Postnatal insulin secretion and sensitivity after manipulation of fetal growth by embryo transfer in the horse

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Abstract

This study examined the effects of intrauterine growth on insulin secretion and resistance in newborn foals. Embryo transfer between small pony and large Thoroughbred mares was used to produce four groups of foals with different birth weights (pony in pony $n=7$; pony in Thoroughbred $n=7$; Thoroughbred in Thoroughbred $n=8$; Thoroughbred in pony $n=8$). On day 2 after birth, glucose (0.5 g/kg) was administered intravenously to the foal and blood samples were taken for 2 h to determine plasma glucose and insulin concentrations. On day 3, insulin sensitivity was assessed by giving insulin (0.75 U/kg i.v.) and measuring the decrement in plasma glucose in the foals. There were no significant differences in

insulin secretion, insulin sensitivity or glucose tolerance between the control and growth-retarded Thoroughbred foals. Overgrown pony foals delivered by Thoroughbred mares had higher basal insulin levels and greater β cell responses to glucose than the other groups of foals. The relationship between plasma glucose and insulin was also significantly steeper in overgrown pony foals than in the other groups. Variations in intrauterine growth rate, therefore, affect postnatal insulin secretion in the horse. More specifically, it is overgrowth, not growth retardation *in utero* that alters equine β cell function in the immediate neonatal period.

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Introduction

Epidemiological studies in man have shown that both high and low birth weight are associated with an increased risk of glucose intolerance and non-insulin dependent Type 2 diabetes in adult life (Hales *et al.* 1991, Phillips *et al.* 1994b, Williams *et al.* 1999, Lindsay & Bennett 2001). These associations have been linked to changes in nutrient provision to the fetus and have led to the hypothesis that adult metabolic diseases are programmed by nutritional conditions *in utero* (Hales & Baker 1992, Hoet & Hanson 1999, Aerts & Van Assche 2003). More specifically, this hypothesis proposes that, when nutrients are either plentiful or scarce, there are metabolic and endocrine adaptations in the fetus which match the intrauterine growth rate to the nutrient supply, with beneficial effects for survival in the short term but which have detrimental effects on the secretion and effectiveness of insulin in the long term and increase susceptibility to metabolic disease in adult life (Hales *et al.* 1996, Hoet & Hanson 1999, Aerts & Van Assche 2003).

Prenatal programming of postnatal metabolic function has been investigated experimentally in a number of species, including rats, guinea pigs, pigs and sheep (Aerts & Van Assche 1979, Holemans *et al.* 1997, Moss *et al.* 2001, Simmons *et al.* 2001, Poore & Fowden 2002, Kind

et al. 2003). In these studies, nutrient availability to the fetus was either increased by inducing maternal diabetes or reduced using a range of techniques including restriction of dietary intake, uterine blood flow and placental size (Aerts *et al.* 1990, Hales *et al.* 1996, Holemans *et al.* 1997, Hoet & Hanson 1999, Simmons *et al.* 2001). These experiments all support the hypothesis that adult metabolic disease originates *in utero* and confirm that the pattern of fetal growth and development has consequences for glucose metabolism long after birth. Similarly, when fetal growth is altered spontaneously in man and other species during twin or multiple pregnancies, the resultant low birth weight is associated with perturbed glucose metabolism in later life (Poulsen *et al.* 1997, Poore & Fowden 2002, Clarke *et al.* 2000). In rats, the postnatal abnormalities in glucose tolerance observed after both fetal micro- and macrosomia appear to be caused primarily by changes in pancreatic β cell function and insulin secretion (Hoet & Hanson 1999, Aerts & Van Assche 2003) whereas in humans and pigs, the associations between birth weight and adult glucose intolerance are more closely related to insulin resistance than insulin deficiency (Phillips *et al.* 1994a, Plagemann *et al.* 1997, Ravelli *et al.* 1998, Poore & Fowden 2002).

In species such as the pig and horse, in which placentation occurs over the entire uterine surface

(Steven 1975), the placental supply of nutrients and oxygen to the fetus depends on the size of the uterus, which, in turn, is determined by the size of the mother. Experimental studies of embryo transfer or of breeding reciprocal crosses between small and large breeds of these species have shown that fetal growth is determined by the uterine capacity of the mother (Walton & Hammonds 1938, Tischner 1987, Allen *et al.* 2002a). In the horse, manipulation of intrauterine growth by embryo transfer between small pony and large Thoroughbred mares leads to altered cardiovascular and endocrine function in the foals shortly after birth (Giussani *et al.* 2003). However, the extent to which postnatal glucose handling is affected by these embryo transfer techniques remains unknown. Hence, this study investigated the secretion of, and sensitivity to, insulin in newborn foals with different intrauterine growth rates caused by embryo transfer between pony and Thoroughbred mares.

Materials and Methods

Establishment of pregnancy

Within-breed insemination Mares from a pony (P) herd and a Thoroughbred (TB) herd were artificially inseminated once during oestrus when ultrasound examination of their ovaries revealed a dominant follicle of >35 mm in diameter (Allen *et al.* 2002a). Mares were inseminated with 5–10 ml fresh semen collected from a stallion of the same breed using an artificial vagina. The semen was diluted with an equal volume of skim milk glucose extender containing antibiotics. At the time of insemination, each mare was given an intravenous injection of human chorionic gonadotrophin (Chorulon; Intervet Laboratories, Milton Keynes, UK; 1500 I.U. for ponies and 3000 I.U. for Thoroughbreds) to induce ovulation, which was diagnosed by ultrasound and confirmed by measurement of plasma progesterone concentrations (Allen *et al.* 2002a). Pregnancy was diagnosed ultrasonically between 11 and 14 days after ovulation (Allen *et al.* 2002a).

Between-breed embryo transfer Other mares from the two herds were examined, treated and had blood samples taken as above but were divided arbitrarily into donor and recipient groups for embryo transfer. The donors were inseminated with semen from the stallion of the same breed while the recipient mares were not inseminated. On day 7 after ovulation (day 0) the uteri of the donor mares were flushed three times with embryo flushing medium. The infused fluid was recovered by gravity flow through an in-line embryo filter (Em-Com, Immuno Systems, Wisconsin, WI, USA) using the final 20 ml fluid to search for the presence of an embryo. Recovered embryos were washed three times in fresh medium before being transferred non-surgically via the

cervix into the uteri of recipient mares that had ovulated 1–3 days after the respective donors. Pregnancy in the recipient mares was diagnosed ultrasonographically 7–9 days after transfer and was monitored at frequent intervals thereafter until approximately day 60.

These manipulations resulted in four experimental groups: pony fetuses carried by pony mares (P in P, $n=7$), pony fetuses carried by Thoroughbred mares (P in TB, $n=7$), Thoroughbred fetuses carried by Thoroughbred mares (TB in TB, $n=8$) and Thoroughbred fetuses carried by pony mares (TB in P, $n=8$).

Postnatal measurements and experimental procedures

All births were supervised and occurred spontaneously at gestational ages ranging from 313 days to 348 days. Occasionally, manual assistance was given either during delivery and/or immediately thereafter to help the foal to stand and suck. A blood sample was taken by venipuncture from the jugular vein immediately after birth for the determination of packed cell volume (PCV) and the haemoglobin concentration. Within 30 min of delivery, all the foals were weighed and assessed for maturity using standard clinical criteria (Rossdale *et al.* 1984). The placenta was collected immediately after delivery and, after it was weighed and measured, random samples were fixed for determination of the percentage of chorion (Allen *et al.* 2002a). The total microscopic area of the placenta was calculated by multiplying the surface density of the microcotyledons (Sv) by the volume of the chorionic portion (Vc) of each placenta. The surface density of the microcotyledons (Sv) was measured stereologically on 10 randomly selected samples of the allantochorion. The volume of the chorion (Vc) was determined by multiplying the volume of allantochorion (Va) by the percentage of allantochorion that was just chorion ($Pc/100$) (Allen *et al.* 2002a). The volume of allantochorion (Va) was obtained by fluid displacement at birth, while the chorionic percentage was estimated histologically from the 10 necropsy samples (Allen *et al.* 2002a). The total microscopic area of feto-maternal placental interface (S) in m^2 was calculated using the following formula: $S = Sv \times Vc$ where $Vc = Va \times Pc/100$. The data on gestational length, foal birthweight and the morphometry of the placenta for the four groups of animals has been published previously (Allen *et al.* 2002a, Giussani *et al.* 2003).

On the day of birth (day 1), a long-term catheter (16 g, central venous catheter, Arrow International Inc., Reading, PA, USA) with an extension tube (Tset; Mila International Inc., Florence, KY, USA) was inserted into a jugular vein of each foal. On day 2, the foals were infused intravenously with glucose (0.5 g/kg 50% dextrose, Arnolds, Harlescote, Shrewsbury, Shropshire, UK) over 5 min to assess glucose clearance and the pancreatic β cell response to glucose. The catheter was flushed with 10–20 ml saline (0.9% w/v) immediately after glucose

administration. Blood samples (3 ml) were taken through the jugular catheter at -30 and 0 min (immediately before) and 5, 15, 30, 45, 60, 90 and 120 min after glucose administration for measurement of plasma glucose and insulin concentrations. An extra 1 ml blood was collected at 0 min and added to a chilled EDTA tube containing a protease inhibitor (Trasylol, Bayer UK Ltd, Newbury, Berks, UK; 2000 Kallikrein inactivator units per ml blood) for glucagon analysis. On day 3, the insulin sensitivity of the foals was assessed by monitoring the fall in the plasma glucose concentration in response to a bolus administration of insulin (0.75 U/kg i.v. Novo Acta rapid human insulin). Blood samples were taken at -30 and 0 min before and at 5 and 15 min after insulin administration for the measurement of plasma glucose. An extra 1 ml blood was taken at 0 min and added to a chilled heparin tube containing reduced glutathione (4 nmol/tube) and EGTA (5 nmol/tube) for the measurement of catecholamine concentrations. The foals were muzzled to prevent suckling from -30 min to the end of the sampling period in both experiments. All blood samples were centrifuged immediately at 3000 g and 4 °C for 5 min and the plasma was decanted and stored at -20 °C.

All procedures were carried out under the UK Animals (Scientific Procedures) Act 1986.

Biochemical analyses

Plasma glucose concentrations were measured using a Yellow Springs glucose analyser (YSI 2300 Stat Plus, Yellow Springs, Ohio, USA). Plasma insulin and glucagon concentrations were determined by radioimmunoassay validated for use with equine plasma as described previously (Fowden *et al.* 1980, 1999). The minimum detectable quantity of insulin was 2.0 µU/ml and the intra- and interassay coefficients of variation were 7.5% and 11.5% respectively. The minimum detectable concentration of glucagon was 30 pg/ml and the intra- and interassay coefficients of variation were 6% and 8% respectively. Plasma catecholamine concentrations were determined using high performance liquid chromatography with electrochemical detection (Silver *et al.* 1987). Briefly, 250 µl plasma were absorbed onto acid-washed alumina and 20 µl aliquots of the 100 µl perchloric acid elutes were injected onto the column. Dihydroxybenzylamine was added as the internal standard to each plasma sample and its concentration used to correct for recovery. The minimum detectable quantity of adrenaline and noradrenaline was 20 pg/ml.

Calculations and statistical analyses

For each glucose tolerance test, the areas under the glucose and insulin response curves (AUC) were calculated by integrating the increment in plasma concentration after

glucose administration (from 5 to 120 min) above the mean pre-infusion baseline (30 and 0 min). To calculate the glucose half time ($t_{1/2}$), an exponential (e) decay curve was fitted to the plasma glucose concentrations during the response (5–120 min). The $t_{1/2}$ was calculated using the formula: $t_{1/2} = 0.69 \times b^{-1}$ where glucose concentration = peak glucose concentration $\times e^{-b(\text{time})}$ + basal glucose concentration. The glucose decrement over the first 15 min after insulin administration was used to assess insulin sensitivity, as the counter regulatory response is minimal at this time (Forhead & Dobson 1997). All results are expressed as means $\pm$ S.E. Comparisons within and between groups were assessed using either one-way or two-way ANOVA with or without repeated measures followed by the Tukey *post hoc* tests, as appropriate. The slopes of the relationship between plasma insulin and glucose concentrations were analysed by linear regression and their statistical significance assessed using the method of Armitage and Berry 1994. When required, data were log transformed to normalise distribution.

Results

Morphometry and pregnancy outcome

The mean body weight and placental area of the foals at birth are shown in Table 1. The mean birth weight of the TB in TB foals was significantly higher than that of the P in P foals ($P < 0.01$, Table 1). The mean birth weight of the P in TB foals was significantly higher than that of the P in P control animals and, conversely, the mean birth weight of the TB in P foals was significantly lower than that of the TB in TB foals ($P < 0.01$ both cases, Table 1). Two-way ANOVA showed significant effects of both maternal ($P < 0.01$) and fetal genomes ($P < 0.01$) on foal birth weight, and greater birth weights were associated with a TB genome regardless of whether maternal or fetal in origin. The total placental area was highest in the TB in TB pregnancies and lowest in the P in P pregnancies with intermediate values in the other two groups (Table 1). Two-way ANOVA showed that both the maternal and fetal genomes were significant influences on total placental area ($P < 0.002$ both cases).

All the mares delivered within the range of gestational ages considered full term for their breed (Rossdale 1976). The mean duration of gestation was longest in the TB in TB pregnancies and shortest in the P in P pregnancies with intermediate values in the other two groups (Table 1). Two-way ANOVA of the data showed significant effects of both the maternal ($P < 0.029$) and fetal ($P < 0.015$) genome on the duration of pregnancy with a longer gestation with a TB genome irrespective of whether this was the mare or the foal. All foals were categorised as mature immediately after birth, whatever their gestational age at delivery.

Table 1 Mean ($\pm$ S.E.) values of birth weight, placental area, gestational age packed cell volume (PCV), basal concentrations of blood haemoglobin (Hb), plasma glucose, insulin and glucagon and the ratio of the plasma concentrations of insulin to glucose in the different groups of foals (Thoroughbred in Thoroughbred, TB in TB, $n=8$; Thoroughbred in pony, TB in P, $n=8$; pony in pony, P in P, $n=7$; pony in Thoroughbred, P in TB, $n=7$ except for glucagon where $n=5$ in each group)

	TB in TB	TB in P	P in P	P in TB
Body weight (kg)	52.7 $\pm$ 2.3 ^a	33.0 $\pm$ 2.4 ^b	24.0 $\pm$ 1.3 ^c	37.9 $\pm$ 2.2 ^b
Placental area (m ²)	42.0 $\pm$ 4.5 ^a	29.4 $\pm$ 2.6 ^{b,c}	19.4 $\pm$ 1.5 ^c	34.2 $\pm$ 2.0 ^{a,b}
Gestational age (days)	338.0 $\pm$ 3.0 ^a	332.0 $\pm$ 2.8 ^{a,b}	324.6 $\pm$ 3.0 ^b	331.1 $\pm$ 2.7 ^{a,b}
PCV (%)	44.0 $\pm$ 1.1	42.7 $\pm$ 1.4	44.2 $\pm$ 1.3	47.2 $\pm$ 2.5
Hb (g/dl)	14.7 $\pm$ 0.5	14.3 $\pm$ 0.4	14.6 $\pm$ 0.5	16.0 $\pm$ 0.9
Insulin (μ U/ml)	5.1 $\pm$ 0.6 ^a	6.6 $\pm$ 1.2 ^a	13.9 $\pm$ 4.1 ^{a,b}	31.5 $\pm$ 5.5 ^b
Glucose (mmol)	10.89 $\pm$ 1.00	12.48 $\pm$ 1.49	12.90 $\pm$ 0.71	13.91 $\pm$ 0.68
Ratio of insulin:glucose	0.49 $\pm$ 0.05 ^a	0.52 $\pm$ 0.07 ^a	1.06 $\pm$ 0.31 ^{a,b}	2.03 $\pm$ 0.38 ^b
Glucagon (pg/ml)	769 $\pm$ 214	1027 $\pm$ 473	852 $\pm$ 125	1499 $\pm$ 455

^{a-c}Values within rows with different superscripts are significantly different from each other (one-way ANOVA $P<0.05$). Two-way ANOVA of the data is presented only in the text.

Basal metabolite and hormone concentrations

There were no significant differences in the basal concentrations of plasma glucose between the four groups of foals (Table 1). However, basal concentrations of plasma insulin differed between the groups and were highest in the P in TB foals (Table 1). Two-way ANOVA showed that the fetal ($P<0.01$), but not the maternal ($P>0.05$), genome was a significant influence on the plasma insulin level, with higher insulin levels in P than TB foals irrespective of the maternal genome. There was also a significant interaction between the fetal and maternal genomes ($P<0.01$) in determining plasma insulin levels with higher insulin levels in P foals delivered by TB mares than in those carried by P mares. The ratio of basal insulin and glucose concentrations was also highest in the P in TB foals (Table 1). Two-way ANOVA of the data showed that the

fetal and maternal genomes were both significant factors ($P<0.001$, both cases) in determining this ratio and that there was a significant interaction between the two genomes ($P<0.02$) with a greater ratio in P foals carried by TB mares than in those gestated by P mares.

There were no significant differences in the plasma concentrations of glucagon and adrenaline between the four groups of foals using either one-way (Tables 1 and 2) or two-way ANOVA ($P>0.05$ for both fetal and maternal genomes in all cases). Using two-way ANOVA, the fetal ($P<0.025$) but not the maternal ($P>0.05$) genome was a significant influence on the plasma noradrenaline concentration with higher concentrations in P than TB foals. No significant differences in the PCV or blood haemoglobin concentrations were observed between the four groups of foals using either one-way (Table 1) or two-way ANOVA ($P>0.05$, all cases for both fetal and maternal genomes).

Table 2 Mean ($\pm$ S.E.) values of basal concentration of plasma adrenaline, noradrenaline and glucose and the mean change (Δ) in plasma glucose 5 and 15 min after insulin administration expressed in absolute values (mmol/l) and as a percentage of the basal value (%) in the four groups of foals, (Thoroughbred in Thoroughbred, TB in TB, $n=6$; Thoroughbred in pony, TB in P, $n=7$; pony in pony, P in P, $n=7$; pony in Thoroughbred, P in TB, $n=6$)

	TB in TB	TB in P	P in P	P in TB
Adrenaline (pg/ml)	227 $\pm$ 92	202 $\pm$ 104	107 $\pm$ 43	181 $\pm$ 53
Noradrenaline (pg/ml)	258 $\pm$ 59	243 $\pm$ 35	422 $\pm$ 87	316 $\pm$ 25
Basal glucose (mmol/l)	10.96 $\pm$ 1.00	9.96 $\pm$ 1.00	10.70 $\pm$ 0.70	11.90 $\pm$ 0.50
Δ Glucose (5 min)				
mmol/l	-2.22 $\pm$ 0.34	-2.93 $\pm$ 0.40	-3.11 $\pm$ 0.79	-3.21 $\pm$ 0.58
%	-21.3 $\pm$ 3.0	-31.3 $\pm$ 4.2	-27.4 $\pm$ 6.0	-26.9 $\pm$ 4.1
Δ Glucose (15 min)				
mmol/l	-7.72 $\pm$ 1.05	-7.25 $\pm$ 0.50	-6.25 $\pm$ 8.6	-7.40 $\pm$ 0.69
%	-70.4 $\pm$ 2.1	-73.9 $\pm$ 3.4	-57.3 $\pm$ 5.5	-62.9 $\pm$ 5.3

Figure 1 Mean ($\pm$ S.E.) changes in the plasma concentrations of (A) glucose and (B) insulin in response to glucose administration (grey box) in the four groups of foals ($\blacktriangle$, Thoroughbred foals in Thoroughbred mothers (TB in TB), $n=8$; $\triangle$, Thoroughbred foals in pony mothers (TB in P), $n=8$; $\bullet$, pony foals in pony mothers (P in P), $n=7$; $\circ$, pony foals in Thoroughbred mothers (P in TB), $n=7$). * $P<0.05$, significant change in concentration from the 0 min (pre-infusion) value (one-way ANOVA with repeated measures).

Insulin secretion in response to exogenous glucose

Administration of glucose evoked a rapid release of insulin with peak insulin concentrations at the end of the 5-min infusion of glucose in all four groups of foals (Fig. 1). The maximum increment in plasma insulin was significantly greater in P in TB foals ($+49.5 \pm 6.1$ μ U/ml, $n=7$) than in the other three groups of foals (P in P, $+31.5 \pm 7.3$ μ U/ml, $n=7$; TB in TB, $+18.9 \pm 2.1$ μ U/ml, $n=8$; TB in P, $+16.8 \pm 2.0$ μ U/ml, $n=8$, $P<0.05$ all cases, one-way ANOVA). The insulin AUC was also greatest in the P in TB foals (Fig. 2A). Two-way ANOVA using fetal and maternal genomes as factors showed significant effects of the fetal, but not the maternal, genome on the maximum increment in plasma insulin (fetus $P<0.001$, mother $P>0.05$) and on the insulin AUC (fetus $P<0.003$, mother $P>0.05$), with greater values in P than TB foals,

Figure 2 Mean ($\pm$ S.E.) values of the area under the curve (AUC) of (A) insulin concentration, (B) glucose concentration and (C) the half time ($t_{1/2}$) of glucose disappearance after glucose administration in the four groups of foals (TB in TB, $n=8$; TB in P, $n=8$; P in P, $n=7$; P in TB, $n=7$). Bars with different superscripts are significantly different from each other ($P<0.05$, one-way ANOVA). Two-way ANOVA of the data is presented only in the text.

irrespective of the maternal genome. However, there was a significant interaction between the fetal and maternal genomes in determining these variables, with larger insulin responses in P foals gestated in TB mares than in those carried by P mares ($P<0.04$). There were no significant differences in either the maximum increment in plasma glucose (Fig. 1A) or the glucose AUC (Fig. 2B) between the four groups of foals. Neither was there any significant difference in the half time for glucose disappearance between the groups (Fig. 2C).

The relationships between the plasma insulin and glucose concentrations in the four groups of foals are shown in Fig. 3. The slope of this relationship was greater in P than in TB foals irrespective of the maternal genome ($P<0.05$

Figure 3 The relationship between the plasma concentrations of insulin and glucose before, and in response to, exogenous glucose administration in (A) eight TB foals in TB mothers ($\blacktriangle$; $y=0.93x-4.4$, $n=72$, $r=0.749$, $P<0.001$), (B) eight TB foals in P mothers ($\triangle$; $y=1.08x-5.4$, $n=72$, $r=0.807$, $P<0.001$), (C) seven P foals in P mothers ($\bullet$; $y=2.38x-16.3$, $n=63$, $r=0.632$, $P<0.001$) and (D) seven P foals in TB mothers ($\circ$; $y=5.59x-48.75$, $n=63$, $r=0.837$, $P<0.001$).

all cases) and was significantly steeper in the P in TB foals than in the other three groups of animals (Fig. 3, $P<0.01$ all cases). No significant difference in the slope of the relationship between the plasma insulin and glucose concentrations was observed between the two groups of TB foals (Fig. 3A and B).

Insulin sensitivity

Administration of insulin caused a rapid fall in the plasma glucose concentration in all four groups of foals (Table 2). Plasma glucose levels were significantly lower than baseline values 5 min after insulin administration and were still falling 10 min later in all the groups of foals (Table 2). There were no significant differences in the absolute or percentage decrease in plasma glucose at 5 min after insulin administration between the four groups of foals

(Table 2). However, at 15 min, the percentage fall in plasma glucose tended to be greater in TB than in P foals (Table 2, $P=0.09$). Two-way ANOVA of the data showed a significant effect of the fetal ($P<0.012$), but not the maternal ($P>0.05$), genome in determining the percentage decrease in plasma glucose 15 min after insulin administration, with a greater insulin sensitivity in TB than in P foals irrespective of the maternal genome. The basal concentration of plasma glucose was not significantly different between the four groups of foals (Table 2).

Discussion

These results demonstrate that variations in the intra-uterine growth rate affect insulin secretion by the foal in the period immediately after birth. Fetal growth was

manipulated by transferring embryos between small pony mares and larger Thoroughbred mares, and was related directly to the total area of placenta that developed within the differently sized uteri. However, in contrast to findings in other species (Holemans *et al.* 1997, Moss *et al.* 2001, Simmons *et al.* 2001, Poore & Fowden 2002, Kind *et al.* 2003), it was only the overgrown P in TB foals, and not the growth retarded TB in P foals, that showed abnormalities in insulin secretion after birth. Similarly, it was the overgrown P in TB, not the growth retarded TB in P foals that had elevated blood pressure, enhanced adrenal adrenocorticotrophin sensitivity during hypotension and altered baroreflex function in the 6–7 days after birth (Giussani *et al.* 2003). These differences in postnatal function are unlikely to be due to the slight variations in gestational length between groups as the foals were all classified as mature at birth (Rossdale *et al.* 1984). Taken together, these findings indicate that, in the horse, enhancing fetal growth above the norm has a greater range of effects on postnatal cardiovascular and endocrine function than intrauterine growth retardation, at least in the immediate neonatal period.

The growth retarded TB in P foals had normal basal concentrations of plasma insulin and glucose and their β cell responses to exogenous glucose were similar to those of the TB in TB control animals. There were also no significant differences in the half time for glucose disappearance, or in the sensitivity to exogenous insulin between the two groups of TB foals. In other species, impaired intrauterine growth is associated with changes in postnatal insulin secretion and/or insulin resistance of several tissues including the liver, fat and skeletal muscle (Holemans *et al.* 1997, Hoet & Hanson 1999, Ozanne & Hales 2002). The specific nature and extent of these changes depend on postnatal age and on the type and cause of the intrauterine growth retardation (Hales *et al.* 1996, Hoet & Hanson 1999, Ozanne & Hales 2002). In pigs and sheep growth retarded *in utero*, there is no evidence for glucose intolerance until after puberty (Moss *et al.* 2001, Poore & Fowden 2002). In contrast, abnormalities in postnatal insulin secretion and resistance associated with low birth weight have been observed as early as 4 years of age in humans, and within the first week of life in rats following restriction of uterine blood flow during late gestation (Yajnik *et al.* 1995, Simmons *et al.* 2001). However, even in these species, the abnormalities become more pronounced with increasing postnatal age and only lead to overt diabetes in old age (Robinson *et al.* 1992, Phillips *et al.* 1994b, Petry *et al.* 2001, Simmons *et al.* 2001). Indeed, in rats deprived of protein *in utero*, and in the smaller of discordant twin lambs, glucose tolerance and insulin sensitivity can be enhanced during early postnatal life and only deteriorate after puberty (Langley-Evans *et al.* 1994, Clarke *et al.* 2000, Moss *et al.* 2001, Ozanne & Hales 2002). In human populations, the greatest risk of glucose intolerance and Type 2 diabetes occurs in

individuals who are born small and thin but who show rapid catch-up growth during late infancy and become obese as adults (Phillips *et al.* 1994a, Eriksson *et al.* 2003, Hales & Ozanne 2003). The absence of any alteration in insulin secretion or sensitivity in the growth-retarded newborn TB in P foals suggests that it is the combination of pre- and postnatal environments, rather than intrauterine growth retardation *per se*, that conditions the association between low birth weight and subsequent abnormalities in glucose handling. Further studies of the TB in P foals later in life are required to establish whether retardation of fetal growth by embryo transfer may eventually lead to glucose intolerance in this species.

The overgrown P in TB foals had higher insulin levels and greater β cell responses to exogenous glucose than the other groups of foals. The slope of the relationship between the plasma glucose and insulin concentrations was also significantly steeper in the P in TB foals than in the control P in P foals or in the two groups of TB foals. Glucose-stimulated insulin secretion is, therefore, enhanced in the overgrown P in TB foals. These observations are consistent with previous findings of neonatal hyperinsulinaemia and hypersecretion of insulin in the macrosomic offspring of diabetic rats and women (Lindsey & Bennett 2001, Aerts & Van Assche 2003). The increased supply of nutrients provided by the larger placenta of the P in TB foals may have led to β cell proliferation and/or changes in the mechanisms of insulin synthesis and secretion. Neogenesis of β cells occurs throughout adult life in the horse (Helmstaedler *et al.* 1976), although relatively little is known about the intrauterine development of the endocrine pancreas in this species (Fowden *et al.* 1984). The pancreatic endocrine cells of the fetal horse are functional from mid gestation and are known to become progressively more responsive to stimuli during late gestation in normal pony pregnancies (Fowden *et al.* 1980, 1984, 1999). Certainly, the elevated insulin levels in the P in TB foals may explain, in part, their larger size, since insulin is known to be an important growth factor in the fetus (Fowden 1995).

In humans and rats, fetal macrosomia induced by increasing the nutrient supply is often accompanied by increased fat deposition, a known risk factor for adult insulin resistance (Phillips *et al.* 1994a, Eriksson *et al.* 2003, Hales & Ozanne 2003). Nothing is known about the body composition of the foals in the present study although the gross morphometry of the foals did differ between groups (Allen *et al.* 2002a,b). In humans, the expression of specific polymorphisms in the promotor region of the insulin gene is associated with enhanced birth weight, hyperinsulinaemia and an increased risk of adult glucose intolerance (Ong & Dunger 2001). The enhanced insulin levels found in the P in TB foals could, therefore, reflect epigenetic programming of the regulatory regions of the insulin gene(s) during early development in the larger TB uterus. Alternatively, the hyperinsulinaemia may have a postnatal

rather than a prenatal origin. Gastrointestinal peptides are known to enhance insulin secretion by the adult pancreas and their concentrations rise rapidly after feeding in the newborn foal (Ousey *et al.* 1995). Exposure to these peptides per kg bodyweight may have been greatest in the P in TB foals as these foals were small relative to the milk supply offered by the larger TB mammary gland.

Although the insulin response to glucose was enhanced in the P in TB foals, there was no significant difference in the half time for glucose disappearance between any of the groups of foals. This observation, coupled with the hyperinsulinaemia and elevated insulin to glucose concentration ratio in the P in TB foals, suggests that these animals were more resistant to the hypoglycaemic actions of insulin than the other groups of foals. Enhanced insulin secretion may, therefore, have been a compensatory mechanism to maintain glucose homeostasis in the relatively insulin resistant P in TB foals. However, insulin sensitivity, measured as glucose decrement to exogenously administered insulin, was not significantly different between the P in TB foals and their P in P controls. Further studies using more sensitive indices of insulin sensitivity, such as the hyperinsulinaemic euglycaemic clamp, would be needed to confirm if P in TB foals are more insulin resistant than P in P control animals.

The P foals had larger β cell responses to glucose than the TB foals irrespective of maternal breed. In part, this may have been due to differences in the concentrations of other hormones involved in regulating insulin secretion (Fowden 1995, Giussani *et al.* 2003). However, there were no differences in the glucagon or adrenaline concentrations between groups in the current study, and the breed differences in plasma noradrenaline would have tended to suppress, not enhance glucose-stimulated insulin secretion in the P foals (Fowden 1995). Nor was there any indication from the PCV and haemoglobin concentrations at birth of breed differences in prenatal or intrapartum stress that may have accounted for the differences in neonatal β cell function. Since glucose tolerance was similar in TB and P foals, it is more likely that the breed differences in neonatal insulin secretion were directly linked to the reduced insulin sensitivity observed in P compared with TB foals. In turn, this may have reflected the breed differences in plasma noradrenaline. Since adult insulin sensitivity is known to be lower in ponies than in more athletic breeds of horse (Jeffcott *et al.* 1986, Forhead & Dobson 1997), genotype, rather than intrauterine conditions, may also be the major determinant of insulin sensitivity in the newborn foal.

In summary, this study, together with our previous observations (Allen *et al.* 2002a, Giussani *et al.* 2003), show that both enhancement and retardation of fetal growth in the horse leads to altered postnatal cardiovascular, endocrine and metabolic functions. The present findings demonstrate that alterations in fetal growth above but not below the normal trajectory for the breed leads

to abnormalities in the secretion and effectiveness of insulin in the immediate neonatal period. Whether these abnormalities resolve, persist or magnify with increasing postnatal age remains unknown but they are likely to affect neonatal metabolism and growth, which, in turn, may programme glucose handling later in life (Hales & Ozanne 2003). The horse is, therefore, a useful model in which to investigate the intrauterine origin of adult glucose intolerance.

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