Glucocorticoid exposure and tissue gene expression of 11 β HSD-1, 11 β HSD-2, and glucocorticoid receptor in a porcine model of differential fetal growth

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Abstract

Glucocorticoids play a critical role in fetal development, but inappropriate exposure is associated with reduced fetal growth. We investigated cortisol exposure and supply in a porcine model of differential fetal growth. This model compares the smallest fetus of a litter with an average-sized sibling at three stages of gestation. At day 45, small fetuses had reduced plasma cortisol (16.8 ± 3.4 ng/ml) relative to average fetuses (34.4 ± 3.4 ng/ml, $P < 0.001$). At day 65 levels had reduced in small and average fetuses to similar concentrations (5.7 ± 1.0 vs 4.8 ± 0.5 ng/ml, $P = 0.128$). By day 100, elevated levels were found in small fetuses (10.7 ± 1.5 vs 7.6 ± 0.7 ng/ml, $P < 0.001$). Maternal plasma cortisol was unchanged over gestation (day 45, 56.7 ± 21.6 ng/ml; day 65, 57.8 ± 14.4 ng/ml; day 100, 55.7 ± 6.5 ng/ml). We examined the cause of altered cortisol by investigating the fetal hypothalamic–pituitary–adrenal axis through the measurement of adrenocorticotrophic hormone and assessing exposure to maternal cortisol by quantifying placental 11 β -hydroxysteroid dehydrogenase-isoform 2 (11 β HSD-2) gene expression. These data suggest that altered cortisol supply was of fetal origin. We examined organ glucocorticoid (GC) metabolism by the measurement of GC receptor (GR) and 11 β -hydroxysteroid dehydrogenase-isoform 1 (11 β HSD-1) gene expression. We found that fetal organs have different temporal patterns of 11 β HSD-1 and GR expression, with the liver particularly sensitive to cortisol in late gestation. This study examines GC exposure in naturally occurring differential growth and simultaneously explores tissue GC sensitivity and handling, at three key stages of gestation.

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Introduction

In humans and animals, inadequate fetal growth decreases postnatal survival, and is linked to increased risk of adult disease (Barker & Clark 1997). In the pig, birth weight is the major determinant of neonatal survival and is, therefore, of major economic importance (Winters & Stewart 1947). Inappropriate exposure to glucocorticoids (GCs) has been proposed as a common mechanism linking the varied causes of reduced fetal growth to common deleterious outcomes (Fowden & Forhead 2004). GC administration during late gestation can increase fetal exposure to inappropriate levels resulting in fetal growth retardation in rats, sheep, pigs, monkeys, and humans (Sloboda *et al.* 2000, Seckl 2001, Cleasby *et al.* 2003). Despite this, as birth approaches it is normal for fetal GC levels to increase. This natural increase in GC exposure is vital for promoting the organ

maturation required for postnatal survival. Altered GC levels are associated with induced and spontaneous fetal growth restriction. For example, the offspring of protein-deficient rats expressed GC-induced enzymes at an elevated level (Langley-Evans & Nwagwu 1998) and in day 110 pig fetuses, plasma cortisol is negatively correlated with weight (Wise *et al.* 1991). Interestingly, this relationship may not hold earlier in gestation in the pig, as at day 35, fetal size positively correlates with cortisol level (Klemcke *et al.* 1999). Little is known about the role that GC exposure plays in non-induced (i.e. naturally occurring) reduced fetal growth, particularly early in gestation.

There are two main sources of fetal GC that may be altered during growth retardation; the fetal adrenal gland and maternally derived cortisol which diffuses across the placenta. The fetal adrenal gland, regulated by the

hypothalamus and pituitary gland (the HPA axis), synthesizes an endogenous supply of GC. As early as day 50 of gestation, HPA axis cortisol synthesis contributes 77% of total supply, increasing to 94% by day 100, and is, therefore, the major source of fetal cortisol (Klemcke 1995). Adrenocorticotrophic hormone (ACTH) is released by the anterior pituitary and its plasma concentration is an index of HPA axis activation (Challis *et al.* 2001).

The enzymes 11β hydroxysteroid dehydrogenase isoforms 1 and 2 (11β HSD-1 and -2) are critical components of placental and fetal ability to regenerate and deactivate GCs. 11β HSD-2 deactivates cortisol through conversion to the less active cortisone (Stewart *et al.* 1995, Klemcke & Christenson 1996, Clarke *et al.* 2002). Placental 11β HSD-2 is particularly important because maternal GC levels are up to tenfold higher than in the fetus, therefore, 11β HSD-2 acts to prevent placental diffusion of GC (Seckl 2001, Clarke *et al.* 2002). Placental 11β HSD-2 is downregulated in response to maternal undernutrition, protein deficiency, reduced oxygen conditions, to chemicals such as glycyrrhizic acid and through mutation of the 11β HSD-2 gene (Dave-Sharma *et al.* 1998, Bertram *et al.* 2001, Alfaidy *et al.* 2002). In these examples, reduced placental 11β HSD-2 is associated with inadequate fetal growth or increased GC exposure. Placental 11β HSD-2 may, therefore, be an important factor in the regulation of maternal GC supply to the developing fetus. In the porcine placenta, 11β HSD-2 expression and activity are present as early as day 24, and 11β HSD-1 by day 30. It is known that porcine placental 11β HSD-2 expression increases between days 50 and 100 of gestation (Klemcke & Christenson 1996, Klemcke *et al.* 2003), however, the localization of these enzymes within the porcine placenta has not been described.

Although until relatively recently attention has focused on cortisol levels in the developing fetus, it has become evident that organ GC processing and sensitivity may both be altered by reduced fetal growth, and may therefore markedly affect fetal responses to GCs at an organ-specific level. 11β HSD-1 regenerates cortisol from cortisone, acting to locally amplify the action of GCs in various tissues (Seckl & Walker 2001). The importance of this enzyme has been demonstrated using transgenic mice that lack the 11β HSD-1 gene. This model demonstrates that fetal lung development is retarded due to tissue 11β HSD-1 deficiency (Hundertmark *et al.* 2002). Placental 11β HSD-1 may act to enhance the GC supply during the crucial late gestation GC 'surge' (Speirs *et al.* 2004). Relatively little is known about the effect of fetal and placental 11β HSD-1 on differential fetal growth, however, if tissue expression of this enzyme changes, both fetal exposure and sensitivity to GC could be markedly altered.

Tissue abundance of GC receptor (GR) also affects tissue sensitivity to GC. GR expression is critical for development, with transgenic mice carrying a disrupted GR gene suffering severely retarded lung development and death

within hours of birth through respiratory failure (Cole *et al.* 1995). Tissue GR expression in the offspring of rats fed a low protein diet is increased in a number of tissues, including liver and lungs (Bertram *et al.* 2001). Increased GR expression may be involved in the development of metabolic syndrome and obesity in adulthood and is, therefore, a candidate for the chronic programming effects caused by reduced fetal growth (Whorwood *et al.* 2002).

This study tested three main hypotheses: 1) inadequate fetal growth is associated with altered cortisol exposure at key stages of gestation, 2) altered cortisol exposure is caused by a modification in placental capacity for deactivation of maternal cortisol, and 3) inadequate fetal growth is associated with organ-specific alterations in GC handling and sensitivity.

Materials and Methods

Animals and sampling

Sampling experiments were carried out in two periods, each using different protocols for animal euthanasia. During the first period, all blood and tissue samples studied were obtained with the exception of the placenta. The second sampling period was dedicated to obtaining placental RNA.

During the first sampling period, on days 45 ($n=7$), 65 ($n=9$), and 100 ($n=20$) of gestation (term = ~ 114 days) pregnant Large White $\times$ Landrace sows ($n=36$) were anesthetized by an i.m. injection of 5 ml Zoletil (chlorhydrate of toleamine and zolazepam; Zoletil 100, Virbac S. A., Carros, France). Deep anesthesia was induced by inhalation of 8% halothane (Halothane BP, Rhone-Poulanc chemicals Ltd, Bristol, UK) and medical oxygen (BOC gases, Manchester, UK). Euthanasia was performed by exsanguination and a sample of whole maternal blood was collected.

The gravid uterus was removed aseptically for dissection. Each conceptus was removed and the mass of the fetus measured. At day 45, the lightest fetus from each litter and a fetus of approximately average weight were designated the 'small' and 'average' respectively. At days 65 and 100, the identification was initially performed by palpation of the uterus and confirmed by measurement of fetal weight. Fetal blood was sampled by cardiac puncture using a sterile heparinized syringe. Immediately after blood sample collection, days 65 and 100 fetuses received a 1 ml intracardiac injection of sodium pentobarbitone (Euthatal, Rhone Merieux, Essex, UK) to ensure rapid euthanasia. Fetal and maternal blood samples were centrifuged at 1500 g, the plasma was collected and stored at -20°C . The liver, lungs, kidneys, and heart of the small and average fetuses were removed, dissected and frozen in liquid nitrogen before storage at -80°C .

During the second sampling period, four sows per stage of gestation were sampled using a modified euthanasia method. Briefly, after anesthesia was induced using an i.m.

injection of Zoletil, euthanasia was performed by i.v. injection of 15 ml Somulose (sodium quinalbarbitone 400 mg/ml, cinchocaine hydrochloride 25 mg/ml; Arnolds Veterinary Products Ltd, Shropshire, UK). Following euthanasia, placental tissue supplying average and small fetuses was excised, snap frozen in liquid nitrogen, and stored at -80°C . All experimental procedures were conducted in accordance with the UK-Animals (Scientific Procedures) Act, 1986 following local ethical approval.

Measurement of cortisol and ACTH in maternal and fetal plasma

Plasma cortisol was measured by specific RIA according to the manufacturer's instructions (Diagnostic Product Corporation UK, Euro/DPC Ltd, Gwynedd, UK) after validation for porcine plasma (Mwanza *et al.* 2000). The assay sensitivity was 2 ng/ml, mean interassay coefficient of variability (CV) was 7.3% and intraassay CV was 6.0%. ACTH was measured in a single-specific RIA using ^{125}I -human ACTH and rabbit anti-human ACTH antiserum, validated for use on porcine plasma, as described by Jarvis *et al.* (2007). The assay sensitivity was 77 pg/ml and had an average intraassay CV of 7.1%.

RNA extraction and analysis by northern blotting

Total RNA preparation

Total RNA extractions were performed by two methods. The first method (method A) obtained high quality RNA from heart, kidney, lung, and liver. This method proved sub-optimal for placental tissues, because of the presence of substantial lipophilic material. An alternative method (method B) was used to obtain high quality placental RNA. RNA obtained using both methods was of comparable quality when visualized using ethidium bromide staining, and was subsequently used for northern blotting.

Method A

Total RNA was extracted from the tissues of the smallest and an average-sized fetus carried by four to seven randomly selected sows at each stage of gestation. Frozen tissue was transferred directly to 1 ml TRI reagent (Helena Biosciences, Sunderland, UK) and homogenized using 3×20 s bursts with an Ultra Turrux homogeniser, model T25

and left at room temperature for 5 min. Chloroform (0.2 ml) was added, the sample vortex mixed, and centrifuged at 12 000 *g* for 15 min at 4°C . The aqueous phase was then collected, and RNA within this phase precipitated by incubation with 0.5 ml isopropanol for 10 min at room temperature. RNA was then washed with 75% ethanol in RNAase free water, dissolved in RNAase free water, and stored at -80°C before use.

Method B

Placental tissues were homogenized in TRI reagent, and the homogenate was centrifuged at 12 000 *g* for 10 min, in order to remove insoluble contamination. After 5 min at room temperature, 0.2 ml chloroform was added, and the solution mixed vigorously for 15 s. The homogenate was then stored at room temperature for 10 min before phase separation by centrifugation at 12 000 *g* for 15 min at 4°C . The aqueous phase was then transferred to a separate tube, and 1 vol of 85% (v/v) ethanol added. RNA from this was then extracted using the Qiagen RNeasy columns using the standard centrifugation-based method (Qiagen). Briefly, the sample was applied to the silica column provided, washed with provided buffers before the bound RNA was eluted with RNAase free water. RNA was then stored at -80°C before use.

RNA concentration was quantified spectrophotometrically ($\text{OD}_{260:280}$). Ten to twenty micrograms of total RNA were separated on a 1% agarose gel, visualized to confirm integrity, transferred to a nylon membrane (Hybond, Amersham International) using an electrophoretic wet transfer apparatus (Model TE 62, Pharmacia Biotech) and cross-linked with a u.v. cross-linker (U.v. Products, Upland, CA, USA).

cDNA probes

Specific porcine cDNA northern probes for 11 β HSD-1, 11 β HSD-2, and GR were prepared by the following method: cDNA from day 100 porcine fetal liver and kidney was prepared by RT using Superscript II RT kit (Invitrogen Life Technologies) using oligo-dT primers. PCR primers were then designed using the PRIMER3 online software (Rosen & Skaletski 2000) using porcine-specific sequences obtained from the Institute for Genomic Research (TIGR). Primer sequence, predicted product sizes, and TIGR database accession numbers are detailed in (Table 1).

Table 1 PCR primer sequences.

Gene/primer	Sequence	Product size (bp)	TIGR accession number
11 β HSD-1 F	5'-TCCCTATTCTGCAAGCAAGTTT-3'	680	TC219816
11 β HSD-1 R	5'-ACCAGGTGTGGATTGATAAGGT-3'		
11 β HSD-2 F	5'-CACCGTGTGGAGTTGGATAG-3'	577	TC47747
11 β HSD-2 R	5'-CAAGTGCTCGATGTAGTCCTCA-3'		
GR F	5'-AAGTCATTGAACCCGAGGTG-3'	509	TC120388
GR R	5'-TCCTTCCCTCTTGACAATGG-3'		

PCRs were carried out using liver cDNA for 11 β HSD-1 and GR and kidney cDNA for 11 β HSD-2. PCR products were then separated by agarose gel electrophoresis, and products of the predicted size were excised and DNA extracted and purified using the QIAquick gel extraction kit (Qiagen). These gene fragments were then cloned using the pGEM, T-vector system (Promega) using the manufacturer's instructions. The identity of the cloned DNA fragments inserts was confirmed by sequencing. cDNA probes for northern blotting were generated by PCR using purified plasmid from transformed bacteria as the template, and the cloning site flanking, T7 and SP6 primers.

Northern blotting

Probes were radiolabeled with (α - 32 P)-dCTP using the 'Ready-To-Go' DNA labelling kit (Amersham International). Membranes were pre-hybridized at 42 °C for 30 min in Ultrahyb (Ambion, Abingdon, UK). Hybridizations were carried out overnight at 42 °C and the membranes were washed at 42 °C to a stringency of 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate) + 0.1% SDS. Radioactivity on the membranes was imaged using a phosphorimaging system (FLA-3000, Fuji). The mRNA was quantified as the amount of radioactivity hybridizing to the bands, corrected for nonspecific binding. Phosphorimager images were analyzed using ImageJ image analysis software (National Institutes of Health, Bethesda, Maryland, USA). This expression was corrected for any differences in RNA loading by reprobing the membrane for 18S abundance, using a similar method as described above. When required, cross-membrane expression was calculated by the use of a common reference sample present on each membrane analyzed.

Statistical analysis

Fetal weights, plasma cortisol and ACTH concentrations, and GR and 11 β -HSD gene expression, were analyzed for the effect of fetal size and stage of gestation and for any interaction by two-way ANOVA with data blocked for sow to account for the common maternal environment shared by the small and average siblings. Post-hoc analysis was performed using one-way ANOVA blocked for sow identity, to test for differences between fetal sizes at individual gestational stages.

Correlations between maternal and fetal cortisol concentrations, and between fetal cortisol and ACTH concentration were analyzed by Pearson's two-tailed correlation. For all analyses, differences with $P < 0.05$ were considered significant.

Results

Fetal weights

Table 2 shows that at each stage studied, the fetus designated 'small' was significantly lighter than the

Table 2 Fetal weight in the smallest and an average-sized littermate from a subset of sampled dams.

Day	No.	Fetal weight (g)	
		Average	Small
45	10	20.5 $\pm$ 0.8 ^a	16.3 $\pm$ 1.1 ^b
65	10	214.5 $\pm$ 8.2 ^c	156.3 $\pm$ 11.4 ^d
100	10	1027.3 $\pm$ 53 ^e	590.7 $\pm$ 68 ^f

Data are means $\pm$ S.E.M. Weights not sharing superscripts differ significantly ($P < 0.05$).

average fetus from that litter. There was a significant interaction between fetal size and stage of gestation due to the increasing difference between sibling weights as gestation progressed. At day 45 of gestation, the small fetus was 80% of the weight of their average sibling, by day 65 this difference was 73%, and by day 100 the small fetus was 58% of the weight of the average sibling.

Plasma cortisol and ACTH concentrations

Figure 1 shows that fetal plasma cortisol concentrations changed significantly with the stage of gestation ($P < 0.001$) and fetal size ($P = 0.004$). In average-sized fetuses, cortisol concentrations were highest at day 45 (34.4 ± 3.4 ng/ml) decreasing sevenfold by day 65

Figure 1 (A) Fetal plasma cortisol concentrations in small- and average-sized fetuses at three stages of gestation. Days 45, $n = 6-7$ and 65, $n = 9$; day 100, $n = 18-20$. (B) Fetal plasma ACTH concentrations in small- and average-sized fetuses at two stages of gestation. Data were analyzed for the effect of fetal size and stage of gestation and for any interaction by two-way ANOVA with data blocked for sow to account for the common maternal environment shared by the small and average siblings. Potential differences at individual stages were assessed by one-way ANOVA blocked for sow identity.

(4.8 ± 0.5 ng/ml) before again increasing (7.6 ± 0.7 ng/ml) by day 100 of gestation. Although the small fetuses also demonstrated a similar temporal pattern, at day 45 they had <50% of the plasma cortisol levels of their average siblings (16.8 ± 3.4 ng/ml; $P < 0.001$). At day 65, cortisol in small fetuses (5.7 ± 1.0 ng/ml) was not different from average, but at day 100 they had significantly elevated levels (10.7 ± 1.5 ng/ml; $P = 0.011$) relative to their average-sized siblings. Maternal cortisol concentrations did not change with the stage of gestation (day 45, 56.7 ± 21.6 ng/ml; day 65, 57.8 ± 14.4 ng/ml; day 100, 55.7 ± 6.5 ng/ml) and were greater than fetal concentrations at all stages. Maternal cortisol did not correlate with fetal levels at any stage of gestation, in either the small or average-sized fetuses (results not shown). The sex of a subset of fetuses sampled was determined during this experiment. From this subset, we found that out of 19 small fetuses with confirmed sex, 8 were male and 11 were female. Previous data have found that at day 100, 50% of runt fetuses from this species were male (Finch *et al.* 2002). Together, these data suggest that there is no sex bias for the identity of the smallest fetus of the litter. Using day 100 data (with sufficient sex identified fetuses available for analysis), we tested for any affect of sex on plasma cortisol levels, to examine the possibility that sex may have affected the HPA axis. We did not find any effect of sex on plasma cortisol ($P = 0.985$, one-way ANOVA blocked for fetal size, $n = 6-7$ per group). These analyses suggest that fetal sex did not significantly influence the fetal size:cortisol relationship described.

Plasma ACTH data were only obtained from days 65 and 100 fetuses, due to insufficient sample volume at day 45. Figure 1 shows that plasma ACTH concentrations were not different between fetal sizes at either day 65, or day 100. When the ACTH levels of the small fetuses were analyzed independent of average fetuses by one-way ANOVA, the increase in ACTH between days 65 and 100 approached statistical significance ($P = 0.07$). When this post-hoc analysis was repeated using only average-sized fetuses, no such evidence of increased ACTH was detected ($P = 0.47$, days 65 vs 100; average fetuses only). Figure 2 shows that when plasma ACTH and cortisol concentrations of each day 65 fetus were plotted no correlation was revealed, however at day 100, a positive correlation that approached significance was detected ($P = 0.06$).

11 β -HSD-1, 11 β -HSD-2, and GR gene expression

11 β HSD-1 was detected in all tissues except the kidney. No fetal size related differences in 11 β HSD-1 expression were detected in any tissue at the three time points examined, however organ-specific temporal patterns of expression were observed. Placental 11 β HSD-1 decreased significantly through gestation (Table 3; $P = 0.027$), in contrast, lung 11 β HSD-1 expression

Figure 2 Correlation between fetal plasma ACTH and cortisol concentrations at (A) day 65 ($n = 10$) and (B) day 100 ($n = 12$) of gestation.

increased fourfold from days 45 to 100 (Table 4; $P < 0.001$) with the majority of this increase occurring between gestation days 65 and 100. In the liver, 11 β HSD-1 expression increased by >30% between days 45 and 100 (Table 5). In the heart, 11 β HSD-1 tended to increase throughout gestation (Table 6; $P = 0.073$).

Expression of 11 β HSD-2 was detected in the placenta and fetal kidney but not in lung, liver, or heart tissue. Kidney 11 β HSD-2 increased sixfold from days 45 to 100 (Table 7; $P < 0.001$) with expression in the small fetuses at day 45 significantly less than that of average-sized fetuses ($P = 0.048$). No size related expression differences in kidney 11 β HSD-2 were measured at days 65 and 100. Placental 11 β HSD-2 expression also increased through gestation (Table 2; $P = 0.001$), however, no difference in expression was detected between small and average fetuses. This temporal pattern of placental 11 β HSD-2 regulation is opposite to that of 11 β HSD-1.

GR expression was detected in all tissues at the stages examined. The liver demonstrated the greatest temporal change in expression, increasing threefold from days 45 to 100 (Table 2; $P < 0.001$). Overall, hepatic GR expression, when examined across gestation, was similar between small and average fetuses, however, post-hoc

Table 3 Expression of 11 β HSD-1, 11 β HSD-2, and GR in placentas supplying small (s)- and average (a)-sized fetuses at three stages of gestation.

Gene		Day 45		Day 65		Day 100		Probability		
		a	s	a	s	a	s	Stage	Size	Interaction
<i>HSD1</i>	Mean	1.363	1.286	1.199	1.300	0.963	0.970	0.027	NS	NS
	S.E.M.	0.122	0.057	0.083	0.034	0.103	0.088			
<i>HSD2</i>	Mean	0.728	0.726	0.814	0.829	1.056	1.021	0.001	NS	NS
	S.E.M.	0.060	0.094	0.063	0.041	0.052	0.041			
<i>GR</i>	Mean	0.664	0.554	0.689	0.649	0.572	0.548	NS	0.023	NS
	S.E.M.	0.069	0.022	0.091	0.082	0.045	0.040			

Each gene is expressed relative to a reference sample. $n=4$ per group. Effect of gestation stage, fetal size, and any interaction was analyzed by two-way ANOVA.

analysis at individual stages revealed that day 45 small fetuses expressed 30% more GR than their siblings ($P<0.005$). Of the other tissues examined, lung tissue GR significantly increased by $\approx 20\%$ over gestation ($P=0.011$) by day 100. GR expression in the kidney, heart, and placenta was not related to gestational stage, however, placental GR expression was significantly lower in smaller fetuses ($P=0.023$) over the three stages measured. Post-hoc analysis did not detect a difference at specific time points, suggesting a marginal change maintained over gestation.

Discussion

The present study describes the relationship between fetal size and plasma cortisol concentrations at three stages of gestation in the pig and provides new evidence of the factors associated with altered fetal cortisol concentrations. Fetal cortisol concentrations were positively correlated with fetal size on day 45 and negatively correlated with size on day 100. The expression pattern of placental 11 β HSD-1 and -2 and fetal ACTH concentrations suggest altered cortisol was not caused by compromised placental protection from maternal cortisol. Inadequate fetal growth was associated with tissue-specific altered cortisol sensitivity, as assessed by GR expression, but not GC handling as measured by 11 β HSD-1 expression. These data revealed marked, tissue-specific temporal regulation of GR and 11 β HSD-1 through gestation, enabling us to better predict the impact of fluctuating GC levels on different organs through gestation.

At day 45, fetal cortisol levels were at their highest level of gestation, but with lower cortisol levels in the smallest when compared with average fetuses. Fetal cortisol exposure is influenced by altered supply from the mother or synthesis by the fetal adrenal gland. Studies, carried out at gestation day 50 in the pig, show 78% of fetal cortisol was of fetal origin at this early stage (Klemcke 1995). At day 45, placental 11 β HSD-2 expression was detected and did not differ between small and average fetuses. This suggests the presence of placental protection from maternal cortisol, a conclusion supported by the lack of correlation between maternal and fetal cortisol levels. Between days 45 and 65, plasma cortisol reduced markedly, resulting in similar levels between small and average fetuses. At day 65, fetal ACTH did not correlate with cortisol levels, suggesting low adrenal ACTH sensitivity. Reduced mid-gestation adrenal ACTH sensitivity has been observed in other species, and this mechanism may contribute to reduced cortisol levels (Wintour *et al.* 1995). By day 65, placental 11 β HSD-1 and -2 had decreased and increased respectively, in a pattern that predicts reduced cortisol transfer, potentially contributing to reduced fetal cortisol. By gestation day 100, fetal cortisol levels increased in small and average fetuses. The greater increase in small fetuses resulted in their elevated cortisol relative to average fetuses. Increased prenatal cortisol is mediated by HPA-axis activation concurrent with reduced 11 β HSD-2 and increased 11 β HSD-1 in the placenta (Matthews & Challis 1996, Ma *et al.* 2003). The temporal patterns in 11 β HSD-1 and -2 expressions, and the lack of maternal:fetal plasma cortisol correlation suggest the placental barrier to maternal cortisol was patent at day 100. In contrast,

Table 4 Expression of 11 β HSD-1 and GR in lungs from small (s)- and average (a)-sized fetuses at three stages of gestation.

Gene		Day 45		Day 65		Day 100		Probability		
		a	s	a	s	a	s	Stage	Size	Interaction
<i>HSD1</i>	Mean	0.309	0.284	0.441	0.527	1.183	1.231	<0.001	NS	NS
	S.E.M.	0.050	0.040	0.071	0.022	0.142	0.113			
<i>GR</i>	Mean	0.932	0.990	0.889	0.986	1.103	1.171	0.011	NS	NS
	S.E.M.	0.064	0.085	0.032	0.030	0.048	0.086			

Each gene is expressed relative to a reference sample. $n=6$ per group. Effect of gestation stage, fetal size, and any interaction was analyzed by two-way ANOVA.

Table 5 Expression of 11 β HSD-1 and GR in livers from small (s)- and average (a)-sized fetuses at three stages of gestation.

Gene		Day 45		Day 65		Day 100		Probability		
		a	s	a	s	a	s	Stage	Size	Interaction
HSD1	Mean	0.605	0.582	0.589	0.601	0.786	0.810	0.001	NS	NS
	S.E.M.	0.020	0.023	0.014	0.031	0.022	0.024			
GR	Mean	0.093	0.126*	0.200	0.210	0.277	0.286	<0.001	NS	NS
	S.E.M.	0.011	0.010	0.021	0.012	0.009	0.022			

Each gene is expressed relative to a reference sample. $n=7$ per group. Effect of gestation stage, fetal size, and any interaction was analyzed by two-way ANOVA. * significantly different ($P<0.05$) between sizes at that gestational stage.

increasing plasma ACTH in the small fetuses between days 65 and 100, and the correlation between ACTH and cortisol, both observations approaching statistical significance, hint at early HPA axis activation and enhanced adrenal ACTH sensitivity in small fetuses. Our data do not support the hypothesis that altered placental 11 β HSD-2 is the mechanism for altered GC levels. It is interesting to note that cortisol administered to the ovine fetoplacental unit downregulates placental 11 β HSD-2 (Clarke *et al.* 2002). It is tempting to hypothesize that if reduced placental 11 β HSD-2 contributes to the prenatal cortisol surge in pigs, its downregulation may be a consequence of premature HPA axis activation and not a primary cause of increased cortisol.

These conclusions are based on the evidence that 11 β -HSD gene expression levels reflect net placental 11 β -HSD activity (Murotsuki *et al.* 1998, Stulnig *et al.* 2002). It is useful to highlight, however, that posttranslational regulation of 11 β -HSD, as well as physical factors such as placental blood flow, also play a role in determining cortisol supply to the fetus. Furthermore, other mechanisms exist that can influence fetal cortisol abundance, and bioactivity. The liver metabolizes cortisol by glucuronidation (You 2004). The fetal activity of this pathway is unknown, as is its involvement, if any, with differential fetal growth. In addition, the bioavailability of plasma cortisol could be changed if the abundance of the cortisol-binding globulin was altered in small fetuses (Heo *et al.* 2003). Future studies of these mechanisms are required to further increase our understanding of GC metabolism during differential fetal growth.

It is recognized that it is difficult to predict organ responses to plasma GC levels without information about organ-specific GC sensitivity and 'processing'

capacity (Edwards *et al.* 1996, Ghosh *et al.* 2000). The strength of the present study is the ability to combine cortisol exposure data with tissue GR and 11 β HSD-1 expression patterns, to better predict organ responses to cortisol levels. GR was expressed in all tissues at all stages with the liver and the lung expressions increasing through gestation. In contrast, cardiac, placental, and renal GR expression did not change with gestational age. This pattern of expression reflects the central importance of cortisol induced, late gestation lung and liver maturation for postnatal survival. In these tissues, upregulated GR acts synergistically with increased plasma cortisol to amplify organ responses such as induction of hepatic gluconeogenic enzymes, or surfactant production in the lung (Fowden *et al.* 1993, Schmidt *et al.* 2004). In small fetuses at day 100, increased GC sensitivity in the lung and liver could increase the vulnerability of these organs to excess cortisol. Excess cortisol can downregulate growth critical hepatic genes, such as the insulin-like growth factors and cause lung dysfunction (Li *et al.* 1993, Nyirenda *et al.* 1998, Okajima *et al.* 2001).

Throughout gestation, placental GR expression in small fetuses was less than their average siblings. Cortisol promotes the development and function of the placenta, through enhanced differentiation and upregulated nutrient transport (Nacharaju *et al.* 2004, Jones *et al.* 2006). Therefore, reduced cortisol at day 45, and lower placental GR may compromise placental development and function in small fetuses. This hypothesis is supported by our previous data that found reduced placental:fetal weight ratios in small fetuses at day 45. Furthermore, the normal development of amino acid transport mechanisms as gestation progressed was

Table 6 Expression of 11 β HSD-1 and GR in hearts from small (s)- and average (a)-sized fetuses at three stages of gestation.

Gene		Day 45		Day 65		Day 100		Probability		
		a	s	a	s	a	s	Stage	Size	Interaction
HSD1	Mean	0.765	0.729	0.731	0.794	0.867	0.871	0.073	NS	NS
	S.E.M.	0.065	0.051	0.026	0.034	0.066	0.056			
GR	Mean	0.806	0.837	0.848	0.914	0.887	0.891	NS	NS	NS
	S.E.M.	0.072	0.041	0.052	0.047	0.076	0.042			

Each gene is expressed relative to a reference sample. $n=7$ per group. Effect of gestation stage, fetal size, and any interaction was analyzed by two-way ANOVA.

Table 7 Expression of 11 β HSD-2 and GR in kidneys from small (s)- and average (a)-sized fetuses at three stages of gestation.

Gene		Day 45		Day 65		Day 100		Probability		
		a	s	a	s	a	s	Stage	Size	Interaction
HSD2	Mean	0.224	0.141*	0.676	0.654	0.989	1.014	<0.001	NS	NS
	S.E.M.	0.054	0.043	0.050	0.078	0.133	0.147			
GR	Mean	1.604	1.492	1.279	1.266	1.226	1.320	NS	NS	NS
	S.E.M.	0.128	0.301	0.131	0.076	0.156	0.151			

Each gene is expressed relative to a reference sample. Day 45, $n=4$; day 65, $n=7$; day 100, $n=6$ per group. Effect of gestation stage, fetal size, and any interaction was analyzed by two-way ANOVA. * significantly different ($P<0.05$) between sizes at that gestational stage.

retarded in the placentas supplying small fetuses (Finch *et al.* 2004).

All organs examined, with the exception of the kidney, expressed 11 β HSD-1, demonstrating that fetal tissues augment their supply of cortisol through local regeneration by 11 β HSD-1. Lung 11 β HSD-1, in particular, increased markedly by day 100, emphasizing the importance of cortisol for lung maturation. Tissue 11 β HSD-1 expression was not regulated to compensate for altered plasma GC levels, either under conditions of deficit (day 45 small fetuses) or surfeit (day 100 small fetuses). It is not known if cortisone levels, the substrate for 11 β HSD-1, were different between fetal sizes. If this was the case, it is possible that tissue cortisol supply through 11 β HSD-1 could change despite unaltered expression levels.

The function of renal 11 β HSD-2, as a gatekeeper enzyme for the MR, is reflected in its increased expression through gestation in a pattern that follows the development of the kidney as an active regulator of blood pressure (Moritz *et al.* 2005). Decreased renal 11 β HSD-2 expression in small fetuses at day 45 did not persist, and is unlikely to affect postnatal blood pressure.

In conclusion, we have demonstrated for the first time that plasma cortisol levels at 45 and 100 days of gestation in the porcine fetus differ between small and average siblings from the same litter and that these differences probably reflect alterations in fetal cortisol production rather than in maternal supply. We have also found organ-specific differences in markers of GC sensitivity and processing that allow a better understanding of the impact of the changes in fetal cortisol. Together, these findings make an important contribution to our understanding of GC metabolism and differential fetal growth.

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References

- Alfaiy N, Gupta S, DeMarco C, Caniggia I & Challis JR 2002 Oxygen regulation of placental 11 beta-hydroxysteroid dehydrogenase 2: physiological and pathological implications. *Journal of Clinical Endocrinology and Metabolism* **87** 4797–4805.
- Barker D & Clark P 1997 Fetal undernutrition and disease in later life. *Reviews of Reproduction* **2** 105–112.
- Bertram C, Trowern AR, Copin N, Jackson AA & Whorwood CB 2001 The maternal diet during pregnancy programs altered expression of the glucocorticoid receptor and type 2 11beta-hydroxysteroid dehydrogenase: potential molecular mechanisms underlying the programming of hypertension in utero. *Endocrinology* **142** 2841–2853.
- Challis JR, Sloboda D, Matthews SG, Holloway A, Alfaiy N, Patel FA, Whittle W, Fraser M, Moss TJ & Newnham J 2001 The fetal placental hypothalamic–pituitary–adrenal (HPA) axis, parturition and post natal health. *Molecular and Cellular Endocrinology* **185** 135–144.
- Clarke KA, Ward JW, Forhead AJ, Giussani DA & Fowden AL 2002 Regulation of 11 beta-hydroxysteroid dehydrogenase type 2 activity in ovine placenta by fetal cortisol. *Journal of Endocrinology* **172** 527–534.
- Cleasby ME, Kelly PA, Walker BR & Seckl JR 2003 Programming of rat muscle and fat metabolism by *in utero* overexposure to glucocorticoids. *Endocrinology* **144** 999–1007.
- Cole T, Blendy J, Monaghan A, Kriegstein K, Schmid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K & Schutz G 1995 Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes & Development* **9** 1608–1621.
- Dave-Sharma S, Wilson RC, Harbison MD, Newfield R, Azar MR, Krozowski ZS, Funder JW, Shackleton CHL, Bradlow HL, Wei J-Q *et al.* 1998 Examination of genotype and phenotype relationships in 14 patients with apparent mineralocorticoid excess. *Journal of Clinical Endocrinology and Metabolism* **83** 2244–2254.
- Edwards CR, Benediktsson R, Lindsay RS & Seckl JR 1996 11 beta-Hydroxysteroid dehydrogenases: key enzymes in determining tissue-specific glucocorticoid effects. *Steroids* **61** 263–269.
- Finch AM, Antipatis C, Pickard AR & Ashworth CJ 2002 Patterns of fetal growth within Large White x Landrace and Chinese Meishan gilt litters at three stages of gestation. *Reproduction, Fertility and Development* **14** 419–425.
- Finch AM, Yang LG, Nwagwu MO, Page KR, McArdle HJ & Ashworth CJ 2004 Placental transport of leucine in a porcine model of low birth weight. *Reproduction* **128** 229–235.
- Fowden AL & Forhead AJ 2004 Endocrine mechanisms of intrauterine programming. *Reproduction* **127** 515–526.

- Fowden AL, Mijovic J & Silver M** 1993 The effects of cortisol on hepatic and renal gluconeogenic enzyme activities in the sheep fetus during late gestation. *Journal of Endocrinology* **137** 213–222.
- Ghosh B, Wood CR, Held GA, Abbott BD & Lau C** 2000 Glucocorticoid receptor regulation in the rat embryo: a potential site for developmental toxicity? *Toxicology and Applied Pharmacology* **164** 221–229.
- Heo J, Kattesh HG, Roberts MP & Schneider JF** 2003 Plasma levels of cortisol and corticosteroid-binding globulin (CBG) and hepatic CBG mRNA expression in pre- and postnatal pigs. *Domestic Animal Endocrinology* **25** 263–273.
- Hundertmark S, Dill A, Ebert A, Zimmermann B, Kotelevtsev YV, Mullins JJ & Seckl JR** 2002 Foetal lung maturation in 11beta-hydroxysteroid dehydrogenase type 1 knockout mice. *Hormone and Metabolic Research* **34** 545–549.
- Jarvis S, Moirand C, Robson SK, Summer B, Douglas AJ, Seckl JR, Russell JA & Lawrence AB** 2007 Effects of weaning age on the behavioural and neuroendocrine development of piglets. *Applied Animal Behaviour Science* (In Press).
- Jones HN, Ashworth CM, Page KR & McArdle HJ** 2006 Cortisol stimulates System A amino acid transport and SNAT2 expression in a human placental cell line (BeWo). *American Journal of Physiology. Endocrinology and Metabolism* **291** E596–E603.
- Klemcke H** 1995 Placental metabolism of cortisol at mid- and late gestation in swine. *Biology of Reproduction* **53** 1293–1301.
- Klemcke H & Christenson R** 1996 Porcine placental 11 beta-hydroxysteroid dehydrogenase activity. *Biology of Reproduction* **55** 217–223.
- Klemcke HG, McGuire WJ & Christenson RK** 1999 Cortisol concentrations in early porcine embryos. *Life Sciences* **64** 1307–1312.
- Klemcke HG, Sampath Kumar R, Yang K, Vallet JL & Christenson RK** 2003 11beta-Hydroxysteroid dehydrogenase and glucocorticoid receptor messenger RNA expression in porcine placenta: effects of stage of gestation, breed, and uterine environment. *Biology of Reproduction* **69** 1945–1950.
- Langley-Evans SC & Nwagwu M** 1998 Impaired growth and increased glucocorticoid-sensitive enzyme activities in tissues of rat fetuses exposed to maternal low protein diets. *Life Sciences* **63** 605–615.
- Li J, Saunders J, Gilmour R, Silver M & Fowden A** 1993 Insulin-like growth factor-II messenger ribonucleic acid expression in fetal tissues of the sheep during late gestation: effects of cortisol. *Endocrinology* **132** 2083–2089.
- Ma XH, Wu WX & Nathanielsz PW** 2003 Gestation-related and betamethasone-induced changes in 11beta-hydroxysteroid dehydrogenase types 1 and 2 in the baboon placenta. *American Journal of Obstetrics and Gynecology* **188** 13–21.
- Matthews SG & Challis JRG** 1996 Regulation of the hypothalamo-pituitary-adrenocortical axis in fetal sheep. *Trends in Endocrinology and Metabolism* **7** 239–246.
- Moritz KM, Jefferies A, Wintour EM & Dodic M** 2005 Fetal renal and blood pressure responses to steroid infusion after early prenatal treatment with dexamethasone. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **288** R62–R66.
- Murotsuki J, Gagnon R, Pu X & Yang K** 1998 Chronic hypoxemia selectively down-regulates 11beta-hydroxysteroid dehydrogenase type 2 gene expression in the fetal sheep kidney. *Biology of Reproduction* **58** 234–239.
- Mwanza AM, Madej A, Kindahl H, Lundeheim N & Einarsson S** 2000 Plasma levels of cortisol, progesterone, oestradiol-17 beta and prostaglandin F2 alpha metabolite after ACTH (Synacthen Depot) administration in ovariectomized gilts. *Journal of Veterinary Medicine. A, Physiology, Pathology, Clinical Medicine* **47** 193–200.
- Nacharaju VL, Divald A, McCalla CO, Yang L & Muneyirci-Delale O** 2004 11beta-Hydroxysteroid dehydrogenase inhibitor carbenoxolone stimulates chorionic gonadotropin secretion from human term cytotrophoblast cells differentiated *in vitro*. *American Journal of Reproductive Immunology* **52** 133–138.
- Nyirenda MJ, Lindsay RS, Kenyon CJ, Burchell A & Seckl JR** 1998 Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. *Journal of Clinical Investigation* **101** 2174–2181.
- Okajima S, Matsuda T, Cho K, Matsumoto Y, Kobayashi Y & Fujimoto S** 2001 Antenatal dexamethasone administration impairs normal postnatal lung growth in rats. *Pediatric Research* **49** 777–781.
- Rosen S & Skaletski H** 2000 Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, pp 365–386. Eds S Krawetz & S Misener. Totowa: Humana Press.
- Schmidt M, Sangild PT, Blum JW, Andersen JB & Greve T** 2004 Combined ACTH and glucocorticoid treatment improves survival and organ maturation in premature newborn calves. *Theriogenology* **61** 1729–1744.
- Seckl JR** 2001 Glucocorticoid programming of the fetus; adult phenotypes and molecular mechanisms. *Molecular and Cellular Endocrinology* **185** 61–71.
- Seckl JR & Walker BR** 2001 Minireview: 11beta-hydroxysteroid dehydrogenase type 1- a tissue- specific amplifier of glucocorticoid action. *Endocrinology* **142** 1371–1376.
- Sloboda DM, Newnham JP & Challis JR** 2000 Effects of repeated maternal betamethasone administration on growth and hypothalamic-pituitary-adrenal function of the ovine fetus at term. *Journal of Endocrinology* **165** 79–91.
- Speirs HJ, Seckl JR & Brown RW** 2004 Ontogeny of glucocorticoid receptor and 11beta-hydroxysteroid dehydrogenase type-1 gene expression identifies potential critical periods of glucocorticoid susceptibility during development. *Journal of Endocrinology* **181** 105–116.
- Stewart PM, Rogerson FM & Mason JI** 1995 Type 2 11 beta-hydroxysteroid dehydrogenase messenger ribonucleic acid and activity in human placenta and fetal membranes: its relationship to birth weight and putative role in fetal adrenal steroidogenesis. *Journal of Clinical Endocrinology and Metabolism* **80** 885–890.
- Stulnig TM, Oppermann U, Steffensen KR, Schuster GU & Gustafsson J-A** 2002 Liver X receptors downregulate 11beta-hydroxysteroid dehydrogenase type 1 expression and activity. *Diabetes* **51** 2426–2433.
- Whorwood CB, Donovan SJ, Flanagan D, Phillips DI & Byrne CD** 2002 Increased glucocorticoid receptor expression in human skeletal muscle cells may contribute to the pathogenesis of the metabolic syndrome. *Diabetes* **51** 1066–1075.
- Winters LMCJ & Stewart HA** 1947 A study of factors affecting survival from birth to weaning and total weaning weight of the litter of swine. *Journal of Animal Science* **6** 288–296.
- Wintour EM, Crawford R, McFarlane A, Moritz K & Tangalakis K** 1995 Regulation and function of the fetal adrenal gland in sheep. *Endocrine Research* **21** 81–89.
- Wise T, Stone RT & Vernon MW** 1991 Relationships of serum estriol, cortisol and albumin concentrations with pig weight at 110 days of gestation and at birth. *Biology of the Neonate* **59** 114–119.
- You L** 2004 Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. *Chemico-Biological Interactions* **147** 233–246.

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