Antenatal corticosteroids alter insulin signaling pathways in fetal baboon skeletal muscle

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Abstract

We hypothesize that prenatal exposure to glucocorticoids (GCs) negatively alters the insulin signal transduction pathway and has differing effects on the fetus according to gestational age (GA) at exposure. Twenty-three fetal baboons were delivered from 23 healthy, nondiabetic mothers. Twelve preterm (0.67 GA) and 11 near-term (0.95 GA) baboons were killed immediately after delivery. Half of the pregnant baboons at each gestation received two doses of i.m. betamethasone 24 h apart (170 μg/kg) before delivery, while the other half received no intervention. Vastus lateralis muscle was obtained from postnatal animals to measure the protein content and gene expression of insulin receptor β (IRβ; INSr), IRβ Tyr 1361 phosphorylation (pIRβ), IR substrate 1 (IRS1), IRS1 tyrosine phosphorylation (pIRS1), p85 subunit of PI3-kinase, AKT (protein kinase B), phospho-AKT Ser473 (pAKT), AKT1, AKT2, and glucose transporters (GLUT1 and GLUT4). Skeletal muscle from preterm baboons exposed to GCs had markedly reduced protein content of AKT and AKT1 (respectively, 73 and 72% from 0.67 GA control, \( P < 0.001 \)); IRβ and pIRβ were also decreased (respectively, 94 and 85%, \( P < 0.01 \)) in the muscle of premature GC-exposed fetuses but not in term fetuses. GLUT1 and GLUT4 tended to increase with GC exposure in preterm animals (\( P = 0.09 \)), while GLUT4 increased sixfold in term animals after exposure to GC (\( P < 0.05 \)). In conclusion, exposure to a single course of antenatal GCs during fetal life alters the insulin signaling pathway in fetal muscle in a manner dependent on the stage of gestation.

Key Words

- insulin
- fetus
- primate
- muscle
- glucocorticoid
- corticosteroid
- preterm

Introduction

Antenatal corticosteroid administration to pregnant women has become the standard of care for mothers at a risk of delivering a premature infant. Maternal treatment with synthetic glucocorticoids (GCs) decreases the incidence of many morbidities associated with prematurity, including respiratory distress syndrome and intraventricular hemorrhage (‘Effect of corticosteroids for fetal maturation on perinatal outcomes. NIH Consensus Development Panel on the Effect of Corticosteroids for Fetal Maturation on Perinatal Outcomes’ 1995, Brownfoot et al. 2008). The short-term adverse effects seen with postnatal administration of steroids include hypertension, hyperglycemia, and growth
restricted (Ng 1993). Furthermore, animal and human studies have shown that use of GC during the fetal period may increase the likelihood of acquiring adult diseases earlier in life, more specifically hypertension, coronary heart disease, and diabetes (Newnham 2001, Mizuno et al. 2013). Moreover, GC exposure during pregnancy impacts placental growth factors and insulin signaling pathways during late gestation (Ain et al. 2005, Jellyman et al. 2012). In the fetal sheep, glucose transporter 4 (GLUT4) was increased in skeletal muscle when GCs were administered in late gestation, whereas AKT (protein kinase B) and mTOR pathway molecules were only increased in the muscle of those fetuses exposed to cortisol infusion but not GCs (Jellyman et al. 2012).

Extremely low birth weight (ELBW) infants (<1000 g birth weight) have a high incidence of hyperglycemia (Blanco et al. 2006, Beardsall et al. 2010, Liechty 2010), relative insulin resistance, and defective insulin processing; however, the molecular basis for their impaired glucose metabolism remains unclear (Mitanchez-Mokhtari et al. 2004). In human fetuses, insulin promotes skeletal muscle protein synthesis and growth, which peak by the second trimester, and both synthesis and growth decrease progressively thereafter. Variations in insulin concentrations during intrauterine life contribute to differences in fetal growth (Fowden et al. 1989, Philipps et al. 1991, Schwartz & Teramo 2000). Insulin must bind to its receptor and initiate the insulin-signaling cascade through the insulin receptor (IR) and IR substrate 1 (IRS1), which is followed by activation of AKT. Insulin-responsive tissues (i.e. skeletal muscle) are enriched in AKT, which is considered as a key mediator of both cell growth and metabolism (Calera et al. 1998, Cho et al. 2001). We have previously demonstrated a significantly increased content of IR, total AKT, and AKT isoforms in the fetal skeletal muscle of 125-day gestational age (GA) preterm baboons (0.67 gestation) when compared with term baboons (Blanco et al. 2010). These increased protein levels in immature animals are likely related to the accelerated growth that occurs in mid-gestation compared with the period just prior to delivery when differentiation of organs for postnatal function takes precedence over growth and proliferation. Previous studies have shown decreased levels of GLUT1 and GLUT4 in the skeletal muscle of fetal preterm baboons (Blanco et al. 2010); these differences might be developmentally as glucose homeostasis is controlled by passive diffusion through the placenta during fetal life, and insulin-stimulated glucose disposal is decreased in utero. Others have shown perturbations in fetal skeletal muscle GLUTs in sheep after a single dose of GCs, along with fetal hyperglycemia and hyperinsulinemia (Gray et al. 2006). Therefore, information is needed on the precise change in insulin function and signaling at the muscle cell level, resulting from fetal exposure to concentrations of corticosteroids in excess of those appropriate for the current stage of maturation.

Our previous studies have shown that the baboon is a valuable animal model for studying insulin signaling. Baboons have 97% phylogenetic proximity with humans and spontaneously develop insulin resistance when obese (Chavez et al. 2008, Blanco et al. 2013). In addition, baboons develop common complications pertinent to preterm infants, such as glucose abnormalities, bronchopulmonary dysplasia, and patent ductus arteriosus (Escobedo et al. 1982, McCurnin & Clyman 2008). Preterm baboons also have an incidence of hyperglycemia comparable with ELBW neonates and heightened differences in the insulin signaling pathway when compared with term primates (Blanco et al. 2010, 2013).

We hypothesize that prenatal exposure to GCs reduces the content of key signaling molecules in the insulin signal transduction pathway in a manner dependent on the stage of gestation, with differing effects on preterm baboons and term baboons. In this study, we examined the effects of antenatal GC exposure on the insulin signaling proteins of skeletal muscle in fetal baboons delivered extremely premature and at term.

Materials and methods

The study was conducted at the University of Texas Health Science Center (UTHSCSA) in San Antonio, Texas, USA.

Animal maintenance

All animals were obtained from the Southwest National Primate Research Center at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX, USA. Studies were approved by the Institutional Animal Care Committee at the SFBR and conducted in accordance with standard, humane animal care. Twenty-three fetal baboons (Papio hamadryas, 18 males and five females) were delivered prematurely at 125-day GA (n = 12) or 175-day–190-day GA (n = 11); full term is 185-day GA) under general anesthesia via C-section, as described in detail previously (Escobedo et al. 1982, McCurnin & Clyman 2008), or vaginally (term animals only) from 23 healthy, nondiabetic mothers. Half of the pregnant animals at each of the two designated stages of gestation received no intervention (controls (CTR)) and half received two i.m. betamethasone doses (170 µg/kg) 24 h apart, with the second dose 24 h before...
delivery. On the basis of our previous findings, GLUT1 protein content in the skeletal muscle of preterm baboons increases from 33 to 99% when compared with term baboons, with a similar increase after GC exposure in preterm baboons, a 5% significance level and 80% power, a sample size of five animals per group was calculated.

The animals were killed shortly after birth with 1 ml/10 lb of i.v. pentobarbital, followed by exsanguination. Vastus lateralis muscle samples were immediately obtained, and tissues were promptly snap-frozen in liquid nitrogen and stored at −80 °C.

Measurement of insulin signaling and GLUT proteins by western blot analysis and immunoprecipitation

Primary antibodies against IRβ (ISNR), pIRβ, and AKT1 were purchased from Santa Cruz Biotechnology; IRS1, p85 subunit of PI3-kinase (p85), AKT2, GLUT1, and GLUT4 were from Millipore (Chicago, IL, USA); and GAPDH, pIRβ, phospho-Akt Ser473 (pAkt), and total AKT were obtained from Cell Signaling Technology (Danvers, MA, USA). Insulin signaling molecules were measured in the skeletal muscle using our previously described protocol (Blanco et al. 2010). The intensities of the bands were quantified by densitometry using the NIH imaging program with the results reported in arbitrary optical density units. GAPDH and/or Ponceau S from Thermo Fisher Scientific (Walthman, MA, USA) were used as loading controls. For measurement of IRS1 tyrosine phosphorylation (pIRS1), proteins were immunoprecipitated with anti-IRS1 antibody followed by incubation with protein A agarose beads as described previously (Blanco et al. 2010). The gels were normalized using internal controls to ensure comparable gel-to-gel data analysis across groups.

Statistical analysis

Statistical calculations and demographic distributions were carried out with SPSS for Microsoft Windows (version 17.0, SPSS, Inc.). Differences between groups were determined using two-way ANOVA, followed by the Tukey’s test. A 5% significance level and 80% power, a sample size of five animals per group was calculated. Differences between groups were determined using two-way ANOVA, followed by the Tukey’s test. P < 0.05 was considered to be statistically significant.

Results

Animal characteristics

The 23 animals were delivered at two different gestations (125- and 185-day GA). The characteristics of animal are shown in Table 1.

Table 1 Characteristics of fetal baboons used to investigate developmental differences of key insulin signaling proteins in skeletal muscle

<table>
<thead>
<tr>
<th>Group</th>
<th>Gestation (%) of term</th>
<th>n</th>
<th>Male/female</th>
<th>Birth weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>125-day CTR</td>
<td>67</td>
<td>6</td>
<td>4/2</td>
<td>407 ± 47</td>
</tr>
<tr>
<td>125-day GC</td>
<td>67</td>
<td>6</td>
<td>5/1</td>
<td>352 ± 26</td>
</tr>
<tr>
<td>Term CTR</td>
<td>94</td>
<td>6</td>
<td>5/1</td>
<td>1008 ± 132</td>
</tr>
<tr>
<td>Term GC</td>
<td>94</td>
<td>5</td>
<td>4/1</td>
<td>1120 ± 134</td>
</tr>
</tbody>
</table>

CTR, control; GC, glucocorticoid.

Content of IRβ, IRS1, and p85

Figure 1A shows that the protein content of IRβ in the muscle of preterm fetal baboons exposed to GCs was decreased by 94% compared with the levels in preterm fetal baboons not exposed to GCs and was 93% less than term baboons exposed to GCs (P < 0.001). No change in protein content was seen in term fetuses following GC exposure. Consistent with this finding, pIRβ was also decreased (by 85%) in the muscle of premature GC-exposed fetuses but not term fetuses (P < 0.01) (Fig. 1B). Muscle protein content of IRS1 and pIRS1 was similar across GA regardless of antenatal GC exposure (Fig. 1C and D). p85 was twofold higher in term baboons exposed to GCs, as compared with preterm baboons exposed to GC (Fig. 1E).

Muscle AKT content

Baseline total AKT protein content was 54% greater in preterm fetal baboons vs term fetal baboons (P < 0.01). When exposed to GCs, preterm baboons had a 73% decrease in AKT content (P < 0.001), whereas no change was observed after GC administration in term animals (Fig. 2A). In line with AKT protein content, pAkt was 4.5-fold higher in the muscle of preterm control fetuses compared with term controls (P < 0.05). After GC exposure, protein content was decreased by 27% in preterm but not in term fetuses; however, it failed to reach statistical significance (P = 0.2; Fig. 2B). Figure 2C shows that the decrease in AKT1 observed in preterm animals exposed to GCs parallels the responses to GCs seen in total AKT and pAkt protein content. AKT2 was 2.6-fold higher in immature fetal control baboons when compared with term controls; after GC exposure it tended to increase in preterm baboons (P = 0.1), whereas no effect was seen after GC exposure in term baboons (Fig. 2D).
GLUT content in muscle

In contrast to IRβ, IRS1, and AKT, the muscle of 125-day GA fetal baboons had markedly lower GLUT1 protein content compared with term fetuses \((P<0.001; \text{Fig. 3A})\). There was a trend toward increased GLUT1 after GC exposure \((P=0.09)\) in the preterm fetuses, whereas no changes were seen in term fetuses exposed to GCs. GLUT4 protein content increased in term fetuses by sixfold after GC exposure \((P<0.05)\), whereas only an increasing trend was observed in preterm fetuses after exposure to GCs (Fig. 3B).

Discussion

Treatment with GCs is widely used on women with threatening premature delivery to reduce major neonatal morbidities. However, there are minimal data to assist in the determination of the appropriate dose, type, route, and potential differences in responses at different stages of fetal life. As has been with all powerful therapies, GCs can have short-term adverse effects in the fetal and postnatal period that can lead to alterations in utero, resulting in life-long sequelae (Braun et al. 2009, Drake et al. 2011, Long et al. 2013a,b). The purpose of the present study was to examine the effects of fetal exposure to GCs on insulin signaling pathways in the skeletal muscle of preterm fetal baboons compared with term fetal baboons. Although antenatal GCs are not currently given in term pregnancies, they are given up to 85% of gestation. The use of a well-established nonhuman primate model of fetal development aids in translation to human fetal development.

We found significant differences in the effects of a single course of GCs on key insulin signaling and GLUTs in the muscle of fetal baboons. Importantly, some proximal signaling proteins in muscle are affected differently depending on GA at GC exposure. The most striking observation was the 94% of reduction in the protein content of IRβ and 73% of reduction in AKT protein content that occurred after a single course of GCs in preterm baboons, in contrast to the lack of any effect in term baboons. AKT and IR play other roles besides insulin signaling. During embryonic development, insulin-like growth factors (IGFs) promote growth via two receptors, IR and the IGF1 (Louvi et al. 1997). In human fetuses, variations in insulin concentrations contribute to alterations in fetal growth. Elevated insulin concentrations during uncontrolled maternal diabetes promote fetal...
Glucocorticoids (GCs) can affect glucose metabolism in preterm baboons. Overgrowth, while isolated fetal insulin deficiency under a stable maternal environment can cause restriction of fetal growth (Fowden 1989, Philipp et al. 1991). Therefore, the significant decrease in IR and AKT content seen in immature animals exposed to one dose of GCs will likely stunt the normal acceleration in fetal growth that occurs during this critical period of development. Impairment of cell growth and proliferation will tend to restrict attainment of normal muscle mass and hence have potential major effects on lifetime function including glucose metabolism. Skeletal muscle is the principal site for glucose utilization, and it is the primary tissue responsible for insulin resistance in obese and type 2 diabetic subjects (Selak et al. 2003, Lowell & Shulman 2005, Ozanne et al. 2005). Whether changes in the skeletal muscle IR and AKT concentrations persist postnatally and translate into insulin resistance later in life remains to be determined.

Insulin-responsive tissues are enriched in AKT, which is thought to be a key mediator of cell growth and metabolism (Kelly et al. 2012). AKT exists as three isoforms. In muscle, the most abundant are AKT1 and AKT2 (Walker et al. 1998, Datta et al. 1999). It has been proposed that AKT1 is implicated in cell growth, whereas AKT2 is thought to play a more important role in the regulation of glucose metabolism (Bae et al. 2003, Brozinick et al. 2003). We found a decrease in AKT1 in preterm animals after exposure to GCs, suggesting that the major effect of GCs is on growth, whereas AKT2 did not change after GC exposure. It is possible that transient changes in insulin signaling protein content in the muscle of preterm baboons could be due to concurrent variations in fetal insulin and/or glucose concentrations after GC exposure (Jellyman et al. 2005, Gray et al. 2006). However, birth weights were similar within gestational groups and published plasma glucose and insulin levels measured at birth were similar in preterm and term control animals (Blanco et al. 2010). A limitation of this study is the lack of plasma glucose and insulin measurement in the GC-treated animals.

**Figure 2**
AKT (protein kinase B) protein content and phosphorylation in muscle from fetal baboons at 0.67 gestation and term baboons. Total AKT (A), phospho-AKT Ser473 (pAkt) (B), AKT1 (C), and AKT2 (D) were measured by western blotting. n=5–6 per group. Representative blots from three animals per group are also shown. CTR, control; GC, glucocorticoid. Graphical data are mean ± S.E.M. *P<0.05, **P<0.01, and ***P<0.001.

**Figure 3**
Glucose transporter protein content in muscle from fetal baboons at 0.67 gestation and term baboons. GLUT1 (A) and GLUT4 (B) protein content. n=5–6 per group. Representative blots from three animals per group are shown. CTR, control; GC, glucocorticoid. Graphical data are mean ± S.E.M. *P<0.05, **P<0.01, and ***P<0.001.
Consistent with our previous studies (Blanco et al. 2010), we observed reduced GLUT1 content in immature baboons compared with term baboons. Both, GLUT1 and GLUT4 tended to increase in preterm animals after a single dose of GCs but did not reach statistical significance, whereas GLUT4 increased in term counterparts. A limitation to our study is that a larger sample size may have led to statistical significance on differences in GLUTs as they had a tendency to increase after exposure to GCs in preterm animals; unfortunately, due to the extremely high expenses for nonhuman primates, the sample size could not be increased.

GLUT4 is the predominant insulin-sensitive transporter in muscle. As GLUT4 responds to insulin by promoting the intracellular transport of glucose, the increases we found in GLUT4 in term animals after GC exposure will likely play an important role in alterations in glucose homeostasis later in life. Similarly, GLUT4 was found to be increased in the skeletal muscle of fetal sheep exposed to dexamethasone in late gestation (Jellyman et al. 2012). Furthermore, fetal hyperglycemia and hyperinsulinemia, along with perturbations in skeletal muscle GLUTs of fetal sheep delivered in late gestation, have been found following a single course of GCs (Gray et al. 2006).

While GLUT4 is the main GLUT involved in post-prandial glucose transport, when circulating insulin concentrations are the highest, GLUT1 is responsible for the constitutive, insulin-independent glucose transport that takes place in all cells, including muscle (Pessin & Bell 1992). GLUT1 overexpression results in a three- to fourfold increase in basal glucose transport in muscle ex vivo and improves glucose tolerance (Marshall et al. 1993), suggesting that GLUT1 also plays an important role in the maintenance of whole-body glucose homeostasis. Therefore, the trends seen only in preterm baboons with increased GLUT1 expression after GC exposure will likely improve glucose homeostasis in premature infants exposed to GCs. Our findings indicate that alterations in GLUTs differ in the skeletal muscle of preterm and term baboons, which may explain the gestational differences in abnormal glucose homeostasis during early-postnatal life.

A pitfall of this study is the larger proportion of males, as there are numerous reports of gender differences in various neonatal disease processes, which may have influenced the results (Long et al. 2013a). It is unlikely that the differences found in insulin signaling between groups are due to gender, as all of the groups had a greater proportion of males with no gender differences between groups.

Recently, human studies showed that young adults born preterm, and exposed to antenatal GCs, had decreased aortic distensibility as well as reduction in β cell function expressed by lower insulin, altered homeostasis model assessment, and higher glucose levels (Kelly et al. 2012). Therefore, it is extremely important to investigate the effects of GCs in GLUTs and insulin signaling pathways during critical periods of development, as this therapy may have long-lasting consequences in multiple tissues.

In this study, we demonstrated that exposure to a single course of GCs during fetal life alters the insulin signaling pathway in the muscle of fetal baboons in a manner dependent on the stage of gestation. The changes in some molecules take place in the same direction as those that occur at term with normal maturation. In several species, endogenous fetal corticosteroids increase as parturition approaches (Fowden 1998). Thus, the exposure to GCs may bring those normal changes forward, stimulating differentiation instead of proliferation and resulting in depressed growth and alterations of signaling pathways. Future studies will be needed to evaluate whether baboons exposed to GCs during fetal life have defects in the insulin-transduction pathways under insulin-stimulated conditions in the immediate postnatal period and later life.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported by grants from the Robert Wood Johnson Foundation (C L B, UTHSCSA CTSA (UL1RR025767; C L B), American Diabetes Association (C L B and N M), the National Institutes of Health (HL52636 to BPD resource center, AG030979 to N M), P51RR13986 for facility support at the Southwest Foundation for Biomedical Research, the San Antonio Nathan Shock Center (N M), the UTHSCSA Executive Research Committee (N M), and the South Texas Health Research Center (N M).

Author contribution statement
C L B, P N, and N M designed the study. D G A and L L M carried out key experiments. C L B, A G M, L L M, D G A, P N, and N M have participated in planning of the work, the interpretation of the results, and the writing of the paper. All authors have approved the manuscript.

Acknowledgements
The authors thank the personnel from the Veterinarian Services at UTHSCSA, the Oklahoma Primate Center, and the Texas Biomedical Research Institute for their dedication and support for this project. Study was conducted at the University of Texas Health Science Center (UTHSCSA) in San Antonio, Texas, USA.
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Received in final form 7 February 2014
Accepted 25 February 2014