscle of normally growing fetal sheep (Fig. 1). Activation of insulin signaling in ovine fetal skeletal muscle with insulin stimulation was assessed by measuring the amount of tyrosine phosphorylation of the insulin receptor (IR). Phosphorylation of downstream proteins common to the protein synthetic and metabolic pathways, the insulin receptor substrate-1 (IRS-1), the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase), and Akt protein kinase (Akt), was also measured. Finally, phosphorylation of proteins unique to the protein synthetic and the mitogenic pathway, S6 protein kinase (p70 S6K) and extracellular signal-regulated kinases 1 and 2 (ERK1,2), respectively, were measured. Additionally, as a test of downstream activation of the metabolic pathway, glucose transporter protein 4 (GLUT4) translocation was measured.

We also wanted to test whether serial muscle biopsies from fetuses under deep, general anesthesia would, at least qualitatively, allow experimental determination of the time course of signal transduction responses to acute, sustained hyperinsulinemia. If so, this would provide an important new tool for the assessment of fetal metabolic responses to changes in nutrients and hormones under in vivo conditions.

Two sets of experiments were conducted in late-gestation fetal sheep. In the first, we measured the time course of acute changes in insulin signal transduction in skeletal muscle tissue in response to a hyperinsulinemic, euglycemic clamp. In the

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second, we determined the correlation between the degree of translocation of GLUT4 from cytosolic storage sites to the plasma membrane in fetal hindlimb skeletal muscle and glucose uptake by the skeletal muscle in response to graded insulin infusions.

Our results show that the pattern of mitogenic, protein synthetic, and metabolic insulin signaling is intact and robust in the late-gestation fetus, providing insight into pathways triggering both GLUT4 translocation and fetal growth with hyperinsulinemia. The results also show that the serial muscle biopsy procedure with the fetus under deep anesthesia could provide qualitatively and perhaps quantitatively useful data defining acute hormone signaling responses in vivo in the fetal sheep.

MATERIALS AND METHODS

Studies were conducted in healthy, pregnant 2- to 3-yr-old Columbia-Rambouillet mixed-breed sheep. Hyperinsulinemic-euglycemic clamp studies were performed in two groups of animals. In experiment 1, the biopsy studies were performed in ewes with twin gestation. Glucose uptake and GLUT4 transporter measurements were performed in experiment 2 in ewes carrying a single fetus. All in vivo procedures and studies were performed at the University of Colorado Health Sciences Center (UCHSC) Perinatal Research Center (PRC). The PRC is accredited by the United States Department of Agriculture, the National Institutes of Health, and the American Association for the Accreditation of Laboratory Animal Care. All procedures and studies were approved by the UCHSC Institutional Animal Care and Use Committee.

Biopsy studies for timed, serial measurement of insulin signaling transduction proteins. Three twin-bearing ewes at 134 days gestation underwent standard surgical vascular catheterization procedures (20, 21). The ewes and fetuses were then studied over 4 h under deep isoflurane anesthesia supplemented with oxygen to maintain maternal pulse oximetry saturation values between 90 and 100%. The instrumented twin fetuses were partially delivered by cesarean section from the sedated and anesthetized ewe. The fetuses received anesthesia through the intact placenta. This procedure allowed in vivo fetal metabolic measurements during the glucose clamp and serial skeletal muscle biopsies of the biceps femoris to be taken from each fetus in vivo. The surgical incisions in each limb and in the horn of the uterus were held closed with clamps, except during tissue biopsies. Between biopsies, the partially exposed uterus was covered with warm saline-soaked drapes, and the ewe and the draped uterus were covered with dry drapes that enclosed a heating pad that maintained normal temperatures in both ewe and fetus, as determined by assessment of maternal vital signs.

Maternal glucose clamp technique was used to keep the fetal glucose concentration at control values (17, 18, 20). After control arterial blood draws for fetal glucose and insulin concentrations, hematocrit, pH, Pco₂, oxygen saturation, oxygen content, and lactate, insulin in 0.9% wt/vol NaCl in water was infused at 4 mU·min⁻¹·kg⁻¹ estimated fetal weight in the study twin, and 0.9% wt/vol NaCl in...
water was infused in the control twin. Mean control period arterial plasma glucose concentration in the insulin-infused fetus was maintained by glucose clamp (17, 18, 20). At 5, 30, 60, 120, and 240 min of this hyperinsulinemic-euglycemic period, the hindlimb of each fetus was exposed, and a 1 cm × 0.25 cm (~200 mg) biopsy of the biceps femoris muscle was obtained by sharp dissection and immediately frozen in liquid nitrogen. When bleeding occurred, it was controlled by pressure or electrocautery. A fresh site in the same muscle was used for each biopsy. At the same times, fetal arterial blood samples were obtained to measure insulin and glucose concentrations and blood gases. At the end of the study, the ewe and fetuses were killed immediately with intravenously injected Sleepaway pentobarbital sodium (Fort Dodge Laboratories, Fort Dodge, IA).

Clamp studies to measure glucose transporter translocation. Ten singleton pregnant ewes at ~128 days gestation underwent standard surgical vascular catheterization (40, 41). In the “study” hindlimb, a venous sampling catheter was placed in the pudendoepigastric trunk with the tip advanced 1 cm in the external iliac vein. A 3-mm transit time ultrasonic blood flow transducer (Transonic Systems, Ithaca, NY) was positioned around the external iliac artery of the study limb for continuous measurement of blood flow. An arterial blood sampling catheter was placed in the pedal artery of the nonstudy hindlimb, contralateral to the flow probe, with the tip in the external iliac artery (40, 41). All catheters were filled with 0.9% wt/vol sodium chloride in water with sodium heparin at 100 U/ml. The catheters were tunneled subcutaneously to exit the ewe through a flank incision and kept in a plastic pouch secured to the ewe’s flank. The catheters were flushed every other day with the heparinized sodium chloride solution. For infection prophylaxis, the ewe was given intramuscular injections of gentamicin (80 mg) and procaine penicillin G (600,000 units) before induction of anesthesia and samples of the fetal biceps femoris skeletal muscle were immediately frozen in liquid nitrogen and kept in a −70°C freezer. The chemiluminescence was captured on X-ray film over a density range of 1.1, 22, 36).

Glucose clamp technique was used to produce experimental hyperinsulinemia with euglycemia in the fetus (17, 18, 20). During the clamp, fetal arterial plasma glucose concentration was measured every 5–10 min, and the maternal dextrose infusion was adjusted to keep the fetal arterial plasma glucose concentrations at the mean control period value. Before insulin infusion (control) and after 40 min of fetal insulin infusion (experimental), fetal arterial insulin and glucose concentrations, hematocrit, oxygen saturation, oxygen content, and lactate were measured every 5 min over 20 min. Hindlimb blood flow was measured directly with the flow probe and recorded for later analysis with the Flow-0 software (DTS, Columbus, OH). The resulting supernatant was used in the Western blot analysis. Optimal protein concentrations of the homogenate (25 µg) and the PM (15 µg) and LDM (5 µg) fractions were determined. Samples were subjected to discontinuous 10% SDS-PAGE followed by electroblot transfer to nitrocellulose (Nytran; Schleicher and Schuell, Keene, NH). The nitrocellulose filters were rinsed three times with PBS-Tween 20 (PBS-T) and then blocked for 1 h in 5% nonfat dry milk at 22°C. The filters were washed three times in PBS-Tween 20 (PBS-T) (1 × 15 min and 2 × 10 min). Incubation was with an affinity-purified rabbit anti-rat antibody (0.5 µg/ml) that was raised against rat GLUT4 (1:2,500 dilution) COOH-terminal 16 amino acids, which were synthesized as oligopeptides. The filters were washed three times with PBS-T and then were treated with peroxidase-linked goat anti-rabbit IgG. They were exposed to a chemiluminescence reagent (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK), and the chemiluminescence was captured on X-ray film over exposure times of 1–5 min to determine the optimal exposure time. GLUT4 protein concentrations were measured by densitometry (1, 22, 36).

Phosphorylation and Western blotting of IR, IRS, p85α, Akt, ERK1/2, and p70 S6K. Frozen muscle biopsies (200 mg) were homogenized in 2 ml homogenization buffer (20 mM Tris, 5 mM MgCl2, 1 mM MOPS, 2 µM leupeptin, and 2 µM pepstatin, pH 8.7) at 4°C using a Polytron PTA 205 generator at maximum speed for 30 s. Triton X-100 was added to a final concentration of 1%. The homogenates were allowed to sit on ice and solubilized for 60 min, followed by centrifugation at 100,000 g for 60 min at 4°C. The supernatant was collected and stored at −70°C. Protein content was measured by Bradford assay using BSA as a standard (30). For tyrosine phosphorylation of the IR, IRS, and Akt (phospho-specific antibodies), and mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK), samples were subjected to discontinuous 10% SDS-PAGE followed by electroblot transfer to nitrocellulose (Nytran; Schleicher and Schuell, Keene, NH). The chemiluminescence was captured on X-ray film over exposure times of 1–5 min to determine the optimal exposure time. GLUT4 protein concentrations were measured by densitometry (1, 22, 36).

Biochemical analysis. Plasma glucose and lactate concentrations were measured in duplicate using a Yellow Springs Instrument model 2700 analyzer (Yellow Springs Instrument, Yellow Springs, OH). To measure plasma insulin concentrations, sampled blood was 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, oxygen content, pH, and Pco2 concentrations were measured using Radiometer (Copenhagen, Denmark) OSM3 and ABL520 Hemoximeters.

Calculations. Hindlimb blood flow was measured directly, and hindlimb glucose uptake (HGLU) was calculated, using the Fick principle as:

\[
\text{HGLU} = \frac{(\mu\text{mol} \cdot \text{min}^{-1})}{(100 \text{ g tissue}^{-1})}
\]

where \(G_a\) is the glucose concentration in the contralateral external iliac artery, and \(G_t\) is the glucose concentration in the venous effluent of the study hindlimb.

Subcellular localization of glucose transporter proteins in the fetal skeletal muscle. Cell membrane fractions of fetal skeletal muscle were obtained by differential centrifugation. Frozen skeletal muscle (1 g) was crushed on dry ice and then placed in 30 ml of ice-cold sucrose buffer (250 mM sucrose, 20 mM HEPES/Tris, 1 mM EDTA, and 100 µM phenylmethylsulfonyl fluoride (PMSF), pH 7.4). The sample was homogenized in ice with a Polytron homogenizer (30 mm probe, at setting 3) for 15 s burst. The homogenate was filtered through two layers of cheesecloth to remove residual connective tissue. A sample of the homogenate was saved for Western blot analysis. The remaining homogenate was centrifuged at 3,000 g for 10 min at 4°C. The supernatant was saved for preparing the low-density microsome (LDM) enriched fraction, and the pellet was used to prepare the plasma membrane (PM) as previously described (22, 36). PM and LDM fraction samples were frozen at 70°C pending Western blot analysis.

Western blot analysis. The skeletal muscle homogenates and the PM and LDM fraction samples were sonicated (60 sonic; Dismembrator, Fisher Scientific, Pittsburgh, PA) using two 50-s cycles of 5–7 watts. The suspension was centrifuged at 10,000 g for 10 min at 4°C. The resulting supernatant was used in the Western blot analysis. Optimal protein concentrations of the homogenate (25 µg) and the PM (15 µg) and LDM (5 µg) fractions were determined. Samples were subjected to discontinuous 10% SDS-PAGE followed by electroblot transfer to nitrocellulose (Nytran; Schleicher and Schuell, Keene, NH). The nitrocellulose filters were rinsed three times with PBS-Tween 20 (PBS-T) and then blocked for 1 h in 5% nonfat dry milk at 22°C. The filters were washed three times in PBS-Tween 20 (PBS-T) (1 × 15 min and 2 × 10 min). Incubation was with an affinity-purified rabbit anti-rat antibody (0.5 µg/ml) that was raised against rat GLUT4 (1:2,500 dilution) COOH-terminal 16 amino acids, which were synthesized as oligopeptides. The filters were washed three times with PBS-T and then were treated with peroxidase-linked goat anti-rabbit IgG. They were exposed to a chemiluminescence reagent (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK), and the chemiluminescence was captured on X-ray film over exposure times of 1–5 min to determine the optimal exposure time. GLUT4 protein concentrations were measured by densitometry (1, 22, 36).

Fetal insulin signal transduction and GLUT4 translocation.
TBS-Tween (TBS-T) for 1 h at room temperature and incubated with anti-IR, anti-IRS1, or anti-p85 antibody (Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. After being washed, the membrane was washed three times in TBS-T and incubated with anti-mouse IgG-horseradish peroxidase (HRP; 1:2,000 dilution in TBS-T) or HRP-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature. Membranes were washed again, and enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Arlington Heights, IL) were added for 1 min and immediately exposed to X-ray film according to the manufacturer’s instructions.

To determine the level of Akt, ERK1/2, and p70 S6K phosphorylation, 75 µg protein were subjected to 10% SDS-PAGE. After being transferred and blocked, the membrane was incubated with phospho-Akt (Ser473), phospho-p70 S6K (Thr^421/Ser^422) antibody, or active ERK1/2 (1:1,000 in TBS-T with 3% BSA; Cell Signaling Technology) overnight at 4°C followed by washing, and the signal was detected using ECL as above. To determine the total protein levels, 50 µg muscle tissue homogenate protein were treated with Laemmli sample buffer, boiled for 5 min, resolved on a 7% or 10% denaturing SDS-PAGE gel, and transferred to PVDF membranes as above. Membranes were blocked with 5% nonfat milk (Bio-Rad) or 3% BSA for 1 h at room temperature. The membrane was washed three times with TBS-T and probed with a monoclonal anti-p70S6K antibody (1:2,000 dilution in TBS-T; Transduction Laboratories), anti-Akt antibody (1:1,000 dilution in TBS-T; New England Biolabs), or a monoclonal anti-ERK1/2 antibody (1:3,000 dilution in TBS-T).

Data analysis. All results are expressed as means ± SE. Physiological measurements were analyzed using a two-way mixed-effects ANOVA for a partially balanced incomplete block experimental design. For the insulin signal transduction protein measurements, repeated-measures ANOVA was employed. Transporter protein measurements were analyzed with a one-way ANOVA followed by a post hoc Newman-Keuls test. P values <0.05 were considered significant.

RESULTS

All animals were studied at 135 ± 0.4 days gestation. Fetal weight (3.20 ± 0.1 kg), control fetal arterial plasma glucose concentration (20.4 ± 1.2 mg/dl), and control fetal arterial plasma insulin concentration (6.63 ± 0.8 µU/ml) were not different among animals. All measurements of fetal hematocrit, arterial blood oxygen saturation, arterial oxygen content, and lactate were within the normal range for late-gestation fetal sheep (2) and were unchanged by any experimental conditions in either experimental group (Tables 1 and 2).

Western blot analysis of the changes in insulin signal transduction produced in experiment 1 is shown in Fig. 2. Tyrosine phosphorylation of the IR doubled at 30 min (P < 0.05) and gradually returned to the nonstimulated control level over the course of the experiment. A similar pattern was seen for tyrosine phosphorylation of IRS-1 and the phosphotyrosine-associated p85 regulatory subunit of PI 3-kinase. Akt phosphorylation was also doubled at 30 min but, in contrast, remained elevated throughout the experiment. The most dramatic change was seen in the phosphorylation of p70 S6-kinase, which showed a fourfold increase at 30, 60, and 120 min of insulin stimulation (P < 0.01). Phosphorylation of members of the mitogen-activated protein kinase family, the ERK1 and -2, was increased fivefold during the insulin infusion. The increase was sustained for up to 120 min but decreased by 240 min.

Figure 3 shows steady-state insulin and glucose concentrations during the experimental clamp infusions. The clamp data for the twin fetuses in experiment 1 are included in the control and high insulin infusion data in Fig. 3. Glucose was held constant at control concentrations (21.8 ± 1.7 mg/dl) during the intermediate insulin infusion (20.7 ± 2.34 mg/dl) and during the high insulin infusion (18.5 ± 3.2 mg/dl). The fetal arterial insulin concentration was 5.4 ± 1.0, 32.2 ± 7.7, and 106.8 ± 31.1 µU/ml under control, intermediate, and high insulin infusion. Fetal hindlimb glucose uptake, measured in experiment 2, was 3.35 ± 0.13 µmol min^-1·100 g tissue^-1 under baseline conditions. With intermediate insulin infusion, fetal hindlimb glucose uptake was slightly, but not significantly, increased to 3.61 ± 0.18 µmol min^-1·100 g tissue^-1. Only at the high fetal insulin concentration was hindlimb glucose uptake significantly increased to 4.54 ± 0.31 µmol min^-1·100 g tissue^-1 (Fig. 4).

In experiment 2, translocation of GLUT4 from intracellular storage vesicles to the PM was clearly seen in response to both intermediate and high-dose insulin stimulation (Fig. 5) GLUT4 protein was increased fourfold in the PM relative to control PM-associated GLUT4 concentrations at 1 h, consistent with the timing of the increases in insulin signal transduction proteins discussed above. Interestingly, there was no additional increment of translocation measured at the high fetal plasma insulin concentration, although fetal hindlimb glucose uptake increased significantly only with the higher insulin stimulation.

DISCUSSION

Many natural and experimental conditions have shown that insulin actively regulates fetal metabolism and growth, acutely and chronically and in response to increases and decreases in insulin concentration (13, 18, 19). The critical cellular signaling mechanisms underlying the effects of insulin on fetal metabolism, particularly glucose uptake and utilization, and

| Table 2. Fetal arterial hematocrit, pH, PCO₂, O₂ saturation, O₂ content, and lactate concentration in control, intermediate-insulin and high-insulin infusion groups (experiment 2) |
|-----------------|-----------------|-----------------|
|                 | Control         | Intermediate    | High Insulin   |
| Hematocrit, %   | 34.3 ± 1.6      | 32.3 ± 1.9      | 35.3 ± 1.6     |
| Arterial pH     | 7.362 ± 0.01    | Not measured    | 7.363 ± 0.02   |
| Arterial PCO₂, mmHg | 49.8 ± 1.0     | Not measured    | 52.7 ± 2.4     |
| Arterial O₂ saturation, % | 44.2 ± 3.5 | 36.7 ± 3.8 | 33.7 ± 5.4 |
| Arterial O₂ content, mmol/l | 2.7 ± 0.2 | 2.2 ± 0.2 | 1.8 ± 0.5 |
| Arterial lactate, mmol/l | 2.436 ± 0.22 | 2.596 ± 0.32 | 3.0 ± 1.0 |

Values are means ± SE. Control vs. experimental, not different.

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Fig. 2. Experiment 1 data showing insulin-induced changes over time in tyrosine phosphorylation of the insulin receptor (Ty-ph-IR; A), phosphorylated extracellular signal-related kinases 1,2 (ph-ERK1,2; B), tyrosine phosphorylation of the insulin receptor substrate 1 (Ty-ph-IRS1; C), tyrosine phosphorylation of the p85 regulatory subunit of PI 3-kinase (Ty-ph-p85; D), serine phosphorylation of Akt protein kinase (Ph-Ser473-Akt; E), and threonine phosphorylation of p70 S6-kinase (Ph-Thr412-p70S6K; F). Representative photographs of Western blots with data graphed to show the degree of increases above time 0 over time. Values are means ± SE. *P < 0.05 in hyperinsulinemic compared with control animals.
fetal growth have not been completely defined. Our results show a robust increase in the principal insulin signal transduction proteins with acute insulin stimulation, indicating that the pathways to glucose uptake and metabolism, initiation of mRNA translation, protein synthesis, and growth are developed in the late-gestation fetal sheep. The results also show that the in vivo time course of insulin signal transduction effects is rapid, beginning almost immediately after an abrupt increase in plasma insulin concentration, and variable, with several proteins showing rapid increases in concentration followed by decreases of some and sustained values for others.

In these studies, we have used techniques that allow hindlimb-specific measurements of glucose flux and blood flow, providing accurate measurement of the skeletal muscle contribution to total fetal glucose uptake. We have also developed a new differential centrifugation technique for fetal sheep skeletal muscle to separate PM from intracellular storage vesicle membranes. Although in previous studies we identified normal intracellular glucose transporter distribution in the fetal sheep skeletal muscle using immunohistochemistry, the immunohistochemical technique was not sensitive enough to measure translocation of transporter to active sites in the PM (1). Separation of PM-associated transporter from intracellular transporter using differential centrifugation allowed accurate measurement of glucose transporter translocation, and we have been able to correlate GLUT4 translocation with increases in skeletal muscle glucose uptake in response to fetal hyperinsulinemia.

These studies also provide an assessment of acute, time-dependent changes in insulin signaling by performing serial muscle biopsies of skeletal muscle of fetuses under deep anesthesia. The lack of differences between the basal conditions of the insulin-infused and control twins for biochemical and physiological variables indicates that these preparations were stable. Furthermore, the similarity of the biochemical and physiological values between the twins in this study and those of many historical singleton fetuses studied under nonanesthetized, chronic conditions supports the value and reliability of the acute, anesthetized, serial muscle biopsy model to provide normative data about basal and insulin-stimulated conditions in vivo (1, 20, 40, 41).

Glucose tolerance and protein synthesis have been shown to be altered during surgery, and the type of anesthesia provided may well impact the mechanisms and extent of the metabolic change. For example, isoflurane anesthesia, as was used during experiment 1, may stimulate gluconeogenesis and decrease glucose clearance, leading to a more pronounced intraoperative hyperglycemia. In contrast, epidural anesthesia may block hepatic glucose production and blunt surgery-associated hyperglycemia. Fentanyl and midazolam have no effect on gluconeogenesis but do seem to impair glucose utilization and in that way contribute to intraoperative hyperglycemia (25).

In our glucose clamps, fetal serum glucose concentration was maintained at control concentration, and fetal insulin...
infusion reliably elevated fetal insulin concentration to the projected low and high concentration goals. Any stimulation of gluconeogenesis would have been balanced by decreased exogenous glucose infusion based on clamp procedures to control glucose concentration, as verified by measurements made during the experiment. It may be that, despite reassuring serum measurements, the effect of insulin to increase glucose uptake by the skeletal muscle cell could have been blunted. If this occurred, our very significant increases in signaling protein activation represent an underestimate of the unperturbed in vivo situation.

In these studies, we have shown the time course of acute changes in fetal insulin signaling proteins with insulin stimulation in vivo by measuring representative phosphorylation changes in 1) the mitogenic pathway, 2) the protein synthesis pathway, and 3) the metabolic pathway of insulin signal transduction in response to acute insulin stimulation. The response to increases in insulin concentration shows that the insulin signaling pathways are intact and robust in the late-gestation fetus, providing mechanistic insight into both GLUT4 translocation and fetal growth with hyperinsulinemia.

Previously, we have reported insulin-induced effects on the mitogenic pathway in fetal sheep skeletal muscle, measured by increases in the activity of farnesyltransferase and in the amount of farnesylated p21 Ras after 4 h of insulin stimulation (32). The current study and a previous in vitro study (12) suggest a more rapid effect of insulin in this pathway, since the peak in downstream phosphorylated ERK proteins occurred after 2 h of insulin infusion. Increased availability of farnesylated Ras with activation of ERK1 and ERK2 proteins would be anticipated to lead to phosphorylation and activation of transcription factors regulating genes necessary to promote cell growth, adaptation, and differentiation. Insulin may also act through Ras to potentiate the effects of other growth factors, such as IGF-I (16, 26). Our data suggest that the growth-promoting actions of insulin by signaling through ERKs to DNA expression are developed in the late-gestation sheep fetus and are separate from the pathways to mRNA translation and GLUT4 translocation.

The effect of insulin to activate the IR and to initiate phosphorylation of signaling proteins early in the metabolic and protein synthesis pathways was rapid, but transient. Downstream, Akt showed similar early increases in phosphorylation, but, in contrast, remained elevated throughout the study and, in fact, increased further between 120 and 240 min of insulin stimulation. Insulin-responsive tissues are enriched in Akt (4), and Akt is thought to be a key mediator of both cell growth (3, 8) and metabolism (3, 5, 7, 23). The current results support these roles for Akt in the fetal sheep for the first time.

The downstream effect of insulin stimulation on p70 S6 kinase was rapid and sustained. Phosphorylated p70 S6 kinase concentration increased dramatically by 30 min, and the increase was maintained for 2 h. This substantial upregulation of activated p70 S6 kinase suggests the possibility of increased initiation of mRNA translation and protein synthesis as a result of insulin stimulation. These findings are consistent with previous studies in fetal sheep, which showed increased 4E-BP1 phosphorylation in skeletal muscle after 7 h of insulin infusion (31).

GLUT4 translocation to the PM was increased with insulin stimulation, but did not increase beyond the degree of translocation produced by the intermediate rate of insulin infusion. In contrast, hindlimb glucose uptake increased significantly only at the higher rate of insulin infusion. The lower insulin infusion rates, while producing translocation of GLUT4 to the PM, may not have affected some of the downstream regulatory steps in glucose metabolism, such as phosphorylation of glucose and metabolism of glucose into various specific pathways, thereby not affecting glucose utilization. This discrepancy might indicate that, although GLUT4 translocation is highly responsive to insulin, downstream regulatory steps in glucose metabolism might require higher concentrations of insulin to produce increased glucose utilization.

Alternatively, at the high fetal insulin concentration, activation of the metabolic pathway through phosphorylation of Akt, resulting in negative regulation of glycogen synthase kinase, might have been higher at the high fetal insulin concentration than at the intermediate fetal insulin concentration, leading to a lower intracellular glucose concentration. This would have enabled a faster rate of glucose uptake in the muscle cells, since glucose transport is driven by the concentration gradient across the membrane.

It also is possible that higher rates of insulin infusion might have augmented glucose utilization by insulin’s capacity to increase microvascular perfusion via capillary recruitment in the skeletal muscle, without measured evidence of increased total hindlimb blood flow (9, 10, 15, 28).

Additionally, higher rates of insulin infusion and higher plasma insulin concentrations might increase production of and/or translocation of other glucose transporter isoforms, including GLUT1, which our previous studies did show was responsive to insulin with increased expression and abundance, and GLUT3, which has been found in fetal skeletal muscle (6, 14, 29, 33–35, 37–39).

Perspectives

The results of this study indicate that insulin is critical to fetal metabolism, protein synthesis, and growth. Although it is clear that the signaling pathways to these effects are well developed and robust in the late-gestation sheep fetus, it is unclear how the relative proportion of effect compares with similar pathways in the adult. Our results indicate that the relative contribution of insulin signaling to fetal glucose uptake and metabolism is much less than is seen in the adult where the primary function of insulin seems to be glucose homeostasis. Protein synthesis and growth are likely the critical functions of insulin in the developing fetus. Our previous studies, in fact, showed a decrease in total fetal glucose uptake and in GLUT4 transporter protein concentration in fetal sheep skeletal muscle after extended periods (2 and 24 h) of high glucose availability (1). Similarly, high fetal insulin concentrations increased GLUT4 protein in fetal skeletal muscle for a limited period of time, followed by an apparent downregulation of transporter concentration by 24 h of stimulation. Under those hyperinsulinemic conditions, however, total fetal glucose uptake remained significantly elevated above control, indicating that insulin signaling mediated increased glucose utilization independently of its effect to translocate GLUT4. This possibility is further supported by the present studies that show increased glucose utilization rate with increasing insulin infusion and plasma concentrations, despite the lack of further increase in...
GLUT4 translocation. Finally, although further studies will be required to substantiate the validity of the anesthetized, serial biopsy model, these studies provide an optimistic entree into the assessment of acute, time-dependent changes in hormonal signaling and metabolic responses at organ, tissue, cellular, and subcellular levels during controlled, in vivo conditions in the sheep fetus.

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