Peripheral Insulin Resistance and Impaired Insulin Signaling Contribute To Abnormal Glucose Metabolism in Preterm Baboons

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Premature infants develop hyperglycemia shortly after birth, increasing their morbidity and death. Surviving infants have increased incidence of diabetes as young adults. Our understanding of the biological basis for the insulin resistance of prematurity and developmental regulation of glucose production remains fragmentary. The objective of this study was to examine maturational differences in insulin sensitivity and the insulin-signaling pathway in skeletal muscle and adipose tissue of 30 neonatal baboons utilizing the euglycemic hyperinsulinemic clamp. Preterm (67% gestation) baboons had reduced peripheral insulin sensitivity shortly after birth (*M* value 12.5±1.5 vs. 21.8±4.4 mg/kg·min in term baboons) and at 2 weeks of age (*M* value 12.8±2.6 vs. 16.3±4.2, respectively). Insulin increased Akt phosphorylation, but these responses were significantly lower in preterm baboons during the first week of life (3.2-fold versus 9.8-fold). Preterm baboons had lower GLUT1 protein content throughout the first 2 weeks of life (8–12% of term). In preterm baboons, serum free fatty acids (FFA) did not decrease in response to insulin whereas FFA decreased by >80% in term baboons; the impaired suppression of FFA in preterm animals was paired with decreased GLUT4 protein content in adipose tissue. In conclusion, peripheral insulin resistance and impaired non-insulin dependent glucose uptake play an important role in hyperglycemia of prematurity. Impaired insulin signaling (reduced Akt) contributes to the defect in insulin-stimulated glucose disposal. Counter-regulatory hormones are not major contributors.

Prematurity is one of the two leading causes of perinatal morbidity and mortality. In 2011, premature infants with Low Birth Weight (LBW) infants (<2500 g) accounted for 8.1% of all births in the United States, and they have been found to have increasing incidences of type 2 diabetes mellitus, essential hypertension, and coronary artery disease (CAD) (1–7). A large proportion of premature infants have impaired glucose control with hyperglycemia during the first few weeks of life. This condition has been associated with hyperosmolar dehydration, intraventricular hemorrhage, retinopathy of prematurity (ROP), brain damage, and death (8–12). Hyperglycemic premature infants are commonly treated with glucose restriction until their glucose tolerance improves, adversely affecting their nutritional status and growth (13). Recently, neonatologists have allowed permissive hyperglycemia in the neonatal period, tolerating serum glucose levels of 120–150 mg/dL and classifying hyperglycemia only if the glucose is > 150 mg/dL with the ultimate goal of promoting growth with higher glucose infusions. Often,

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serum glucose is tolerated at levels greater than 150 mg/dL and if glucose is > 200 mg/dL, insulin therapy is typically used. Once hyperglycemia persists, neonates require significantly higher and variable insulin infusion rates than older children and adults to achieve euglycemia (14, 15). In many cases, insulin therapy remains ineffective or causes hypoglycemia, which can result in impaired neurologic function (16).

Very Low Birth Weight (VLBW) premature infants (<1500 g) have elevated plasma insulin levels suggesting peripheral insulin resistance (15, 17). Furthermore, humans and animals born with intrauterine growth restriction demonstrate peripheral insulin resistance (18, 19). In adults, peripheral insulin resistance primarily reflects impaired glucose uptake by skeletal muscle, which is the main site for insulin-stimulated glucose disposal (20). Resistance to the action of insulin in muscle has predictive value for the development of type 2 diabetes (21–23). Several abnormalities distinguish insulin-resistant muscle from normal muscle, including: decreased insulin-stimulated GLUT4 translocation, impaired insulin receptor (IR) and IR substrate-1 (IRS-1) tyrosine phosphorylation, decreased insulin-stimulated Akt (Protein Kinase B) phosphorylation, and PI3-kinase activity (20). GLUT1 is the predominant fetal glucose transporter isoform that facilitates basal glucose transport into fetal cells that are proliferating and is found in abundance in most fetal rat and sheep tissues. GLUT4 transporters are found in insulin-responsive tissues and are expressed in low amounts compared to GLUT1 in fetal rats (24). Studies in fetal sheep at term gestation have shown up-regulation of insulin-signaling molecules in response to insulin, although it remains unclear how this effect compares with similar pathways in children and adults (24). Insulin stimulation causes translocation of GLUT4 from the intracellular compartment to the sarcolema and the transverse tubules mediating insulin-induced glucose transport; some studies have reported reduced GLUT4 translocation in the neonatal rat muscle compared to adult animals (25). Recently, our group demonstrated reduced muscle content of key glucose transport-regulating proteins (GLUT1/GLUT4) in the basal state and up-regulation of upstream insulin signaling molecules in preterm baboons when compared to mature counterparts (26). It remains to be determined if these alterations in key insulin-signaling molecules play a role in the pathogenesis of neonatal hyperglycemia and reduced insulin-stimulated glucose disposal.

Baboons are an appropriate animal model to study insulin signaling during the neonatal period because they develop hyperglycemia of prematurity spontaneously (27), are long-lived animals, have close (98%) phylogenetic proximity with humans, and develop insulin resistance/hyperglycemia when obese (28). In the present study, we have utilized the euglycemic hyperinsulinemic clamp (29) to quantitate insulin sensitivity in neonatal baboons as it has been demonstrated that molecular mechanisms involved in muscle and adipose insulin resistance of adult baboons are similar to those in man (28).

The biological basis for the insulin resistance of prematurity remains unclear and our understanding of the developmental regulation of glucose production and its mechanisms remains fragmentary. No previous study has examined insulin sensitivity in an extremely preterm animal in a serial manner, and, to date, no study has evaluated insulin sensitivity in preterm humans. The primary objective of this study was to examine maturational differences in insulin sensitivity and in the insulin-signaling pathway in skeletal muscle during the early postnatal period utilizing the euglycemic hyperinsulinemic clamp. A secondary objective was to investigate maturational differences in adipose tissue.

RESEARCH DESIGN AND METHODS

Animal Care

A total of 30 baboons were studied, 16 were delivered at 185 days (d) gestational age (GA)(full term), and 14 were delivered at 125 ± 2d GA (67% of gestation) at the University of Texas Health Science Center in San Antonio, Texas (UTHSCSA), the Texas Biomedical Research Institute (TBRI) in San Antonio, Texas, or at the Oklahoma Primate Center, Oklahoma. The animals were delivered prematurely or at term via Caesarean section under general anesthesia from healthy, nondiabetic mothers or by vaginal delivery (only term for postnatal survival); fetal animals were sacrificed immediately after birth at the same gestational age and were utilized as shared controls. All preterm baboons were of an appropriate weight for their gestational age. The Institutional Animal Care Committee at the TBRI and UTHSCSA approved studies. Animal experiments were conducted in accordance with accepted standards of humane animal care. Primary analysis was based on the rate of insulin-stimulated glucose metabolism (*M*) to determine differences in insulin sensitivity between preterm and term baboons. Based on our preliminary data, the *M* value for term infants at 5 days of life (DOL) was 23 ± 1.6 mg/kg·min, whereas the *M* value in preterm baboons was 11 ± 2.7 mg/kg·min. We calculated that 5 animals per group were sufficient to detect a significant difference between groups with 80% power and alpha of 5%.

Routine care for preterm animals was done as previously described with the exception of IV nutrition and glucose management (27). To prevent hypoglycemia, a

2.5% IV dextrose solution was started after birth at a rate of 150 mL/kg/d. The glucose infusion rate (GIR) was titrated to maintain a target glucose of 50–100 mg/dL using a sliding scale which allowed adjustment of the GIR every 2–4 hours by 1–2 mg/kg/min. Measurements of glucose levels were obtained shortly after birth and then at a minimum of every 4 hours using the AU640 Immuno Analyzer (Olympus Inc., San Diego, CA) or GM9 Glucose analyzer (Analox Instruments Ltd, London, UK). Additional glucose measurements were obtained if serum glucose was out of target. Urine glucose was measured at least every 12 hours by Multistix 10 SG reagent strips for urinalysis (Siemens [Bayer] Medical Solutions Inc., Pittsburg, PA). IV insulin could be given if the serum glucose was greater than 200 mg/dL despite stopping the IV dextrose infusion. Parenteral nutrition was initiated at 24 hours of life (HOL) with a mixture of pediatric amino acids at a dose of 1.75 g/kg/d and increased to 3.5 g/kg/d by 48 HOL, and maintained at this rate until 12 hours prior to the initiation of the insulin clamp, at which time the amino acid infusion was stopped. IV lipids were not administered. Enteral feedings were initiated on day 3 of life with Similac formula (Abbot Laboratories, North Chicago, Illinois) and increased as tolerated by 10–20 mL/kg/d. Feeds were stopped 24 hours prior to the insulin clamp.

Routine Care for Term Animals. Animals were fed by their mother until their transfer to the nursery and then fed following a veterinarian protocol with Similac (Abbot Laboratories) 4–5 times a day, until 12 hours prior to experiments when they were placed *nil per os* (NPO). For experiments, animals were sedated with Ketamine (10 mg/kg, IM) and isoflurane gas anesthesia (1%-2%) and intubated. Two peripherally inserted central catheters were placed, or if necessary, femoral arterial or venous cutdowns were performed to insert the central catheters. Normal saline was started at a rate of 120 mL/kg/d. A glucose infusion was initiated to avoid hypoglycemia if serum glucose was $<$ 50 mg/dL. Measurements of glucose levels were obtained hourly using the AU640 Immuno-Analyzer (Olympus Inc). Additional glucose measurements were obtained if serum glucose was out of target (50–100 mg/dL). Analgesia (Meloxicam 0.2 mg/kg lM) was given for potential pain in biopsy and catheter placement sites. Full term animals that served as gestational controls were fed by their mothers and sedated with Ketamine (10 mg/kg) prior to euthanasia without any interventions. All animals were euthanized with pentobarbital followed by exsanguination.

Euglycemic Hyperinsulinemic Clamp Procedure

Insulin sensitivity was measured by performing serial euglycemic hyperinsulinemic clamps as previously described (29). Two 120-minute insulin clamps were performed; one at 5 $\pm$ 2 DOL and at 14 $\pm$ 2 DOL. Animals received a prime (150 mU kg^{-1} min^{-1}) plus constant infusion of insulin at a rate of 15 mU kg^{-1} min^{-1} (Novolin; Novo Nordisk Pharmaceuticals, Princeton, NJ). At the same time, glucose (25% dextrose in water) was infused at a variable rate to clamp blood glucose concentration at 60–80 mg/dL. Blood samples were obtained at 5–10min intervals throughout the clamp to monitor plasma glucose concentration and to adjust the glucose infusion to maintain euglycemia. Plasma samples were collected to determine serum insulin at –180, 0, +30, +60, +90, and +120min during the insulin clamp by the Ultrasensitive Insulin ELISA kit from ALPCO Diagnostics (Salem, NH; Intra-assay CV% 5.2–11.1). Insulin sensitivity was calculated as previously described (29).

Plasma glucagon (EIA Kit from ALPCO Diagnostics, Salem, HN; Intra-assay CV% 3.3–5.1), plasma catecholamines (2-CAT (A-N) Research ELISA TM Enzyme Immunoassay, Labor Diagnostika Nord GMBH&Co., Nordhorn, Germany; Intra-assay CV% 8.4–10.5), and serum free fatty acids (FFA)(HR Series NEFA-HR(2) kit, Wako Diagnostics, Mountain View, CA) were measured at time 0 and +120min. Animals were normotensive, euglycemic, and off medications that may alter glucose metabolism for $>$ 24 hours prior to clamp.

Muscle biopsies. Two muscle biopsies (basal and insulin-stimulated) were performed during each insulin clamp (total of four biopsies). Muscle biopsies were obtained from the biceps femoris muscle via sharp dissection with a sterile technique from one leg for the first biopsy and from the contralateral leg for the second biopsy. Animals were anesthetized and received 1% lidocaine locally prior to the biopsies to obtain ~10 mg of muscle tissue. The tissues were snap-frozen in liquid nitrogen and stored at –80°C.

Adipose tissue was collected immediately after euthanasia and was snap-frozen and stored as above.

Measurement of Insulin-signaling and Glucose Transporter Proteins

Western Blot Analysis

Insulin signaling molecules were measured in skeletal muscle and adipose tissue using our previously described protocol and the antibodies used are listed on Table 1 (26). The intensities of the bands were quantified by densitometry using the NIH imaging program and the results reported in arbitrary optical density units. GAPDH and/or

Table 1. Animal Characteristics

Group	Gestation (% of term)	Gestational Age (days)	n	Female/Male	Birth Weight (grams)
Preterm Control	67%	125	6	2/4	389 ± 40
Preterm +14d	67%	125	8	5/3	375 ± 42
Term Control	94%	185	6	3/3	845 ± 133
Term +28d	94%	185	10	6/4	921 ± 141

Means ± SD are shown.

Ponceau S from Thermo Fisher Scientific (Walthman, MA, USA) were used as loading controls (26). The gels were normalized using internal controls to ensure comparable gel-to-gel data analysis across groups. Phosphorylated proteins were normalized to their total protein content.

PCR Analysis

Total RNA was extracted from the tissues using the RNeasy Mini Kit from Qiagen (Venlo, Netherlands). Relative quantitation of gene expression was accomplished with the TaqMan (Life Technologies, Foster City, CA) methodology using the relative standard curve method. The following TaqMan gene expression assays for PCR primers/probes were obtained from Life Technologies: GLUT4/(Hs00168966_m1), GLUT1/(Hs00892681_m1), Akt-1/(Hs00178289_m1), IRS-1/(Hs00178563_m1), and INSR/(Hs00961554_m1). The quantity of the mRNA for each gene of interest was normalized to IPO8/(Hs00183533_m1). Real-time quantitative PCR reactions were performed as previously described and sequences from primers were similar to those previously published by our group (28).

Statistical Analysis

Statistical calculations were performed with SPSS for Microsoft Windows®, (Version 17.0, SPSS, Inc., Chicago, IL). Two-way ANOVA, followed by the Bonferroni and Tukey test, repeated measures ANOVA, paired and unpaired Student's *T* test, and chi-squares were performed when appropriate. A P value of < 0.05 was considered statistically significant.

Results

Animal characteristics

Animal characteristics are shown in Table 1. Plasma insulin concentrations were similar between groups at birth and while fasting during the first week of life (Table 2). During the second week of postnatal life, the fasting plasma insulin concentration was higher in preterm infants (Table 2). In contrast, serum glucose concentrations remained similar at fast during the first 2 weeks of life; of note, we utilized fetal samples for the comparisons at birth for some animals born at term, which may explain the tendency for a lower fasting glucose level (Table 2). Serum glucose and daily GIRs levels from preterm baboons are depicted in (Figure 1A and 1B). One preterm baboon received dopamine transiently to treat hypotension but experimental procedures were not performed until > 48 after dopamine was stopped. None of the animals received insulin or medications that could alter glucose metabolism.

Table 2. Laboratory Values

Group	Preterm	Term	P value
Fasting Insulin (μIU/mL)			
Birth	**1.1 ± 1.0**	**2.4 ± 2.8***	**0.2**
DOL5	6.4 ± 7.8	4.6 ± 6.2	0.6
DOL14	9.9 ± 9.1	1.1 ± 0.9	0.05
Fasting Glucose (mg/dL)			
Birth	50 ± 20	35 ± 5*	0.07
DOL5	68 ± 13	68 ± 18	0.9
DOL14	68 ± 15	64 ± 22	0.6
Fasting Glucagon (pg/mL)			
DOL5	30 ± 24	86 ± 71	0.07
DOL14	30 ± 36	199 ± 141	0.03

Serum concentrations ± SD are shown.*Fetal term animals.

Insulin Sensitivity

Serum insulin was significantly increased from baseline to the end of the clamp to a target of > 1,000 μU/mL and was similar between groups (Figure 2A and 2B); this level was chosen as it had been previously shown to achieve 100% suppression of endogenous glucose production in newborn beagles (30). Preterm baboons had reduced peripheral insulin sensitivity compared to their term counterparts on DOL5 (M value 12.5 ± 1.5 vs. 21.8 ± 4.4 mg/kg·min, $P < .001$) and DOL14 (M value 12.8 ± 2.6 vs. 16.3 ± 4.2 mg/kg·min, $P = .04$)(Figure 2C). Insulin sensitivity did not improve with postnatal age in preterm ba-

boons. Term baboons tended to be more sensitive to insulin during the first week of life than during the second week of life but failed to reach statistical significance (Figure 2C).

Free Fatty Acids and Counter-regulatory Hormones

Fasting serum FFA (time 0) was significantly lower in preterm baboons during the first week of life ($P < .01$) and was similar to term by the second week of life (Figure 3A). In response to insulin, serum FFA did not decrease (from time 0 to +120) in preterm baboons on DOL5 or DOL14, (Figure 3A); in term baboons, serum FFA decreased by 88% after insulin stimulation on DOL5 and by 80% on DOL14 ($P < .05$, Figure 3A).

Plasma glucagon was significantly lower in preterm baboons compared to term baboons both at baseline and after maximal insulin stimulation during the first 2 weeks of life (Figure 4A). Fasting glucagon measurements are shown in Table 2. During the insulin clamp, plasma glucagon decreased in term baboons from 85 ± 71 to 26 ± 25 pg/mL (time 0 to +120) at DOL5, and from 199 ± 140 to 135 ± 113 pg/mL (time 0 to +120) at DOL14 ($P < .05$ and $P = .05$, respectively). In preterm baboons, plasma glucagon did not decrease after insulin stimulation on DOL5 (30 ± 23 to 24 ± 21 pg/mL, time 0 to +120 respectively); plasma glucagon tended to decrease at DOL14 in preterm baboons (30 ± 35 to 0.5 ± 1.1 pg/mL, time 0 to +120

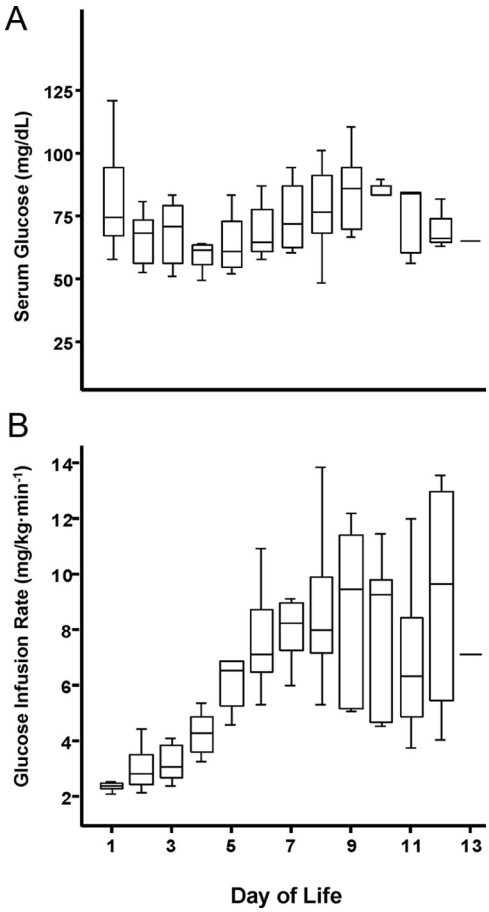

Figure 1. Daily serum glucose concentrations and glucose infusion rates in preterm baboons. Serum glucose measurements are represented as medians with interquartile ranges shown as black lines (A). Daily median glucose infusion rates are expressed as mg/kg/min and compared each day of life with interquartile ranges shown as black lines (B).

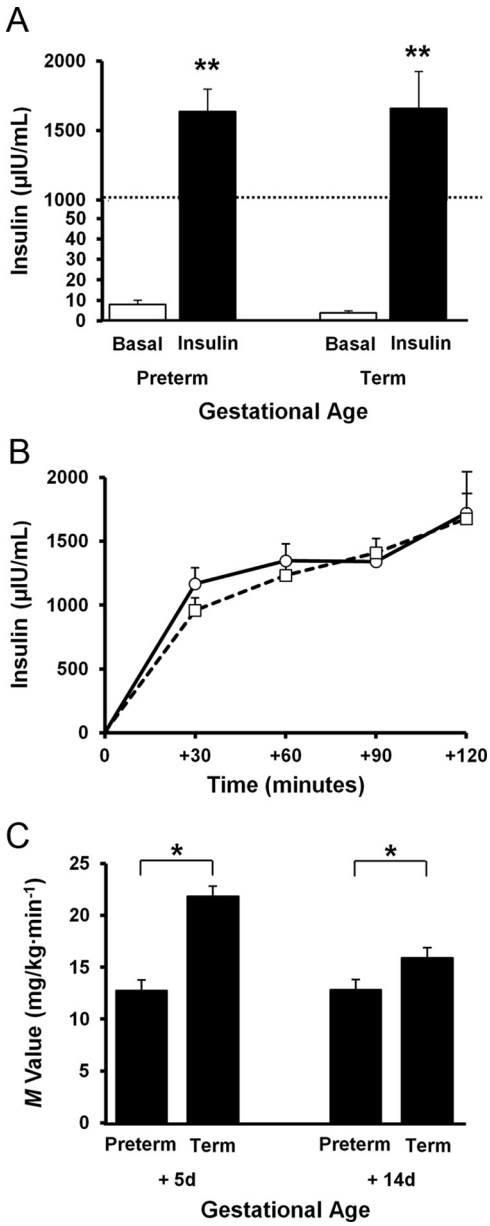

Figure 2. Plasma insulin concentrations during euglycemic hyperinsulinemic clamp and peripheral insulin sensitivity. Serum insulin levels at baseline are shown in white boxes, while insulin levels are shown in dark boxes in both preterm and term baboons. Dotted line is depicted to mark where the graph was superimposed to decrease the height of the bar (A) $**P < .01$. Serum insulin levels are shown at 30 minutes intervals during the 120 minute insulin clamp (B). Preterm baboons are shown with a dotted line and square markers, while term animals are shown with a solid line and circle markers. Error bars show mean +/- 1.0 standard error. Insulin sensitivity (M value) is shown in preterm and term animals at 5 and 14 days of life (C).

respectively, $P = .1$). Plasma epinephrine was similar between preterm and term animals at DOL5 and DOL14 and did not change during the insulin clamp (Figure 4B). Plasma norepinephrine was higher in preterm baboons compared to their term counterparts, and similar to fetal baboons (Figure 4C).

DEVELOPMENTAL DIFFERENCES OF KEY INSULIN SIGNALING MOLECULES IN SKELETAL MUSCLE AND ADIPOSE TISSUE

Muscle Akt content. Tyrosine phosphorylation of Akt (Ser473) increased after insulin stimulation in preterm and term baboons on DOL5 and DOL14 ($P < .05$); however, the response of pAkt to insulin stimulation was significantly lower in preterm baboons on DOL5 with a 3.2-fold increase in preterm animals vs. 9.8-fold increase in term animals (Figure 5A). By DOL14, preterm baboons had similar responses in tyrosine phosphorylation of Akt when compared to term counterparts (Figure 5A). Akt-1 mRNA expression was similar between groups (Figure 5B).

Content of IR-β, IRS-1, and p85 subunit of PI3-kinase. IR-β gene expression tended to be lower in premature animals compared to term animals at DOL5, but failed to reach statistical significance ($P = .08$)(Supplemental Figure). IRS-1 mRNA expression in skeletal muscle under basal and insulin-stimulated conditions was similar between preterm and term animals (Supplemental Figure). The protein content of pIRS-1, IR-β, pIR-β and the p85 subunit of PI3-kinase was similar in the muscle of both, preterm and term animals (N.S, not shown).

Glucose transporters in skeletal muscle. The muscle of preterm baboons had markedly lower GLUT1 protein content at DOL5 (12% of term newborns, $P < .05$) and remained low at DOL14 (8% of term, $P = .04$)(Figure 6A). In contrast, GLUT1 mRNA expression was similar across gestational ages and did not change over time (Figure 6C). GLUT4 protein content was similar between preterm and term animals at DOL5 and was significantly lower in term animals at DOL14 (Figure 6B). GLUT4 gene expression was similar between groups (Figure 6D).

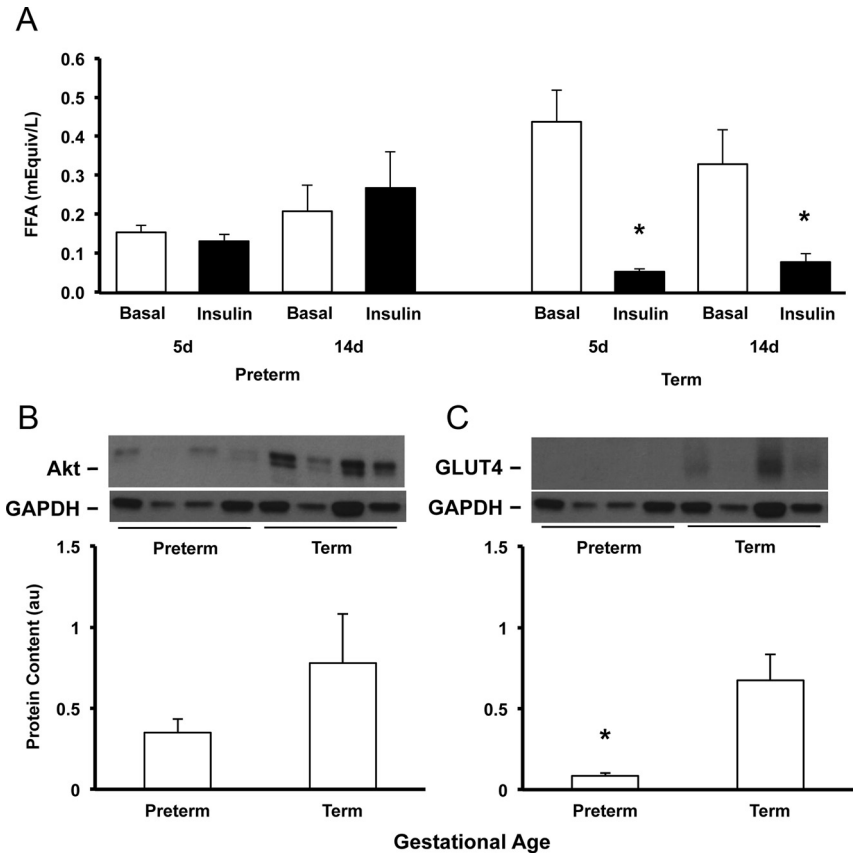

Figure 3. Developmental differences in adipose tissue. Serum concentrations of free fatty acids (FFA) of preterm and term animals at baseline are shown in white boxes, while response of FFA to insulin are shown in dark boxes at 5 and 14 days of life (A). Protein content and gene expression of Akt (B) and GLUT4 (C) were measured by Western blotting and in preterm and term baboons at necropsy. Representative blots from each group are also shown. Data are means ± SE. *$P < .05$. d=day.

Content of Akt, IRS-1 and GLUT4 in Adipose Tissue. Total Akt tended to be lower in preterm baboons when compared the term counterparts, but failed to reach statistical significance ($P = .2$, Figure 3B). Tyrosine phosphorylation of Akt (Ser473), pIRS-1 and IRS-1 were almost undetectable in adipose tissue in both, preterm and term animals. The adipose tissue of preterm baboons had markedly lower GLUT4 protein content at DOL14 as compared to term baboons (Figure 3C).

Discussion

Premature infants are the largest clinical population in the neonatal intensive care unit (NICU) and hyperglycemia remains a prevalent condition increasing their risk of brain injury and death (8, 12). To further complicate their outcomes, young adults born preterm are at high risk for abnormal glucose metabolism with decreased β-cell function (31) and increased incidence of type 1 and 2 diabetes. There is a large

amount of evidence of developmental programming where metabolic disturbances early in life might have long lasting consequences (31–33). Therefore, it is important to understand the biological basis of abnormal glucose homeostasis in the neonatal period.

In this study, we demonstrated marked peripheral insulin resistance in muscle and adipose tissue of preterm baboons compared to term baboons soon after birth. This insulin resistance was accompanied with alterations in the insulin signaling pathway in the muscle of preterm baboons during the first 14 days of postnatal life. Insulin resistance was evidenced by a significantly lower *M* value in preterm baboons under maximal insulin stimulation during the first 2 weeks of life as compared to their term counterparts. This insulin resistance did not improve from the first to the second week of life evidenced by an almost identical *M* value at DOL5 and DOL14. We chose a high insulin infusion rate to ensure that hepatic glucose production would be completely suppressed (30) and, thus, the glucose infusion rate reflects glucose uptake by peripheral tissues.

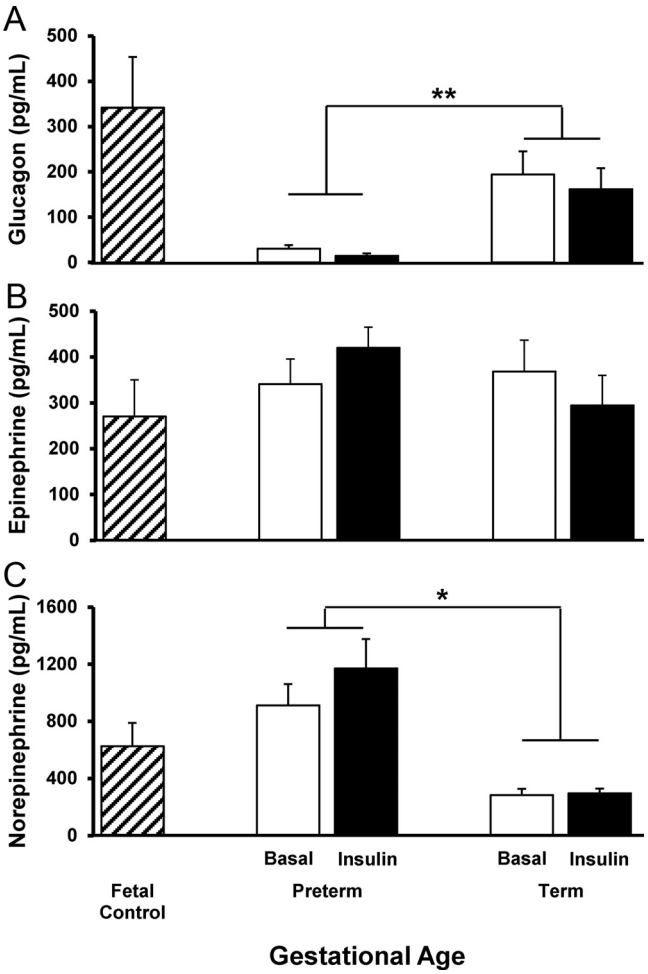

Figure 4. Counterregulatory hormones. Plasma concentrations of glucagon (A), epinephrine (B) and norepinephrine (C) in fetal control, preterm and term baboons are depicted. Fetal controls are shown in boxes with diagonal lines, basal concentrations are shown in white boxes, and concentrations during the insulin clamp are shown in dark boxes. Data are means ± SE. $*P < .05$, $**P < .01$.

To further elucidate the peripheral insulin resistance seen in preterm animals, we investigated the insulin-signaling cascade. In skeletal muscle, Akt is a key mediator of insulin-stimulated glucose disposal and cell growth (34). In both preterm and term baboons insulin increased Akt phosphorylation, but these responses varied depending on gestational age. The pAkt fold increase was significantly lower in preterm vs term baboons during the first week of life (3.2-fold vs 9.8-fold) which is consistent with the strikingly decreased peripheral insulin sensitivity in preterm baboons during the first week of life. During the second week of life, insulin sensitivity remained the same in preterm infants, but term infants tended to have a decrease in insulin sensitivity but remained higher than in preterms and this difference was still significant. The lack of differences at the molecular level (pAkt in particular) during the second week of life might be due to other insulin signaling molecules not measured playing a role during the second week of life. Akt exists as several isoforms. In muscle, the most abundant isoforms are Akt-1 and Akt-2 (35). No differences were found in Akt-1 mRNA expression between preterm and term animals. Unfortunately, due to limited amount of tissues available in these small animals, Akt-2 was not quantified.

Along with the reductions in pAkt responses, we found a tendency for a lower gene expression of IR-β in the muscle of preterm baboons during the first week of life but then normalized to the mRNA expression found in their term counterparts during the second week of life. This mild decrease in IR-β mRNA in skeletal muscle might contribute to the insulin resistance of prematurity but no differences were found in protein expression nor in IRS-1 or PI3K. We previously demonstrated that preterm infants had a significant increase in IR-β immediately after birth compared to term counterparts (26). The lack of consistency of these results might be explained by high variability of insulin signaling molecules suggesting a need of a larger sample size but due to the expensive nature of nonhuman primates, additional animal experiments were not performed. In this study, we demonstrated reduced GLUT1 protein content in premature baboons compared to term baboons. These changes are consistent with our previous studies, in which we found a significant decrease of GLUT1 protein content in immature fetal baboons (26). GLUT1 is responsible for the constitutive, insulin-independent glucose transport that takes place in all cells, including muscle (36). GLUT1 overexpression results in a three-to-four fold increase in basal glucose transport in muscle ex vivo and improves glucose tolerance (37), sug-

gesting that GLUT1 also plays an important role in maintaining whole-body glucose homeostasis. Therefore, the significant decrease in GLUT1 protein in the muscle of preterm baboons may contribute to the impairment in noninsulin dependent glucose metabolism. Gene expression remained unchanged, which is not uncommon as correlations could be low due to variable rates of mRNA transcription and protein translation.

Muscle GLUT4 protein content was similar in preterm and term animals during the first week of life and higher in preterm baboons by the second week of life. GLUT4 is the main glucose transporter involved in postprandial glucose transport, when circulating insulin concentrations are the highest (36). Studies in fetal sheep have shown transient increases and decreases of glucose transporters in insulin sensitive and insulin insensitive tissues in response to hyperglycemia and hyperinsulinemia (24). These responses are variable depending on the type of exposure and tissue exposed (24). We speculate that preterm infants may develop alterations in insulin signaling molecules as a result of developmental disruption of fetal glucose metabolism. Perhaps the increases in the expression of GLUT4 by the second week of life in preterm baboons may be a neonatal adaptation in an attempt to improve insulin sensitivity. On the other hand, it could be due to tissue responses to the increasing fasting insulin levels observed in preterm baboons by 14 days of age (Table 2). Our findings indicate that alterations in glucose transporters differ in skeletal muscle of preterm and term baboons, and this may explain the gestational differences in glucose homeostasis during early postnatal life.

To our knowledge, this is the first study to demonstrate insulin resistance in a preterm animal model along with impairments of insulin signaling in the skeletal muscle and we were able to perform two insulin clamps longitudinally in an extremely preterm animal (67% gestation). Human studies are not possible since muscle biopsies cannot ethically be performed in the neonatal period. Although sheep or mice could be used, no other preterm animal at borderline viability can survive for greater than 48 hours days like the baboon.

Our group recently demonstrated that hyperglycemia is common in the preterm baboon and increases their risk of death (27). In that study, 91% of preterm baboons developed hyperglycemia and had poor glucose control. In this study, we had a tight glucose control from birth by limiting the glucose infusion using a sliding scale to target serum glucose instead of targeting growth. In human premature infants this study could not be possible as glucose infusion is increased to increase caloric intake regardless of serum glucose level (unless is $>$ 150 mg/dL) and intralipids are given from birth on. Therefore, in this study under tight normoglycemic conditions, hyperglycemia does not contribute to the insulin resistance observed. Furthermore, lipid emulsions were not infused since they are known to cause insulin resistance. Preterm baboons had similar insulin levels to term baboons at birth; therefore, hyperinsulinemia could not be responsible for the insulin resistance observed but rather, developed overtime as evidenced by the increases in fasting insulin levels by DOL14 in preterm animals. Of note, there were not deaths in our preterm

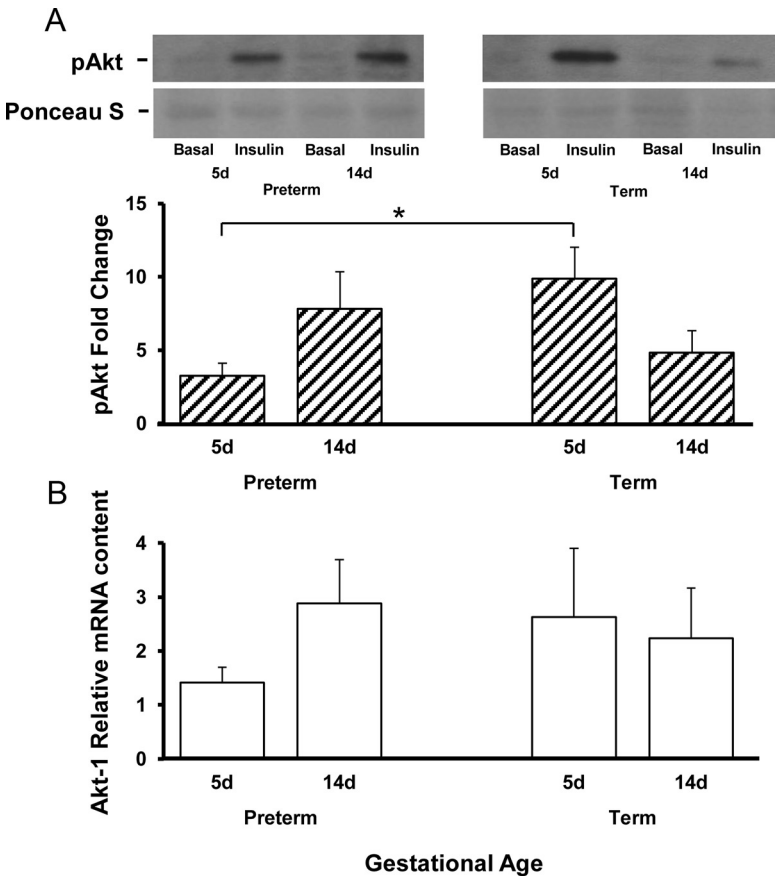

Figure 5. Developmental differences in pAkt and AKt-1 in skeletal muscle. Fold change in protein content of pAKt (A) in preterm and term animals at 5 and 14 days of life were measured by Western blotting and qRT-PCR respectively. Akt-1 gene expression is shown at 5 and 14 days of life (B). Representative blots are also shown. d=day. Data are means ± SE. *$P <$ .05.

baboons in the present study as compared to the high risk of death observed in our previous published studies in hyperglycemic baboons (27). We attribute this to the tight glucose control during the early prenatal period and believe this observation has important implications for the management of human premature infants.

It is possible that counterregulatory hormone surges (cathecolamines, glucagon), at the time of birth may cause insulin resistance and worsen hyperglycemia in premature infants. Epinephrine levels were not increased compared to term infants, but norepinephrine levels were higher in preterm baboons. However, norepinephrine, unlike epinephrine, infusions have been shown not to adversely affect insulin sensitivity in humans (38). Additionally, previously published plasma levels of norepinephrine in the chronically ventilated preterm baboon model peaked at ~7,000 pg/mL at 24 hours of age, suggesting that our experiments were performed after norepinephrine had peaked and gone back to the baseline levels seen in fetal preterm baboons of ~1,000 pg/mL (39). Furthermore, the serum glucagon concentration was significantly lower in preterm baboons throughout the first two weeks of life during fasting and during the clamp compared to term baboons, indicating that preterm baboons had a significant impairment in glucagon secretion. Therefore, hyperglucagonemia cannot contribute to the insulin resistance in the present study or the development of hyperglycemia. Our group has found the presence of pluripotential pancreatic cells in preterm fetal baboons (40). These maturational differences may have long-term consequences when born preterm as we have now seen an increased incidence of type 1 diabetes in preterm infants (32).

Lastly, we found insulin resistance in the adipose tissue, as demonstrated by impaired suppression of free fatty acids after maximal insulin stimulation in preterm baboons (Figure 3A). This was paired with decreased GLUT4 protein content in adipose tissue (Figure 3C) in preterm baboons. We need to further elucidate if these alterations in GLUT4 protein content are reversible and if they persist into adulthood. Insulin resistance in type 2 diabetes is in part, due to impaired stimulation of GLUT4 translocation and trafficking in the adipose tissue and has been a target for therapeutic interventions to improve insulin sensitivity (41). In premature infants, the therapeutic options available to improve glucose control in the neonatal period are limited to insulin. Understanding the specific mechanisms for insulin resistance of prematurity may improve glucose control in the neonatal period with a long-term goal of

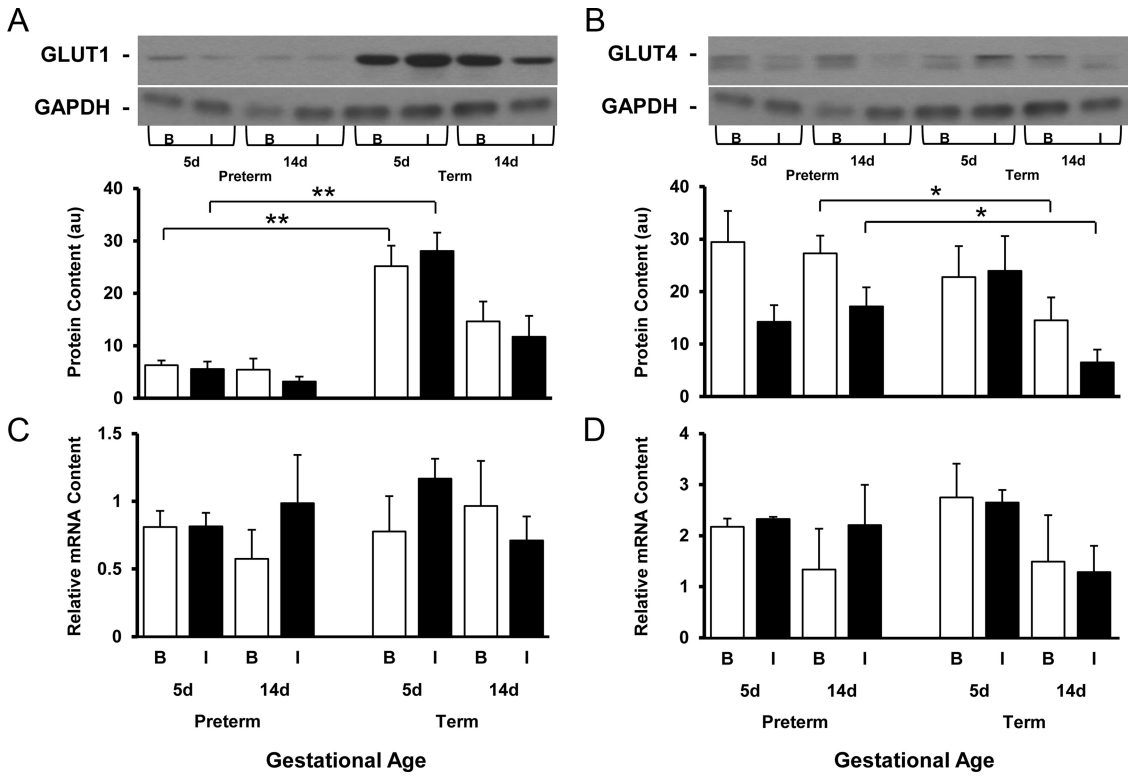

Figure 6. Protein content and gene expression of key glucose transporters in skeletal muscle. Protein content and gene expression of GLUT1 and GLUT4 were measured by Western blotting (A,B) and qRT-PCR (C,D) in preterm and term baboons at 5 and 14 days of life. Data from muscle samples taken at baseline are shown in white boxes, while those samples taken under insulin stimulation are shown in dark boxes in both preterm and term baboons. Representative blots from each group are also shown. GAPDH served as a loading control. d=day. Data are means ± SE. $*P < .05$, $**P < .01$.

decreasing the emergence of type 2 diabetes that appears early in life in surviving adults born preterm.

The protein content of Akt in adipose tissue tended to be lower in preterm baboons but failed to reach significance (Figure 3B); this was likely due to the small sample size, as the differences seemed significant to the naked eye. These alterations in insulin signaling molecules may contribute to the peripheral insulin resistance of the adipose tissue found in preterm baboons. The alterations of adipose tissue are consistent with findings in human adipocytes of diabetic adults who have significant reductions in the protein content of Akt (42). Furthermore, Akt and IRS-1 have been shown to be altered in adipose tissue of adult rats with altered fetal growth, which in humans, has been shown to be associated to metabolic syndrome (43). A limitation to this study was the lack of adipose tissue collection during insulin stimulation; as expected, there was a lack of detection of phosphorylated Akt and IRS-1 since adipose tissue was collected after necropsy and therefore, was no longer under maximal insulin stimulated conditions. Additional studies utilizing insulin stimulated adipose tissue responses are needed but due to limited amount of adipose tissue in the neonatal period, those studies will need to be performed later in life.

These findings are of extreme importance, since infants born prematurely are at high risk for diabetes and cardiovascular disease (44, 45). Furthermore, type 2 diabetes in children is more aggressive than in adults, with signs of kidney disease and hypertension just a few years after diagnosis (46). We postulate interventions that improve insulin resistance and glucose control in premature infants may lead to improved cardiovascular outcomes and decreased incidence of diabetes and obesity later in life. It is, therefore, of utmost importance to understand developmental differences between premature and term infants in order to prevent these long-term sequelae.

In conclusion, peripheral insulin resistance and impaired noninsulin dependent glucose disposal play an important role in prematurity. Impaired insulin signaling in both, muscle and adipose tissue contribute to the defect in insulin-stimulated glucose disposal. Counter-regulatory hormones are not major contributors to the insulin resistance and hyperglycemia of prematurity. Understanding the underlying mechanisms responsible for the development of insulin resistance and hyperglycemia in the neonatal period likely will identify novel interventions to ameliorate the insulin resistance and correct hyperglycemia in premature infants and requires further investigation.

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