Estrogen deprivation in primate pregnancy leads to insulin resistance in offspring

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Abstract

This study tested the hypothesis that estrogen programs mechanisms within the primate fetus that promote insulin sensitivity and glucose homeostasis in offspring. Glucose tolerance tests were performed longitudinally in prepubertal offspring of baboons untreated or treated on days 100 to 165/175 of gestation (term is 184 days) with the aromatase inhibitor letrozole, which decreased fetal estradiol levels by 95%. Basal plasma insulin levels were over two-fold greater in offspring delivered to letrozole-treated than untreated animals. Moreover, the peak 1 min, average of the 1, 3, and 5 min, and area under the curve blood glucose and plasma insulin levels after an i.v. bolus of glucose were greater ($P<0.05$ and $P<0.01$, respectively) in offspring deprived of estrogen *in utero* than in untreated animals and partially or completely restored in letrozole plus estradiol-treated baboons. The value for the homeostasis model assessment of insulin resistance was 2.5-fold greater ($P<0.02$) and quantitative insulin sensitivity check index lower ($P<0.01$) in offspring of letrozole-treated versus untreated animals and returned to almost normal in letrozole plus estradiol-treated animals. The exaggerated rise in glucose and insulin levels after glucose challenge in baboon offspring deprived of estrogen *in utero* indicates that pancreatic beta cells had the capacity to secrete insulin, but that peripheral glucose uptake and/or metabolism were impaired, indicative of insulin resistance and glucose intolerance. We propose that estrogen normally programs mechanisms *in utero* within the developing primate fetus that lead to insulin sensitivity, normal glucose tolerance, and the capacity to metabolize glucose after birth.

Key Words
- estrogen
- insulin
- sensitivity
- offspring
- primate

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Introduction

Estrogen has a fundamentally important role in the adult in regulating pancreatic islet beta-cell integrity and function and consequently the secretion and action of insulin important for the control of metabolism of glucose (reviewed in Liu & Mauvais-Jarvis 2010, Mauvais-Jarvis *et al.* 2013, Gupte *et al.* 2015). Thus, estrogen stimulates proliferation (Choi *et al.* 2005) and protects against apoptosis (LeMay *et al.* 2006) of beta-cells in

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adult rodents and improves glucose-stimulated insulin secretion (Godsland 2005) and decreases the incidence of diabetes (Margolis *et al*. 2004) in postmenopausal women. Indeed, a sex-specific effect exists in which the incidence of diabetes mellitus is lower in women than in men, a benefit that is lost after menopause (Gale & Gillespie 2001, Louet *et al*. 2004, Moran *et al*. 2008, Geer & Shen 2009). Moreover, in several animal models, estradiol at physiological levels inhibits, and androgens stimulate, oxidative stress-induced pancreatic beta-cell glucolipotoxicity and apoptosis (Paik *et al*. 1982, Le May *et al*. 2006, Nadal *et al*. 2009).

In addition to its effects on pancreatic beta-cell integrity and insulin secretion, estrogen also promotes insulin action. Thus, mice lacking estrogen receptor α (ERα (ESR1)) exhibit insulin resistance within skeletal muscle and liver (Bryzgalova *et al*. 2006, Ribas *et al*. 2011, Manrique *et al*. 2012). In humans and rodents, mutation of the aromatase gene results in insulin insensitivity and glucose intolerance (Jones *et al*. 2000, Rochira *et al*. 2000, Takeda *et al*. 2003, Belgorosky *et al*. 2009). ERα is highly expressed in insulin-sensitive target tissues, including skeletal muscle (Deroo & Korach 2006, Heldring *et al*. 2007, Wiik *et al*. 2009). Estradiol stimulates insulin sensitivity and enhances glucose tolerance in skeletal muscle of adult mice (Gao *et al*. 2006, Lundholm *et al*. 2008, Riant *et al*. 2009) and protects ovariectomized mice from high-fat-diet-induced insulin resistance (Camporez *et al*. 2013, Jelenik & Roden 2013). Administration of the ERα agonist propylpyrazoletriyl and estradiol activation of Akt (Vasconsuelo *et al*. 2008, Rogers *et al*. 2009), an essential step in insulin receptor signaling, increases GLUT4 receptor protein transcription and glucose uptake in skeletal muscle of adult rats (Barros *et al*. 2006, 2009, Gorres *et al*. 2011). Estrogen replacement therapy reverses the increased incidence of insulin resistance, which occurs in postmenopausal women (Karjalainen *et al*. 2001, Margolis *et al*. 2004, Manson *et al*. 2013). Estrogen also restores insulin sensitivity and glucose metabolism in ovariectomized rhesus monkeys fed a high-fat diet (Wagner *et al*. 1998).

Although the vast majority of studies of the effects of estrogen on insulin secretion and insulin action have been conducted in the adult, very little is known about the role of the hormonal *milieu in utero* and the mechanisms integral to fetal development that prepare the offspring for controlling insulin secretion and action and glucose homeostasis after birth. We have shown that the baboon provides a superb nonhuman primate translational model for the study of placental, developmental, and perinatal biology (Albrecht & Pepe 1990, Pepe & Albrecht 1995). In the present study, therefore, we used the baboon, and a highly specific aromatase inhibitor, letrozole, was used to suppress placental estrogen production and levels within the fetus during the second half of gestation to test the hypothesis that within the developing fetus estrogen programs mechanisms that promote insulin secretion and action and glucose homeostasis in offspring after birth. Moreover, basal fasting levels of the insulin receptor signaling components within skeletal muscle, where over 80% of total insulin-directed glucose uptake and metabolism occur (DeFronzo *et al*. 1981), were also quantified in the baboon fetus near term to determine the activity of the insulin receptor signaling pathway before delivery of the offspring and postnatal life.

Materials and methods

Animals

Pregnant baboons Female baboons (*Papio anubis*), originally obtained from the Southwest National Primate Research Center, San Antonio, TX, USA were housed individually in large primate cages in air-conditioned rooms with a 12 h light:12 h darkness lighting cycle and fed standard primate chow (Harlan Primate Diet, Madison, WI, USA) twice daily, fresh fruit and vitamins daily, and water *ad libitum*. Female baboons were paired with male baboons for 5 days at mid-cycle and pregnancy was confirmed by ultrasound. Pregnant baboons were then either untreated or treated between days 100 and 165–175 of gestation (term = 184 days) with the aromatase inhibitor letrozole (4,4-[1,2,3-triazol-1-yl-methylene]bis-benzonitrate, Novartis Pharma AG, Basel, Switzerland; 115 µg/kg body weight/day, via maternal s.c. injection in 1.0 mL sesame oil) or with letrozole (115 µg/kg body weight/day) plus estradiol benzoate (beginning at 25 µg/kg on day 100 and increasing to maximum of 115 µg/kg body weight between days 120 and 165–175). Blood samples (2–3 mL) were obtained at 1–3 day intervals during the second half of gestation from a peripheral maternal saphenous vein after brief restraint and sedation with ketamine HCl (10 mg/kg body weight, i.m.) and serum estradiol levels quantified by immulite RIA (Albrecht *et al*. 2000). The use of baboons for this study was approved by the Institutional Animal Care and Use Committees of the University of Maryland School of Medicine and Eastern Virginia Medical School.

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Fetal development On day 165 of gestation, 5 of the fetuses (1 female, 4 males) from untreated baboons and 5 of the fetuses (1 female, 4 males) from letrozole-treated animals were delivered via cesarean section during isoflurane anesthesia, 2 mL blood sample was obtained from the umbilical artery for glucose and insulin assay, and fetuses were immediately killed by an i.v. injection of pentobarbital (100 mg/kg body weight). A section (10×10 mm) of vastus lateralis skeletal muscle was then excised, frozen on dry ice, and stored at −80°C until assayed for insulin signaling molecules.

Postnatal development On days 165–175 of gestation, the remaining offspring from baboon mothers that had been untreated or treated with letrozole ± estradiol were either delivered spontaneously or were anesthetized with isoflurane and after obtaining an umbilical artery blood sample (2 mL) delivered by cesarean section to synchronize the timing of delivery. Baboon newborns that had been untreated (7 females, 3 males) or treated *in utero* with letrozole (3 females, 2 males) or letrozole plus estradiol (2 females, 2 males) were then left with and nursed by their mothers for 8 months at which time they were weaned and placed in pairs in cages immediately adjacent to their respective mothers and fed standard primate chow (Harlan Primate Diet, Madison, WI, USA) twice daily, fresh fruit and vitamins daily, and water *ad libitum*. Every 2–6 months thereafter, baboon offspring were briefly sedated with ketamine HCl (10 mg/kg body weight, i.m.), and body weight and blood pressure (Dinamap Pro 400, GE Medical Systems, Milwaukee, WI, USA) were measured with animals in the supine position. At this time, a blood sample (2–3 mL) was also withdrawn from a peripheral saphenous vein for the purpose of quantifying serum estradiol and testosterone levels by immulite RIA.

Glucose tolerance test

To avoid the potential impact on insulin action/glucose metabolism of the rise in estrogen and testosterone levels associated with the onset of puberty, an i.v. glucose tolerance test was performed, according to the established method of Overkamp and coworkers (Overkamp *et al.* 1997), sequentially, i.e. 3–5 times, on each baboon offspring at 6–12 month intervals between the postnatal ages of 1 and 3¼ years of age, i.e. before puberty which occurs at 3½ years in females and 4½ years of age in males within our baboon colony. The data obtained from the several glucose tolerance tests performed were averaged to yield a single value for each animal. Baboons were fasted overnight and at 08:00 h the following morning sedated with ketamine HCl (initially 5–10 mg/kg body weight, i.m.; then 2 mg/kg body weight, i.v.) and positioned on their left side on a 37°C heating pad. Using a 21-gauge needle, a bolus injection of 0.25 g/kg body weight of dextrose was administered into an antecubital vein at time 0 h. Blood samples (2.5 mL each) were obtained via a sterile catheter (21 gauge) inserted into a peripheral saphenous vein at −2 (i.e. basal fasting), 1, 3, 5, 10, 20, 40, 60, and 90 min before/after dextrose administration.

To determine the potential impact of estrogen deprivation on glucose metabolism in maternal baboons before delivery, an i.v. glucose test was also performed as described above between days 151 and 163 of gestation in ketamine-sedated untreated ($n=4$), letrozole-treated ($n=6$), and letrozole plus estradiol-treated ($n=4$) baboon mothers.

Glucose and insulin assay

Blood glucose levels were determined via an iStat Portable Clinical Analyzer (Model #210003, Abbott Laboratories) on 0.1 mL blood. The remainder of the blood sample was collected into a heparinized tube on ice, centrifuged at 3500***g*** for 15 min and the plasma stored at −20°C for determination of insulin level by solid-phase chemiluminescent immunometric assay via an Immulite System (Siemens Healthcare). The insulin assay employed a monoclonal murine anti-insulin antibody and internal insulin standard curve displayed a sensitivity of 2 µIU/mL and intra- and interassay coefficients of variation of 5.7 and 5.9%, respectively, and exhibited no cross-reactivity with other peptides.

Western immunoblot

Samples of fetal skeletal muscle were homogenized and lysed on ice-cold PBS containing 1% cholic acid, 0.1% SDS, 1 mM EDTA (Sigma-Aldrich), and a protease inhibitor cocktail, essentially as described previously (Zachos *et al.* 2004). Briefly, after determination of protein concentration using the bicinchoninic acid method (Sigma-Aldrich), the samples (50 µg protein) were mixed with 5X Laemmli buffer, heated to 95°C for 5 min, cooled on ice for 2 min, and centrifuged (800***g***). They were then loaded onto 7.5% (IRS-1, AS160) or 10% (Akt/pAkt, GLUT1, GLUT4/pGLUT4) SDS–polyacrylamide gels (PAGE) and electrophoresed using a Bio-Rad

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Mini-Protean electrophoresis chamber (Bio-Rad Laboratories) and SDS–PAGE running buffer comprising 25 mM Tris (pH 8.3), 192 mM glycine, and 0.1% SDS. Proteins were wet-transferred onto an Immobilon-P membrane (Millipore); blocked 1 h at room temperature with 5% BSA in 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.2% Tween-20 buffer (TBST); and then incubated overnight at 4°C with the following primary rabbit polyclonal antibodies diluted in TBST containing 5% BSA: anti-Akt (1:500 dilution, Cell Signaling), anti-pAkt-S^{473} (1:500, Cell Signaling), anti-pAkt-T^{308} (1:500, Abcam), anti-AS160 (1:1,000, Cell Signaling), anti-GLUT1 (1:500, Abcam), anti-pGLUT4-S^{488} (1:1,000, Abcam), anti-IRS-1 (1:5,000, Thermo Fisher), and anti-GAPDH (1:5,000, Abcam), and mouse monoclonal anti-GLUT4 (1:1,000, Abcam). After three washes (6 min) in TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-labeled secondary antibody (Serotec UK) in TBST containing 5% BSA. After washing in TBST, the membranes were developed with enhanced chemiluminescence (GE Healthcare) according to manufacturer's instructions and exposed to Fuji Super RX medical x-ray film (Fujifilm Medical Systems, Inc, Roselle, IL, USA), and band intensities quantified by densitometry using Image J software (National Institutes of Health). The blots were then stripped and reprobed using HRP-conjugated GAPDH as an internal loading control and results, arbitrary densitometric units/μg protein expressed as a ratio to GAPDH. Specificity of the primary antibodies was determined by incubation of samples without primary antibody.

Statistical analysis

Baboons were randomly assigned to the treatment groups and data were expressed as mean ± S.E.M. Data were analyzed by ANOVA with *post hoc* comparison of the means by either Tukey–Kramer multiple comparisons test or Kruskal–Wallis nonparametric test using SAS statistical software (SAS Institute, Cary, NC, USA).

Results

Serum steroid hormone levels

Maternal peripheral saphenous vein serum estradiol levels in untreated baboons increased from a mean ± S.E.M. of 1.0 ± 0.2 ng/mL on day 100 (i.e. midgestation) to 3.6 ± 0.4 ng/mL on days 165/175 of gestation. The administration of letrozole beginning on day 100 resulted in serum estradiol concentrations which rapidly declined within 2 days to and remained at levels of <0.1 ng/mL. Concomitant administration of letrozole and estradiol resulted in a pattern of increasing maternal peripheral serum estradiol levels that was similar to that in untreated animals. Consequently, at the time of delivery on days 165–175 of gestation, serum estradiol concentrations in blood delivered from the fetus (i.e. umbilical artery) of letrozole-treated baboons (46 ± 5 pg/mL) was only 5% of that ($P < 0.001$) in untreated animals (590 ± 72 pg/mL, Fig. 1). Umbilical artery serum estradiol levels in letrozole plus estradiol-treated baboons were increased to a level (133 ± 19 pg/mL) almost three-fold greater ($P < 0.01$) than that in animals treated with letrozole alone, but remained lower ($P < 0.001$) than in untreated animals.

Umbilical artery serum testosterone levels on days 165–175 in letrozole-treated baboons (3.7 ± 0.3 ng/mL) were over three-fold greater ($P < 0.01$) than that in untreated controls (1.1 ± 0.2 ng/mL, Fig. 1). Serum testosterone levels remained elevated in baboons treated with both letrozole and estradiol, because of continued inhibition of aromatization of C_{19} steroid precursors to estrogen in these animals.

Postnatal development

Growth and serum analytes Body weights on days 165–175 of gestation were similar in newborns delivered to untreated, letrozole-treated, and letrozole plus estradiol-treated baboons (Table 1). However, placental weight was

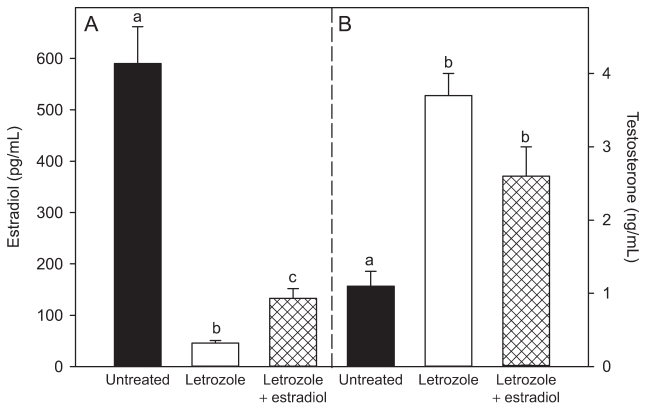

Figure 1
Umbilical artery serum estradiol (A) and testosterone (B) levels (mean ± S.E.M.) on days 165–175 of gestation in baboons untreated or treated on days 100–165/175 with letrozole (115 μg/kg body weight/day via maternal s.c. injection) or letrozole (115 μg/kg body weight) plus estradiol (25–115 μg/kg body weight/day). Data bars marked with different letters are significantly different ($P < 0.01$, ANOVA, Tukey–Kramer multiple comparison statistic) from one another.

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Table 1 Placental weight and newborn body weight and fasting blood glucose and plasma insulin levels in baboons.

Treatment	Placental wt (gm)	Body wt (gm)	Glucose (mg/dL)	Insulin (μIU/mL)
Untreated	183±9	810±27	74±6	3.7±0.3
Letrozole	207±16*	790±62	70±8	4.1±0.4
Letrozole+estradiol	176±12	767±59	75±7	3.9±0.7

Values are expressed as mean±S.E.M. on the day of delivery (days 165–175 of gestation) in newborns from baboons untreated ($n=10$) or treated on days 100–165/175 of gestation (term=184 days) with letrozole (115 μg/kg body weight/day via maternal s.c. injection, $n=5$) or letrozole (115 μg/kg body weight) plus estradiol (25–115 μg/kg body weight/day, $n=4$).
*$P<0.05$ vs untreated.

approximately 10% greater ($P<0.05$) in letrozole-treated than that in untreated animals and returned to normal by letrozole plus estrogen treatment (Table 1).

The body weights of baboon offspring increased ($P<0.01$) progressively throughout postnatal life, reaching levels at 3 years of age that were similar in animals untreated and treated *in utero* with letrozole or letrozole plus estrogen (Table 2). Corresponding with the normal rate of growth, mean arterial blood pressure and levels of serum chemistry analytes, including AST, creatinine, triglycerides, and cholesterol, were similar at birth, throughout postnatal maturation, and at 3 years of age in offspring delivered to baboons untreated or treated during the second half of gestation with letrozole or letrozole plus estradiol (Table 2).

Serum estradiol levels in female baboon offspring that had been untreated or treated *in utero* with letrozole or letrozole plus estradiol were very low (i.e. 15–30 pg/mL) throughout the first 3½ years of postnatal life. Serum testosterone also remained at basal levels (i.e. nondetectable at <0.02 ng/mL) during the first 3½ years of postnatal life in male baboon offspring untreated or treated prenatally with letrozole or letrozole plus estradiol.

Glucose and insulin metabolic parameters in offspring Basal blood glucose and plasma insulin levels at the time of delivery near term were not significantly different in newborns delivered to baboons untreated (74±6 mg/dL and 3.7±0.3 μIU/mL, respectively) or treated *in utero* with letrozole or letrozole plus estradiol (Table 1). Basal levels of glucose immediately before the glucose (dextrose) tolerance test administered postnatally were also not significantly different in offspring from baboons untreated (67±3 mg/dL) or treated throughout the second half of gestation with letrozole (69±2 mg/dL) or with letrozole plus estradiol (69±2 mg/dL). However, basal insulin levels were over two-fold greater ($P=0.06$) in offspring treated prenatally with letrozole (12.3±3.6 μIU/mL) than in untreated animals (5.2±1.0 μIU/mL) and restored after letrozole plus estradiol treatment (6.9±1.9 μIU/mL).

The patterns of glucose and insulin levels during the glucose challenge test in offspring delivered to untreated, letrozole-treated, and letrozole plus estradiol-treated baboons are shown in Fig. 2. Within 1 min of a bolus i.v. injection of dextrose, blood glucose and plasma insulin increased to peak levels, then rapidly declined and were restored within 60–90 min to prechallenge levels. However, in baboon offspring treated *in utero* with letrozole, the peak 1 min postchallenge levels of glucose (210±3 mg/dL, Fig. 3A) and insulin (113.8±18.7 μIU/mL, Fig. 4A) were greater ($P<0.05$ and $P<0.01$, respectively) than in untreated animals (188±6 mg/dL and 47.4±7.4 μIU/mL, respectively), and completely (glucose) or partially (insulin) returned to normal in baboons treated *in utero* with letrozole plus estradiol (192±3 mg/dL and 78.9±9.7 μIU/mL, respectively). Moreover, the net elevations (i.e. 1 min peak minus baseline) in glucose (Fig. 3B) and insulin (Fig. 4B) levels also were greater ($P=0.08$ and $P<0.01$, respectively) in letrozole-treated (141±4 mg/dL and 101.4±16.8 μIU/mL) than in untreated (122±6 mg/dL and 42.3±7.6 μIU/mg/dL) offspring and completely (glucose) or partially (insulin) restored in letrozole plus estradiol-treated animals (123±5 mg/dL and 71.9±11.4 μIU/mL).

When the 1, 3, and 5 min levels in glucose and insulin were averaged, the overall mean levels of glucose (183±4 mg/dL) and insulin (77.7±13.6 μIU/mL) were

Table 2 Body weight, blood pressure, and serum chemistry analytes in baboon offspring.

Treatment	Body weight (kg)	MABP (mmHg)	AST (U/L)	Creatinine (mg/dL)	Triglycerides (mg/dL)	Cholesterol (mg/dL)
Untreated	9.6±0.1	63±9	34.4±7.6	1.4±0.2	52.1±5.0	108±10.1
Letrozole	9.9±0.2	60±10	37.8±5.1	1.3±0.1	53.5±6.8	112±9.2
Letrozole+estradiol	9.6±0.1	62±8	32.7±4.2	1.5±0.3	51.7±4.9	109±7.2

Values are expressed as mean±S.E.M. at 3 years of age in offspring delivered to baboons untreated ($n=10$) or treated on days 100–165/175 of gestation with letrozole ($n=5$) or letrozole+estradiol ($n=4$).

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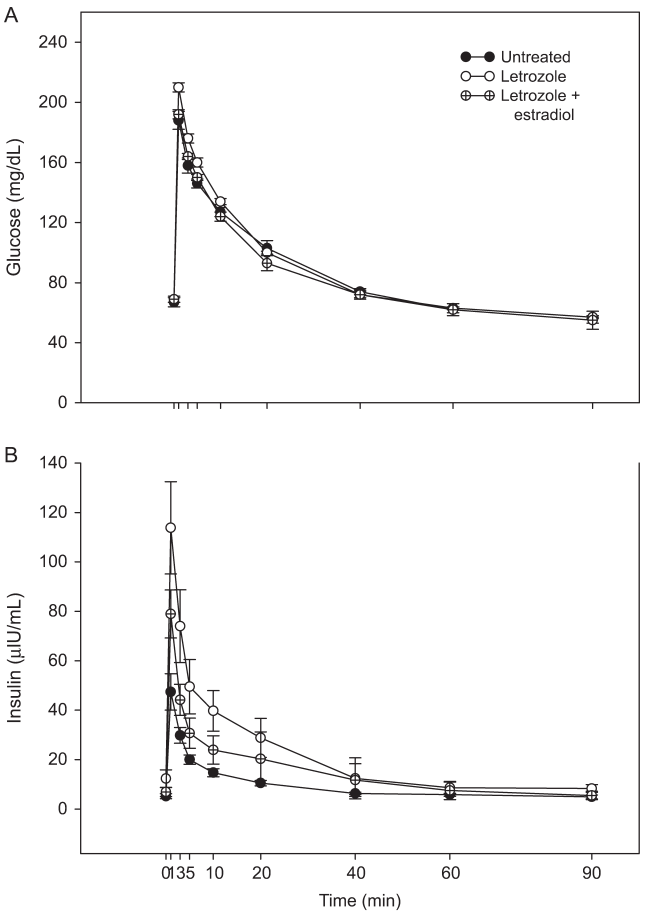

Figure 2
Patterns of blood glucose (A) and plasma insulin (B) levels after i.v. administration at time 0 min of a bolus of dextrose during postnatal life to prepubertal offspring delivered to baboons untreated ($n=10$) or treated with letrozole ($n=5$) or letrozole + estradiol ($n=4$) as detailed in the legend of Fig. 1. Values at each time point are the mean ± S.E.M. of the average of several glucose challenge tests performed longitudinally (i.e. every 6–12 months) in each animal at 1–3¼ years of postnatal life (i.e. before puberty).

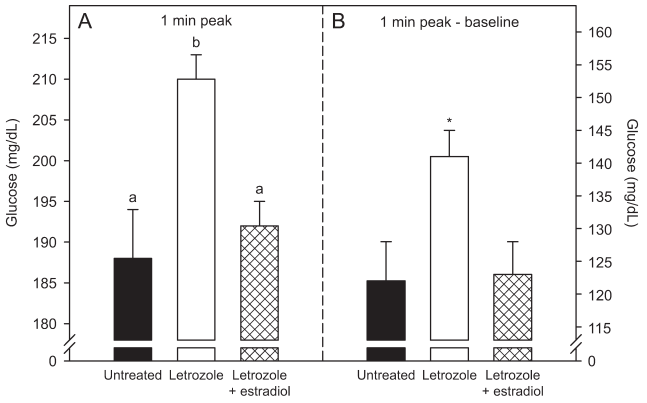

Figure 3
Blood glucose levels, expressed as 1 min peak (panel A) and 1 min peak-baseline (panel B) levels, after administration of an i.v. bolus of dextrose to baboon offspring untreated ($n=10$) or treated *in utero* with letrozole ($n=5$) or letrozole + estradiol ($n=4$). Values of the bars in panel A with different letters are significantly different from each other ($P<0.05$, ANOVA, Tukey–Kramer multiple comparison test). *$P=0.08$ versus untreated or letrozole + estradiol groups (panel B, ANOVA, Kruskal–Wallis nonparametric test).

greater ($P<0.05$ and $P<0.01$, respectively) in baboon offspring treated prenatally with letrozole than that in animals untreated and partially restored by letrozole plus estradiol administration (Table 3). The overall mean levels of blood glucose (114 ± 5 mg/dL) and plasma insulin (65.4 ± 11.7 µIU/mL) at 1, 3, and 5 min minus baseline level also were greater ($P=0.07$ and $P<0.01$, respectively) in baboons treated *in utero* with letrozole than that in untreated animals and restored by treatment with letrozole plus estradiol (Table 3). The values of area under the curve (AUC) for glucose (1,098 ± 21) and insulin (574 ± 85) in offspring from letrozole-treated baboons were also greater ($P<0.05$ and $P<0.001$, respectively) than that in untreated animals and partially restored in letrozole plus estradiol-treated animals (Table 3). The ratio of glucose AUC to insulin AUC was lower ($P<0.01$) in offspring delivered to letrozole-treated baboons (2.04 ± 0.25) than that in untreated offspring, indicative of increased capacity for insulin secretion, and restored to normal with letrozole plus estradiol treatment (Table 3).

The HOMA-IR and QUICKI have not been validated for use in prepubertal and pregnant baboons. However, they are well established as indices of insulin resistance and insulin sensitivity in the human and many animal

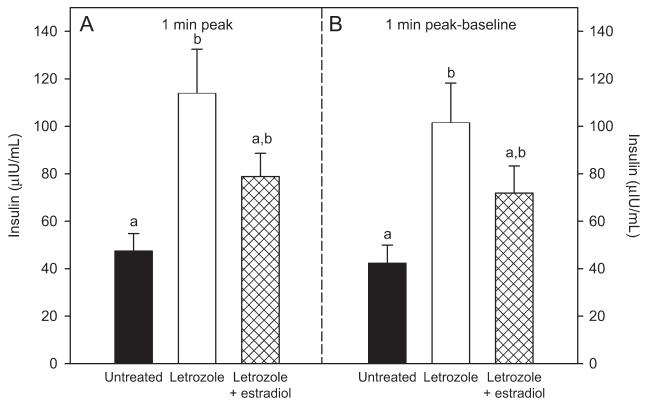

Figure 4
Plasma insulin levels, expressed as 1 min peak (panel A) and 1 min peak-baseline (panel B) levels, after administration of an i.v. bolus of dextrose to the same baboon offspring in which serum glucose levels are shown in Fig. 3. Values of the bars with different letters are significantly different from each other ($P<0.01$, ANOVA, Tukey–Kramer multiple comparison test).

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Table 3 Blood glucose and plasma insulin levels in baboon offspring after an i.v. glucose tolerance test.

Treatment	Glucose (mg/dL)			Insulin (μIU/mL)			
	Mean 1+3+5 min	Mean 1+3+5 min – baseline	Glucose (AUC)	Mean 1+3+5 min	Mean 1+3+5 min – baseline	Insulin (AUC)	Glucose AUC: Insulin AUC ratio
Untreated	165±4[a]	99±5	977±34[a]	32.4±3.8[a]	26.6±4.4[a]	192±23[a]	5.84±0.72[a]
Letrozole	183±4[b]	114±5*	1098±21[b]	77.7±13.6[b]	65.4±11.7[b]	574±85[b]	2.04±0.25[b]
Letrozole+estradiol	169±2[a,b]	99±5	1006±10[a,b]	53.2±3.7[a,b]	44.9±2.2[a,b]	318±22[c]	3.24±0.27[a]

Values are the mean±S.E.M. of the average levels of blood glucose and plasma insulin at 1, 3, and 5 min, area under curve (AUC) for glucose and insulin, and ratio of glucose AUC÷insulin AUC after an i.v. bolus of dextrose to prepubertal offspring from baboons untreated ($n=10$) or treated with letrozole ($n=5$), or letrozole+estradiol ($n=4$). Values with different letters are different from each other ($P<0.05$, glucose: $P<0.01$, insulin; ANOVA, Tukey–Kramer multiple comparison test). *$P=0.07$ vs untreated or letrozole+estradiol-treatment (ANOVA, Kruskal–Wallis test).

models and, therefore, were employed in the present study. The value for the HOMA-IR was over threefold greater ($P<0.05$) in offspring delivered to baboons treated with the aromatase inhibitor letrozole (2.20±0.70) than that in untreated animals (0.65±0.09), and partially restored in animals treated with letrozole plus estradiol (1.14±0.27, Fig. 5A). The value for the QUICKI, an index highly correlated with assessment of insulin sensitivity using the euglycemic glucose clamp method (Katz *et al.* 2000, Chen *et al.* 2005, Lee *et al.* 2011), was lower ($P<0.01$) in offspring from letrozole-treated baboons (0.337±0.012) than that in untreated animals (0.429±0.008) and restored to normal by letrozole plus estradiol administration (0.388±0.025, Fig. 5B).

There was no significant change, as analyzed by repeated measures mixed-model ANOVA, in the various glucose and insulin indices obtained sequentially during postnatal development in offspring delivered to

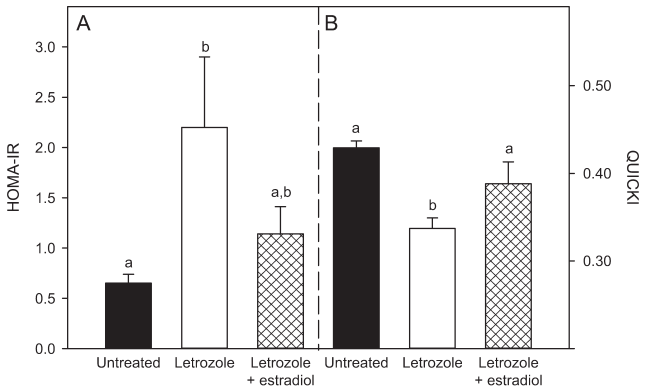

Figure 5
(A) HOMA-IR (i.e. basal glucose×basal insulin levels÷405) and (B) QUICKI (i.e. 1/(log (Io)+log (Go)), where Io is the basal insulin and Go the basal glucose levels) for the same baboon offspring in which serum glucose levels are shown in Fig. 3. Values of the bars with different letters are significantly different from each other ($P<0.05$ for HOMA-IR and $P<0.01$ for QUICKI, ANOVA, Tukey–Kramer multiple comparison test).

untreated, letrozole-treated, or letrozole plus estradiol-treated baboons. For example, mean±S.E.M. values for glucose AUC in untreated offspring were 929±75, 1016±33, and 1017±21 at 1, 2, and 3 years of age, respectively. Mean±S.E.M. insulin AUC values also were similar in untreated offspring at 1 (251±79), 2 (203±37) and 3 (219±35) years of age.

Glucose and metabolic parameters in maternal baboons Maternal body weight near term was similar in baboons untreated (18.6±1.2 kg) or treated with letrozole (18.8±1.3 kg) or letrozole plus estradiol (18.6±1.0 kg). The patterns of blood glucose and plasma insulin levels in maternal baboons during the glucose challenge test are shown in Fig. 6. Basal glucose (mg/dL) and insulin (μIU/mL) levels were similar in untreated (55±6 and 22.5±6, respectively), letrozole-treated (54±3 and 10.7±5) and letrozole plus estradiol-treated (52±5 and 15.8±4) baboons (Fig. 6). The mean (± SE) of the 1+3+5 min levels, AUC of glucose and insulin, and the ratio of glucose AUC/insulin AUC also was similar in each of the groups (Table 4). Moreover, HOMA-IR and QUICKI values (Fig. 7) were similar in untreated (1.86±0.33, 0.37±0.02), letrozole-treated (1.70±0.97, 0.38±0.02), and letrozole plus estradiol-treated (2.24±0.70, 0.35±0.02) baboon mothers.

Fetal insulin signaling components Skeletal muscle of fetuses delivered near term to untreated and letrozole-treated baboons expressed, via Western immunoblotting, the major protein components of the insulin signaling pathway, including IRS1 (approximate molecular size of 185 kDa), total Akt, Akt phosphorylated at threonine 308 or serine 473 (each 60 kDa), GLUT1, GLUT4, GLUT4 phosphorylated at serine 488 (each 55 kDa), and total AS160 (160 kDa). Representative examples of these components of the insulin signaling pathway expressed

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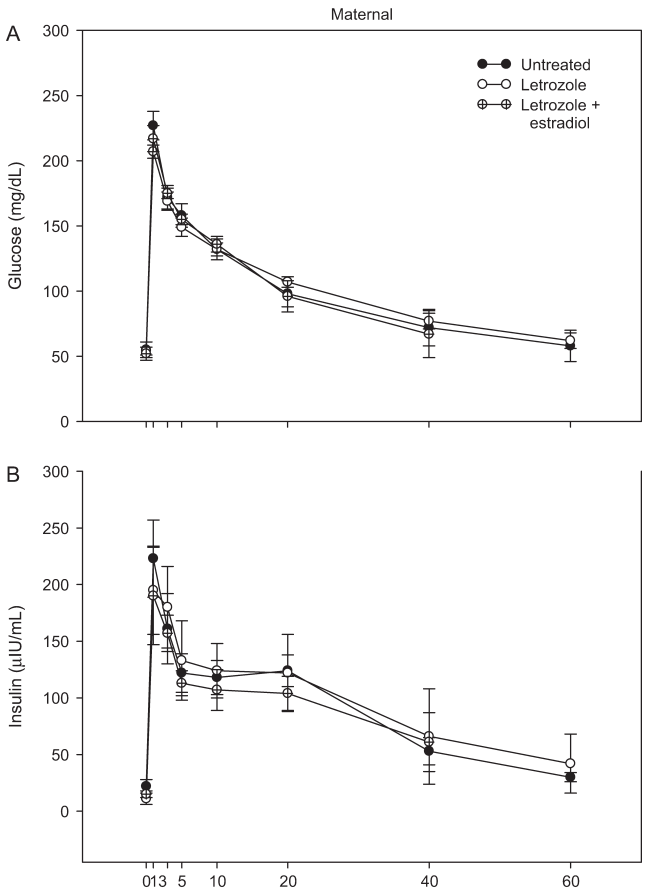

Figure 6
Mean ± S.E.M. levels of blood glucose (A) and plasma insulin (B) after i.v. administration at time 0 min of a bolus of dextrose at 151–163 days of gestation in maternal baboons untreated ($n=4$) or treated with letrozole ($n=6$) or letrozole + estradiol ($n=4$).

in fetal skeletal muscle of untreated and letrozole-treated baboons are shown in Fig. 8. Quantitative densitometric analyses showed that basal levels (i.e. noninsulin stimulated) of each of these proteins, expressed as a ratio to GAPDH, were similar in fetal skeletal muscle of baboons untreated or treated with letrozole (Table 5). Moreover, in the absence of insulin challenge, the mean (±S.E.M.) ratios of p S^{473} Akt/total Akt (0.144 ± 0.031), p T^{308} Akt/total Akt (0.114 ± 0.015), and p Glut-4/total Glut-4 (2.50 ± 0.43) in fetal skeletal muscle of letrozole-treated baboons were not different from values in untreated baboons (0.112 ± 0.020; 0.113 ± 0.030; 2.69 ± 0.68, respectively).

Discussion

The present study shows, for the first time in a nonhuman primate, that the peak rise in blood glucose level induced by a glucose challenge was greater in prepubertal offspring delivered to baboons in which estrogen had been suppressed throughout the second half of pregnancy than in animals exposed *in utero* to the normal elevation in estrogen. Moreover, the exaggerated rise in glucose levels of estrogen-suppressed baboon offspring was associated with significantly greater basal and glucose-induced levels of plasma insulin, as well as lower ratio of glucose AUC/insulin AUC, indicating that the pancreatic beta cells had the capacity to secrete insulin, but that peripheral glucose uptake and/or metabolism were impaired, a condition indicative of insulin resistance or type 2 diabetes. We propose, therefore, that estrogen normally has an important role in programming mechanisms *in utero* within the developing fetus that lead to insulin sensitivity and the capacity to metabolize glucose after birth.

The latter concept is consistent with previous observations of impaired insulin sensitivity and glucose metabolism in aromatase-null laboratory mice (Jones *et al.* 2000, Takeda *et al.* 2003), in the few clinical cases of aromatase deficiency that have been reported in humans (Rochira *et al.* 2007, Zirilli *et al.* 2008, Belgorosky *et al.* 2009, Guercio *et al.* 2009), and in healthy men administered anastrozole to suppress aromatase (Gibb *et al.* 2016). In addition, ERα-null mice develop insulin resistance, glucose intolerance, and decreased glucose

Table 4 Blood glucose and plasma insulin levels in maternal baboons during an i.v. glucose tolerance test.

Treatment	**Glucose** (mg/dL) Mean 1+3+5 min	Mean 1+3+5 min − basal	**Glucose (AUC)**	**Insulin** (μIU/mL) Mean 1+3+5 min	Mean 1+3+5 min − basal	**Insulin (AUC)**	**Glucose AUC: Insulin AUC ratio**
Untreated	185 ± 9	130 ± 5	869 ± 44	175 ± 24	153 ± 30	819 ± 111	1.16 ± 0.27
Letrozole	179 ± 5	129 ± 7	844 ± 28	174 ± 34	166 ± 31	805 ± 158	1.19 ± 0.29
Letrozole + estradiol	182 ± 5	128 ± 5	858 ± 14	164 ± 21	145 ± 20	770 ± 96	1.18 ± 0.15

Values are the mean ± S.E.M. of the average levels of maternal blood glucose and plasma insulin at 1, 3, and 5 min, area under curve (AUC) for glucose and insulin, and glucose AUC ÷ insulin AUC after an i.v. bolus of dextrose on days 151–163 of gestation to baboons untreated ($n=4$) or treated with letrozole ($n=6$), or letrozole + estradiol ($n=4$).

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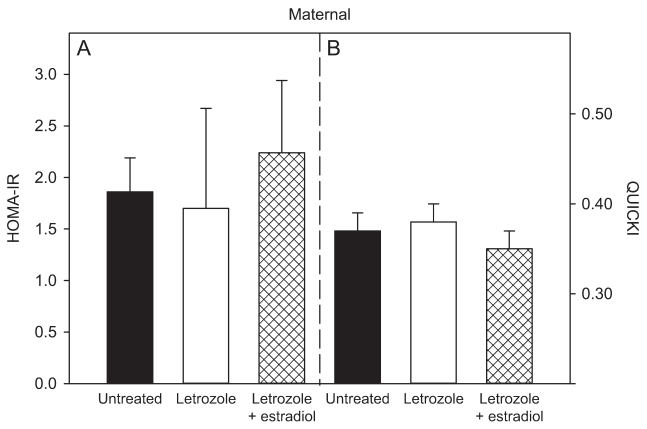

Figure 7
HOMA-IR (A) and QUICKI (B) values for the baboon mothers, in which glucose and insulin levels are shown in Fig. 6.

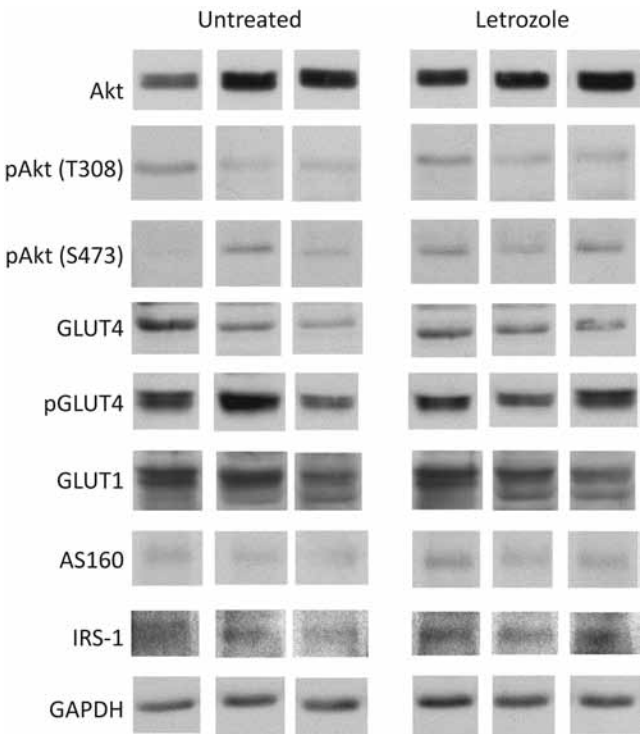

Figure 8
Representative Western immunoblot of total and phosphorylated insulin receptor signaling and GLUT proteins in extracts of skeletal muscle of fetuses obtained on day 165 of gestation from baboons untreated or treated with letrozole. Protein samples from untreated and letrozole-treated animals were applied side-by-side and electrophoresed on the same membrane. For illustrative purposes, each protein band was positioned separately below the untreated and letrozole columns as shown in this figure.

uptake in skeletal muscle (Couse & Korach 1999, Heine *et al*. 2000, Bryzgalova *et al*. 2006, Ribas *et al*. 2011, Manrique *et al*. 2012). In contrast to estrogen deprivation, the administration of phytoestrogen to mice throughout gestation enhanced glucose tolerance in offspring at adulthood (Cederroth & Nef 2009). Estrogen treatment reversed insulin resistance and normalized serum insulin levels in aromatase-deficient mouse offspring (Takeda *et al*. 2003). Moreover, low estrogen levels in adult laboratory rodents have been shown to result in insulin resistance in target tissues (Godsland 2005, Bryzgalova *et al*. 2008, Ropero *et al*. 2008, Nadal *et al*. 2009, Riant *et al*. 2009).

Our study takes on heightened translational clinical significance when considering exposure of the fetus during human pregnancy to endocrine disruptors which interfere with estrogen action (Diamanti-Kandarakis *et al*. 2009, Robins *et al*. 2011, Stel & Legler 2015) and the increasingly high incidence of premature birth in humans (Voelker 2010), a condition that deprives the developing fetus of the normal elevation in estrogen of late gestation. However, preterm birth is also accompanied by lower than normal birth weight. Low birth weight resulting from preterm birth in humans (Barker 2005, Gluckman *et al*. 2008, Ross & Beall 2008, Thompson & Regnault 2011) and nonhuman primates (Blanco *et al*. 2010, 2015) and intrauterine growth restriction induced experimentally by placental insufficiency (Thorn *et al*. 2009), uterine artery ligation/uteroplacental insufficiency (Simmons *et al*. 2001), or maternal nutrient restriction (Ozanne *et al*. 1996, Desai *et al*. 1997, Kind *et al*. 2003, Choi *et al*. 2011) of laboratory animals result in insulin resistance in offspring. However, placental and fetal growth (Table 1), maternal glucose tolerance (Fig. 6 and Table 4), umbilical blood flow (Aberdeen *et al*. 2010), and placental villous vascularization (Robb *et al*. 2007) were not decreased in letrozole-treated/estrogen-deprived baboons. Overweight/obesity also predicts insulin resistance in baboons (Chavez *et al*. 2008, 2009); however, body weight was normal in insulin-resistant offspring delivered to letrozole-treated baboons (Table 2). Therefore, the development of insulin resistance in offspring delivered to estrogen-deprived baboons of the current study was not associated with/caused by a disruption of maternal glucose metabolism, utero-placental perfusion, fetal growth restriction, or overweight. We suggest that this points to the selective role of estrogen in programming processes during fetal development that promote insulin action after birth.

The mechanisms(s) by which estrogen programs processes in the developing primate fetus that prepare the offspring to respond to insulin after birth are unknown at this point. Insulin action requires a sequence of several essential steps, including development of an extensive

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Table 5 Skeletal muscle insulin signaling molecule protein levels in fetuses delivered to baboons untreated or treated with letrozole.

	IRS-1	Akt	pAkt(T)	pAkt(S)	GLUT4	pGLUT4	GLUT1	AS160
Untreated	0.39±0.15	2.82±0.48	0.30±0.06	0.22±0.04	0.60±0.16	3.26±1.33	3.58±1.19	0.16±0.04
Letrozole	0.36±0.20	3.40±1.06	0.38±0.17	0.27±0.06	0.48±0.10	2.73±0.86	2.92±0.78	0.25±0.09

Values are the mean±S.E.M. of skeletal muscle insulin signaling molecule protein levels (arbitrary densitometric units expressed as a ratio to GAPDH) quantified by Western immunoblot in fetuses obtained on day 165 of gestation from baboons untreated ($n=5$) or treated daily on days 100–165 with letrozole (0.115 mg/kg body weight/day, sc, $n=5$).

blood vessel network to deliver circulating insulin to cells of the target tissues (Richards *et al*. 2010); binding of insulin to the insulin receptor; phosphorylation of IRS; expression and phosphorylation/activation of the insulin receptor signaling molecules, e.g. serine/threonine kinase Akt, phosphatidylinositol, and glucose transporters notably GLUT4; and facilitated intracellular transport of glucose (Nystrom & Quon 1999). Insulin receptor is expressed in high level in tissues of the human (Kaplan 1984) and baboon (Blanco *et al*. 2010) fetus. Insulin target tissues, such as skeletal muscle (Barros *et al*. 2006, Wiik *et al*. 2009) and adipose (Dieudonné *et al*. 2004), express estrogen receptor and thus are estrogen responsive. In rodents, estrogen upregulated the expression of GLUT4 (Barros *et al*. 2006, Moreno *et al*. 2010) and Akt (Vasconsuelo *et al*. 2008, Rogers *et al*. 2009) in skeletal muscle, and GLUT4 expression was impaired in insulin-resistant rats (Kahn *et al*. 1991). In the current study, the basal total and phosphorylated levels of several components of the insulin signaling pathway, including IRS1, Akt, and GLUT4, were similar in skeletal muscle of near-term fetuses obtained from baboons untreated or treated with letrozole. However, whether the expression of these insulin signaling molecules in skeletal muscle and other insulin target tissues of fetuses or offspring delivered to estrogen-deprived baboons is altered after insulin challenge is unknown. Additional investigation is required, therefore, to determine the latter question and consequently the mechanisms that underlie the disruption of insulin sensitivity after birth induced by estrogen deprivation during fetal development.

In the present study, the glucose challenge-induced increases in blood glucose and plasma insulin in offspring delivered to baboons treated *in utero* with letrozole were largely but not completely prevented by concomitant administration of letrozole plus estradiol. This may have occurred because estradiol levels within the fetus of letrozole plus estradiol-treated baboons were only partially restored to normal since estradiol was administered to baboon mothers, and is not readily transferred across the placenta into the fetus due to placental metabolism and selective placental secretion of estrogen into the maternal compartment during human and nonhuman primate pregnancy (Albrecht & Pepe 1990). However, testosterone levels were elevated in baboon fetuses treated with both the aromatase inhibitor letrozole and letrozole plus estradiol. Androgens, in high levels, have the capacity to impair insulin sensitivity (Golden *et al*. 2007), and administration of androgen to rhesus monkeys and sheep during early gestation disrupted insulin sensitivity in the offspring (Eisner *et al*. 2000, Bruns *et al*. 2004, Padmanabhan *et al*. 2010). Consequently, endogenous sex hormones may differentially regulate insulin sensitivity in men and women (Ding *et al*. 2006). An additional possibility, therefore, is that the insulin insensitivity and glucose intolerance, which developed in baboon offspring treated prenatally with letrozole, resulted from a combination of estrogen suppression and androgen excess, and thus an alteration in the ratio of estrogen and androgen.

In summary, the present study shows that the rise in serum glucose level induced by glucose challenge was greater in prepubertal offspring delivered to baboons deprived of estrogen during the second half of pregnancy than in animals exposed *in utero* to the normal elevation in estrogen. Since the exaggerated rise in glucose was accompanied by significantly greater levels of insulin, the results suggest a condition of insulin insensitivity and glucose intolerance. We propose, therefore, that estrogen normally has an important role *in utero* during primate pregnancy in programming mechanisms within the developing fetus that lead to insulin responsivity and the capacity to metabolize glucose in offspring after birth.

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.

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