Insulin resistance and glucose transporter expression during the euglycemic hyperinsulinemic clamp in the lamb

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1Department of Pediatrics, Women & Infants Hospital, Brown University School of Medicine, Providence, Rhode Island 02905-2401; 2Robert Schwartz MD Center for Metabolism and Nutrition, and the Division of Neonatology, MetroHealth Medical Center, Cleveland 44109-1998; and 3Department of Neonatology, The Children’s Hospital, Cleveland Clinic Foundation, Cleveland, Ohio 44195-0001

Gelardi, Nancy L., Robert E. Rapoza, Joseph F. Renzulli, and Richard M. Cowett. Insulin resistance and glucose transporter expression during the euglycemic hyperinsulinemic clamp in the lamb. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E1142–E1149, 1999.—Three- to six-day-old lambs infused with 100 mU·kg−1·min−1 insulin required greater amounts of glucose to maintain euglycemia during a euglycemic hyperinsulinemic clamp compared with 31- to 35-day-old insulin-infused lambs (15.87 ± 3.47 vs. 4.30 ± 1.11 mg·kg−1·min−1, P < 0.05, respectively). Endogenous glucose production persisted in both groups; however, the percent decrease compared with age-matched lambs receiving no insulin was greater in the younger group compared with the older group (53%, P < 0.001, vs. 34%, P < 0.01). The younger animals showed greater glucose utilization compared with the older animals (215 vs. 96%, respectively, P < 0.01). No effect of insulin was noted on GLUT-4 protein expression in either group. GLUT-2 expression was increased in older vs. younger lambs. Older insulin-infused lambs showed lower GLUT-2 expression than older 0 insulin-infused lambs (0.94 ± 0.07 vs. 1.64 ± 0.10 (OD) units, P < 0.005). Increased sensitivity to insulin in the younger animals was not related to acute changes in GLUT-4 expression. Increased GLUT-2 expression with age, as well as decreased expression with hyperinsulinemia, is consistent with the development of an insulin-resistant state in the adult.

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EUGLYCEMIC HYPERINSULINEMIC CLAMP PROCEDURE

Euglycemic Hyperinsulinemic Clamp Procedure

Each study lasted 300 min and began after a 1-h recovery from surgery. A blood sample was obtained immediately following the conclusion of the clamp and subsequently at 30, 60, 90, 120, and 150 min.

Insulin resistance and glucose transporter expression during the euglycemic hyperinsulinemic clamp in the lamb

THE EUGLYCEMIC HYPERINSULINEMIC CLAMP TECHNIQUE, as described by DeFronzo et al. (9), is a useful approach to assess the isolated effects of hyperinsulinemia on glucose metabolism without the associated insulin counter-regulatory hormonal effects from hypoglycemia. By combining the clamp with infusion of a stable isotope, endogenous glucose production can be quantified, and the contribution of changes in glucose production vs. altered peripheral sensitivity to insulin can be evaluated.

Farrag et al. (12) applied these techniques to the human preterm neonate and reported that, in contrast to the adult, the neonate has persistent glucose production and greater peripheral sensitivity to insulin. In a subsequent investigation to evaluate the developmental response to insulin, Farrag et al. (11) evaluated a group of preterm infants early in the immediate neonatal period and again at the conclusion of the neonatal period. Greater peripheral sensitivity to insulin was noted in the preterm neonate early in the neonatal period, but not later, compared with the term neonate. The investigators concluded that an adult-like response to insulin requires maturation past the neonatal period in the human neonate.

In this investigation, we combined our experience with the lamb as an animal model for evaluation of glucose homeostasis in the newborn period (6–8) and the euglycemic hyperinsulinemic clamp technique (11, 12) to test the hypothesis that increased insulin sensitivity of the neonate is due in part to an alteration in the expression of glucose transport proteins. We have focused our attention on the effect of euglycemic hyperinsulinemia on 1) endogenous glucose production and GLUT-2 protein expression in the liver and 2) glucose utilization and GLUT-4 protein expression in muscle.

MATERIALS AND METHODS

Animals and Research Study Design

Animal Care Committees at Brown University and Women & Infants Hospital approved all protocols. Mixed-breed pregnant sheep and/or nursing ewes with 20- to 25-day-old lambs were obtained from EM Parsons & Sons (Hadley, MA). All animals were housed at the Brown University Animal Care Facility for ≈5 days before either delivery or study with ad libitum food and water. To ensure that the lambs were in a postabsorptive state for the study, early groups (3- to 6-day-old newborns) were fasted overnight for 12 h. The late groups (31- to 35-day-old lambs) were fasted for 72 h, with water being withheld for the final 12 h. The mean weight of the animals at the time of study was 4.43 ± 0.22 g for the early groups and 11.59 ± 0.76 g for the late groups. Lambs were secured on a surgery table, their necks were shaved, and lidocaine was administered as the anesthetic. Cotton blindfolds were placed on the animals to help calm them. An incision, −1.5 in. long, was made over the jugular vein. A 5-Fr polyurethane catheter was inserted −8 cm into the vessel to facilitate the administration of study solutions. A similar procedure was used for the carotid artery for blood sampling. Animals were placed in a mesh sling for the remainder of the study. No additional sedation was utilized. Animals were kept blindfolded and primarly slept throughout the study.
before the study to measure blood glucose concentration and background isotopic enrichment of glucose. The first 180 min constituted the basal period of the study. This period started with a prime plus constant infusion of deuterated glucose \( (\delta[^{6,6}-2H_2] \text{glucose}) \) at a rate of 0.04 mg·kg\(^{-1}\)·min\(^{-1}\) in 0.45% saline solution. The prime was given at a rate of 50:1 relative to that of the continuous infusion. The clamp period constituted the final 120 min of the study. At 180 min, the subject was infused with a prime plus constant infusion of insulin in a rate of 100 mU·kg\(^{-1}\)·min\(^{-1}\). At the same time, glucose (10% dextrose in water) was infused at various rates to clamp blood glucose concentration at each subject's euglycemic concentration. The glucose infusion contained deuterated glucose \( (\delta[^{6,6}-2H_2] \text{glucose}) \) to maintain a fixed rate of tracer glucose to “cold” glucose. It has been shown that during clamp studies it is necessary to add tracer to the exogenous glucose infusate to maintain steady-state isotopic enrichment throughout the study (2, 22). Control studies consisted of only isotope infusion. Blood samples were obtained at 10-min intervals throughout the study to monitor glucose concentration on a YSI 2700 Select Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH). Samples for basal period measurements were obtained between 150 and 180 min; those for clamp measurements were obtained between 270 and 300 min.

Stable isotope tracer. Deuterated glucose \( (\delta[^{6,6}-2H_2] \text{glucose}) \) was obtained from Cambridge Isotope Laboratories (Woburn, MA) and used as the stable isotopic tracer. The lyophilized material was prepared as a separate stock solution in 0.45% saline solution and tested for sterility and pyrogens by Ethidix Sterilizing (Coventry, RI) according to Food and Drug Administration standards. The solution was stored in sterile standard pharmacy containers at 4°C.

Preparation of infusates. All reagents were prepared on the morning of each study. Regular human insulin (Humulin R, recombinant DNA origin, Eli Lilly, Indianapolis, IN) was prepared in 0.45% saline solution containing 1% albumin to a final concentration of 20 mU/ml. Ten percent dextrose water was used for exogenous glucose delivered by a Medfusion pump model 2010 (Medfusion, Duluth, GA).

Preparation of blood samples. Blood was centrifuged to obtain plasma. Plasma \(^2H_2\) enrichment glucose turnover was determined as follows. Plasma proteins were precipitated with 70% acetone, and the resultant supernatant was passed over an anion (Dowex AG-1-X8), cation (Dowex AG-50-W-X8) exchange column and rinsed with \( H_2O \). The pentacatectate derivative was prepared, and the enrichment was determined by gas chromatography-mass spectrometry (GC-MS) on a Hewlett-Packard (Palo Alto, CA) 5988B GC-MS by electron impact ionization at mass-to-charge ratios (m/z) 200 and m/z 202. Sample enrichment was calculated relative to a standard curve run simultaneously.

The following parameters were measured during the basal and clamp periods. Insulin concentration was measured by radioimmunoassay (DIagnostic Product, Los Angeles, CA). Glucagon concentration was measured by radiolmmunoassay (ICN Pharmaceuticals, Costa Mesa, CA). Cortisol was measured by radiolmmunoassay (INCSTAR, Stillwater, MN). Blood gases were measured on a Ciba-Corning 238 pH/blood gas analyzer (Ciba-Corning, Norwood, MA). Heart rate and systolic and diastolic pressures were recorded on a Corometrics 556 Monitor (Corometrics Medical Systems, Wallingford, CT).

Calculations. The glucose turnover was calculated according to the equations of Steele as they apply to isotopic non-steady-state conditions (21), as we have utilized previously (12). The insulin sensitivity index (ISI) at euglycemia, as reported by Bergman et al. (1), was calculated by the following formula:

\[
\frac{\Delta \text{glucose utilization}}{\Delta \text{insulin concentration}} = \frac{\text{glucose concentration x } \Delta \text{insulin concentration}}{\Delta \text{insulin concentration (A[I])}}
\]

where \( \Delta \text{glucose utilization} \) equals the increment in glucose utilization during the clamp, \( \Delta \text{insulin concentration} \) is the glucose concentration at the time of the clamp, and \( \Delta \text{insulin concentration} \) is the increment in insulin concentration during the clamp.

Metabolic clearance rate (MCR) of insulin was calculated by the following formula:

\[
\text{insulin infusion rate} = \frac{\Delta \text{insulin concentration}}{\Delta \text{insulin concentration} (A[I])}
\]

Western Blot Analysis

At the conclusion of the clamp study, the animals were immediately euthanized, and tissue samples were rapidly dissected. Tissue collection was completed as quickly as possible (i.e., within 3–5 min). The tissues were then snap-frozen in liquid nitrogen and stored at −80°C. Primary antibodies against glucose transporter proteins were purchased from Biogenesis (Sandown, NH). Electrophoresis reagents and standards were purchased from Bio-Rad (Hercules, CA), and enhanced chemiluminescence (ECL) Western blot reagents and film were from Amersham (Arlington Heights, IL).

Tissue samples were homogenized on ice in homogenization buffer (0.25 M sucrose, 0.5 mM EDTA, 50 mM HEPES, pH 7.4, containing aprotonin, leupeptin, and 4-(2-aminoethyl)benzenesulfonyl fluoride). Samples were then centrifuged at 1,200 g for 10 min. The resulting supernatant was collected and saved on ice. The pellet was rehomogenized and centrifuged at 1,200 g for 10 min. The supernatants were pooled and centrifuged at 9,000 g for 10 min. The resulting supernatant was centrifuged at 100,000 g for 2 h (10, 15, 16, 19). The pellet then was resuspended in buffer, and protein was determined by the biochlinonic acid method (Pierce, Rockford, IL). Samples were solubilized for 30 min at room temperature in sample buffer (0.5 M Tris·HCl, pH 6.8, 10% SDS, glycerol, 0.1% mercaptoethanol, \( H_2O \), and 0.5% bromophenol blue) and loaded onto 10% gels, 50 or 100 µg protein/lane. Prestained molecular mass markers, positive controls, negative controls, and a protein pool sample, to assess the efficiency of transfer, were run on each gel. Proteins were transferred to polyvinylidene fluoride membrane using a semi-dry transfer apparatus (Fisher Scientific, Pittsburgh, PA). Equal loading and transfer efficiency were also assessed by Coomassie Blue staining of the gel. Membranes were then blocked at 37°C for 1 h in 5% (wt/vol) nonfat dry milk in Tris-buffered saline with Tween 20 (TBST). This was followed by incubation with either rabbit anti-rat GLUT-2 or rabbit anti-rat GLUT-4 antibody (1:500 dilution with 5% normal donkey serum) for 1 h at room temperature. After extensive washing in TBST, the membranes were incubated with donkey anti-rabbit horseradish peroxidase immunoglobulin G (Amersham, Arlington Heights, IL) at 1:5,000 with 5% nonfat dry milk for 1 h at room temperature. Membranes were again extensively washed in TBST, and immunoreactivity was detected using ECL on Hyper film. Autoradiograms were quantified by scanning densitometry, and the results are reported in arbitrary optical density (OD) units.
Statistics

Statistical analysis was by SAS Proc Mixed (SAS Institute, Cary, NC). The data obtained during the baseline and clamp periods were averaged to determine the mean value for each concentration (i.e., blood glucose) or measurement (i.e., heart rate) for each group. The groups were subsequently compared using between- and within-animal comparisons over time. Statistical significance was at a level of \( P \leq 0.05 \).

RESULTS

Figure 1A depicts the blood glucose concentration over time for the basal and euglycemic hyperinsulinemic clamp periods. Euglycemia was maintained during all of the steady-state insulin infusions. Blood glucose concentrations during the clamp period are listed in Table 1. There were no significant differences between the basal and clamp periods within any group. The early groups did have significantly greater glucose concentrations compared with the late groups in the basal time periods: 88 \( \pm \) 7 mg/dl (early basal 0 insulin) vs. 58 \( \pm \) 7 mg/dl (late basal 0 insulin) (\( P < 0.01 \); 91 \( \pm \) 6 mg/dl (early basal 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin) vs. 61 \( \pm \) 3 mg/dl (late basal 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin) (\( P < 0.01 \)). The early groups also had significantly greater glucose concentrations compared with the late groups in the clamp periods: 88 \( \pm \) 7 mg/dl (early clamp 0 insulin infused) vs. 58 \( \pm \) 1 mg/dl (late clamp 0 insulin infused); 92 \( \pm \) 6 mg/dl (early clamp 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin infused) vs. 61 \( \pm \) 2 mg/dl (late clamp 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin infused), \( P < 0.01 \). Figure 1A depicts the blood glucose concentration over time for the basal and euglycemic hyperinsulinemic clamp periods. Statistics are noted in Table 1.

Figure 1B depicts the total glucose appearance rates, which are listed in Table 1 for the clamp period. The total glucose appearance rates were significantly higher in the early groups vs. the late groups during the basal period: 5.86 \( \pm \) 0.03 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (early basal 0 insulin infused) vs. 2.91 \( \pm \) 0.33 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (late basal 0 insulin infused), \( P < 0.05 \); 7.33 \( \pm \) 0.53 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (early basal 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin infused) vs. 2.93 \( \pm \) 0.04 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (late basal 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin infused), \( P < 0.01 \). There were no significant differences in the clamp periods when 0 insulin was infused compared with the corresponding basal periods: 5.49 \( \pm \) 0.03 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (early clamp 0 insulin infused) vs. 5.86 \( \pm \) 0.03 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (early basal 0 insulin infused); 2.82 \( \pm \) 0.30 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (late clamp 0 insulin infused) vs. 2.93 \( \pm \) 0.04 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (late basal 0 insulin infused). During the hyperinsulinemic clamp period, there were significant increases in glucose appearance rate from basal values in the 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin groups: 22.56 \( \pm \) 2.24 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (early clamp 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin infused) vs. 7.33 \( \pm \) 0.53 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (early basal 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin infused), \( P < 0.01 \); 5.86 \( \pm \) 1.00 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (late clamp 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin infused) vs. 2.93 \( \pm \) 0.04 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (late basal 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin infused), \( P < 0.001 \).

Figure 1C depicts endogenous glucose production (EGP) rates, which are also listed in Table 1. EGP rates were significantly higher in the early groups vs. the late groups during the basal period: 5.83 \( \pm \) 0.33 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (early basal 0 insulin infused) vs. 2.86 \( \pm \) 0.33 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (late basal 0 insulin infused); 7.29 \( \pm \) 0.53 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (early basal 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin infused) vs. 2.89 \( \pm \) 0.04 mg \cdot kg\(^{-1}\) \cdot min\(^{-1}\) (late basal 100 mU \cdot kg\(^{-1}\) \cdot min\(^{-1}\) insulin infused), \( P < 0.001 \).
Table 1. Metabolic data during the euglycemic clamp period

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Insulin Infusion Rate, mU·kg⁻¹·min⁻¹</th>
<th>Blood Glucose, mg/dl</th>
<th>Plasma [Insulin], µU/ml</th>
<th>Glucose Infusion Rate, mg·kg⁻¹·min⁻¹</th>
<th>Rate of Appearance, mg·kg⁻¹·min⁻¹</th>
<th>Endogenous Glucose Production, mg·kg⁻¹·min⁻¹</th>
<th>Plasma [Cortisol], ng/dl</th>
<th>Plasma [Glucagon], pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>5</td>
<td>100</td>
<td>88 ± 7</td>
<td>8 ± 2</td>
<td>0.04</td>
<td>5.49 ± 0.03</td>
<td>5.46 ± 0.33</td>
<td>8.3 ± 1.7</td>
<td>586 ± 150</td>
</tr>
<tr>
<td>Late</td>
<td>6</td>
<td>0</td>
<td>58 ± 1*</td>
<td>12 ± 2</td>
<td>0.04</td>
<td>2.82 ± 0.03*</td>
<td>2.74 ± 0.30*</td>
<td>13 ± 1.7</td>
<td>759 ± 138</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no./group. *P < 0.01 vs. corresponding early group; †P < 0.001 vs. 0 insulin groups; ‡P < 0.05 vs. early 100 mU·kg⁻¹·min⁻¹ insulin group.

kg⁻¹·min⁻¹ (late basal 100 mU·kg⁻¹·min⁻¹ insulin infused), P < 0.01. There were no significant differences in the clamp periods when 0 insulin was infused compared with the corresponding basal periods: 5.46 ± 0.33 mg·kg⁻¹·min⁻¹ (early clamp 0 insulin infused) vs. 5.83 ± 0.33 mg·kg⁻¹·min⁻¹ (early basal 0 insulin infused); 2.74 ± 0.30 mg·kg⁻¹·min⁻¹ (late clamp 0 insulin infused) vs. 2.86 ± 0.33 mg·kg⁻¹·min⁻¹ (late basal 0 insulin infused). During the hyperinsulinemic clamp periods there were significant decreases in glucose production rates from basal values in the insulin-infused groups: 3.61 ± 1.18 mg·kg⁻¹·min⁻¹ (early clamp 100 mU·kg⁻¹·min⁻¹ insulin infused) vs. 7.29 ± 0.53 mg·kg⁻¹·min⁻¹ (early basal 100 mU·kg⁻¹·min⁻¹ insulin infused), P < 0.01; 1.55 ± 0.33 mg·kg⁻¹·min⁻¹ (late clamp 100 mU·kg⁻¹·min⁻¹ insulin infused) vs. 2.89 ± 0.04 mg·kg⁻¹·min⁻¹ (late basal 100 mU·kg⁻¹·min⁻¹ insulin infused), P < 0.05.

Plasma insulin concentrations were not significantly different between the groups during the basal period: 8 ± 2 µU/ml (early basal 0 insulin infused), 17 ± 1 µU/ml (late basal 0 insulin infused), 10 ± 2 µU/ml (early basal 100 mU·kg⁻¹·min⁻¹ insulin infused), 13 ± 2 µU/ml (late basal 100 mU·kg⁻¹·min⁻¹ insulin infused). During the clamp period, plasma insulin concentrations were significantly higher in the insulin-infused groups, as listed in Table 1.

Plasma cortisol concentrations were not significantly different between the groups in the basal period: 8.9 ± 2.1 µg/dl (early basal 0 insulin infused), 14.2 ± 1.3 µg/dl (late basal 0 insulin infused), 9.7 ± 2.7 µg/dl (early basal 100 mU·kg⁻¹·min⁻¹ insulin infused), 6.1 ± 1.9 µg/dl (late basal 100 mU·kg⁻¹·min⁻¹ insulin infused). Plasma cortisol concentrations during the clamp period were also not significantly different between the groups, as listed in Table 1.

Plasma glucagon concentrations were not significantly different between the groups during the basal period: 500 ± 192 pg/ml (early basal 0 insulin infused), 650 ± 93 pg/ml (late basal 0 insulin infused), 526 ± 103 pg/ml (early basal 100 mU·kg⁻¹·min⁻¹ insulin infused), 376 ± 46 pg/ml (late basal 100 mU·kg⁻¹·min⁻¹ insulin infused). There were no differences in the glucagon concentrations during the clamp period, as listed in Table 1.

The late groups of animals had a lower heart rate than the early groups (P < 0.01), but no group showed a change in rate between basal and clamp periods. Systolic and diastolic pressures were measured during both the basal and clamp periods, and there were no significant differences between the groups. No significant differences were detected in pH, PO₂, or PCO₂ between the groups during either the basal or clamp periods.

Figure 2 depicts the percent decrease in EGP and the percent increase in glucose utilization. EGP was significantly reduced at both ages in the insulin-infused groups compared with the age-matched groups receiving no insulin (53% reduction in the early group, P < 0.001, and 34% reduction in the late group, P < 0.01). There was a significant difference in percent reduction between the two insulin-infused groups (P < 0.05).

The increase in glucose utilization was significant in the insulin groups compared with the noninfused groups (P < 0.001, respectively, for early and late infused groups vs. age-matched noninfused groups). The early animals showed a significantly greater increase in utilization compared with the late groups of animals (215 vs. 96%, respectively, P < 0.01).

Figure 3 shows the ISI at euglycemia, as well as the MCR of insulin calculated for the early and late groups during infusion of 100 mU·kg⁻¹·min⁻¹ insulin. The early group had an ISI of 0.115 ± 0.048, which was significantly greater than the ISI for the late group (0.033 ± 0.019), P < 0.005. The MCR was not different between the early and late insulin-infused groups: 6.81 ± 0.61 (early group) vs. 6.65 ± 0.50 (late group).

Figure 4A shows representative Western blots of GLUT-4 protein in muscle and GLUT-2 protein in liver. Fifty micrograms of crude muscle or 100 µg of crude liver membrane protein were loaded per lane. Five-day-old 0 insulin, 5-day-old 100 mU·kg⁻¹·min⁻¹ insulin, 30-day-old 0 insulin, and 30-day-old 100 mU·kg⁻¹·min⁻¹ insulin groups are shown. Positive and negative rat membrane protein controls were run simultaneously on each gel. For muscle gels, the positive controls were rat muscle membrane proteins, and the negative controls were rat liver membrane proteins. For the liver gels, the positive controls were rat liver membrane proteins, and the negative controls were rat muscle membrane proteins.

Figure 4B demonstrates results quantified by scanning densitometry of autoradiograms. Data are means ± SE reported in OD units. GLUT-4 protein concentration was not significantly altered with insulin infusion in any group: 5-day-old 0 insulin, 5.67 ± 0.12; 5-day-old 100 insulin, 5.99 ± 0.01; 30-day-old 0 insulin, 5.67 ± 0.01; 30-day-old 100 insulin, 5.70 ± 0.01. GLUT-2
protein concentration increased with age: 5-day-old 0 insulin, 0.77 ± 0.01 vs. 30-day-old 0 insulin, 1.52 ± 0.12, P < 0.005. Euglycemic hyperinsulinemia decreased GLUT-2 expression in the older animals: 30-day-old 0 insulin, 1.52 ± 0.12 vs. 0.94 ± 0.07, P < 0.005.

Table 1 shows the metabolic data during the clamp period of the studies. The amount of glucose infused to maintain euglycemia in the late group was significantly lower than that in the early group (P < 0.05).

**DISCUSSION**

In this investigation we examined glucose homeostasis throughout the neonatal period, utilizing our published lamb model (6–8). To evaluate glucose homeostasis during this developmental period at the cellular level, we also examined changes in the glucose transport system. We studied lambs at 3–6 days of age, early in the neonatal period, and at 31–35 days, late in the neonatal period. Muscle and liver tissue samples were harvested at the conclusion of euglycemic hyperinsulinemic clamp studies for glucose transporter evaluation. Kliegman et al. (14) first used the euglycemic hyperinsulinemic clamp in newborn beagle puppies. At comparable plasma insulin concentrations, glucose production in the adult group was completely suppressed, whereas in the newborns complete suppression was not achieved. The investigators attributed the persistent glucose production to hepatic insulin resistance. Farrag et al. (12) recently reported the use of the hyperinsulinemic euglycemic clamp for the first time in the human neonate. Persistent glucose production was apparent across a wide range of insulin infusion rates (0.2–4.0 mIU·kg⁻¹·min⁻¹). EGP was sensitive to low insulin concentrations, plateaued quickly, and became nonresponsive to higher insulin concentrations. Beginning at the insulin infusion rate of 0.5 mIU·kg⁻¹·min⁻¹ in the human preterm neonate, there was a significant reduction in EGP ranging from 41–58% in the groups studied. If suppression of glucose production is the maximal effect of insulin on the liver, then this effect was not achieved in the human preterm neonate. Peripheral glucose utilization increased over basal
rates only at insulin infusion rates of 2 and 4 mU·kg⁻¹·min⁻¹. The investigators were not able to determine the maximal effect on glucose utilization because a plateau was not reached at the insulin infusion rates employed. However, the neonatal glucose utilization response to insulin far exceeded the maximal response reported in the adult.

In the present study, we have successfully employed the euglycemic hyperinsulinemic clamp in the neonatal lamb. The choice of 100 mU·kg⁻¹·min⁻¹ insulin infusion rate was based on a series of preliminary studies that used a range of rates from 2.0 to 500 mU·kg⁻¹·min⁻¹. At lower infusion rates no effects of insulin were detected. Not until rates of 25–50 mU·kg⁻¹·min⁻¹ insulin were used did effects on glucose production and utilization begin to appear. Responses at 100 and 500 mU·kg⁻¹·min⁻¹ insulin were not significantly different, and therefore we concluded that a maximal insulin response could be obtained at the 100 mU·kg⁻¹·min⁻¹ insulin infusion rate. This rate is comparable to that used by Kleigman et al. (14) in the newborn beagle puppy model but considerably higher than the rate used by Farrag et al. (12) in the human neonate.

Similar to data in the human neonate, EGP was not completely suppressed in the lamb despite very high plasma insulin concentrations. Interestingly, the early neonatal group appeared to be more responsive to insulin, resulting in a significantly greater percent decrease in EGP than in the late group (i.e., 53 vs. 38%, respectively, P < 0.05). Relative to peripheral glucose utilization, at comparable insulin infusion rates, the early group responded with a 215% rise over basal. This was significantly higher than the 96% increase in the late group. The late group also required significantly lower glucose infusion rates to maintain euglycemia compared with the early group, as noted in Table 1. These data are consistent with data from the human preterm neonate compared with the adult, because the neonate showed persistent glucose production as well as greater peripheral sensitivity to insulin.

The late groups had significantly lower blood glucose concentrations compared with the early groups. We attribute this to the fact that, for the older animals, food was withheld for 72 h, as advised by the Food and Drug Administration and with the approval of the animal care committees, because by 30 days these animals can be weaned and considered true ruminants. Because of this difference in basal and, therefore, clamp glucose concentrations between the groups, we utilized the ISI to compare the peripheral sensitivity to insulin among the groups (1). As was noted with the early preterm human neonate vs. the late preterm neonate (11), the early group of lambs had significantly greater peripheral sensitivity to insulin compared with the late group. The MCR for insulin was not different between the groups. It may be that at an infusion rate of 100 mU·kg⁻¹·min⁻¹ insulin there is a plateau in the clearance rate.

The appropriate distribution of whole body glucose is, at least in part, regulated by the tissue-specific expression and regulation of several glucose transporter isoforms with distinct kinetic properties. GLUT-2 is the major glucose transporter isoform expressed in hepatocytes, β-cells, and kidney. The distinguishing feature of this isoform is that it is a low-affinity high-turnover transport system. Coupled with the kinetically similar hexokinase, glucokinase in hepatocytes, and β-cells, GLUT-2 forms part of a glucose-sensing apparatus that responds to subtle changes in blood glucose with alterations in the rate of glucose uptake into the cell. GLUT-4 glucose transporter is expressed in adipocytes and muscle cells. These are the "insulin-sensitive" cell types, so called because they respond to insulin with a rapid and reversible increase in glucose uptake.
in glucose transport. Glucose transport in insulin-sensitive tissues has received attention because of the importance of this process in the maintenance of whole body glucose homeostasis (15, 19).

In this study there appeared to be a developmental increase in GLUT-2 in the late groups vs. the early groups (P < 0.05). This increase may signal the onset of an insulin-resistant state in the ruminant (3, 4, 13, 20). Similar patterns of increased expression have been noted in studies of rats made diabetic by streptozotocin administration. After an initial decrease, GLUT-2 expression increased with time (5, 17). The reduction in expression of GLUT-2 with euglycemic hyperinsulinemia is in agreement with clamp studies in the diabetic rat. Forty eight to 72 h after streptozotocin injection, GLUT-2 protein levels increased in the liver of the diabetic rat. Physiological insulin infusion decreased GLUT-2 protein to levels below control levels (5). It should also be noted that, in ruminant liver, a high level of GLUT-5 mRNA has also been detected, implying that GLUT-5 may also be involved in the uptake and release of glucose (13). We have yet to explore this possibility in our model.

Acute euglycemic hyperinsulinemia caused no change in the expression of GLUT-4. This is consistent with previous studies showing that acute hyperinsulinemia is not a regulator of GLUT-4 expression (18). We speculate that changes in GLUT-4 expression are not directly responsible for the changes in insulin sensitivity.

In summary, we found persistent glucose production during the infusion of 100 mU · kg⁻¹ · min⁻¹ of insulin in both groups of animals. We noted that the late group of insulin-infused animals were not sensitive to the effects of insulin in that they 1) required very little glucose infused to maintain euglycemia, 2) showed a lesser percent decrease in EGP, and 3) had a lower percent increase in glucose utilization compared with the early group. At the level of the glucose transporter, GLUT-4 protein expression was not significantly different between the two ages and was not affected by acute euglycemic hyperinsulinemia. The expression of GLUT-2 appeared to change with the age of the animals, increasing significantly as they matured. Insulin infusion resulted in no change in the early animals but a significant decrease in the late group.

In conclusion, in the newborn lamb as in the human neonate: 1) persistent glucose production during insulin infusion was noted in all lambs studied, 2) an adult-like response to insulin requires maturation beyond the neonatal period. Although conclusions for other species should be made with caution because of differences in the regulation of glucose transporters between ruminants and nonruminants, insulin-sensitive GLUT-4 does not appear to be a pivotal protein in the control of glucose uptake and metabolism during acute euglycemic hyperinsulinemia in the neonatal period in the lamb. GLUT-2 expression increases with age and decreases with acute insulin infusion in the older animals. This appears to be consistent with the development of insulin resistance in the adult sheep.

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