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Lack of Effect of Metyrapone and Exogenous Cortisol on Early Porcine Conceptus Development

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Experimental Physiology

Lack of effect of metyrapone and exogenous cortisol on early porcine conceptus development

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A study was conducted to evaluate the influence of maternal cortisol on early conceptus development in pigs (*Sus scrofa*). The corticosteroid synthesis inhibitor metyrapone was injected daily during days 14–19 of pregnancy, without ($n = 6$) and with commensurate administration of cortisol ($n = 6$). Blood samples were taken via an indwelling jugular catheter on days 14 and 18, and conceptuses were harvested during surgery on day 20. Compared with vehicle-injected control dams ($n = 7$) plasma cortisol and aldosterone concentrations were decreased ($P < 0.01$) by 52 and 29%, respectively, by metyrapone treatment. Cortisol administration reversed decreases in plasma cortisol by day 18. There were no treatment-associated effects on conceptus survival or size. Nor were there treatment-associated effects on allantoic fluid volume or content. Trophodermal glucocorticoid receptor (GR) mRNA expression decreased by 34% ($P < 0.05$) in metyrapone-treated pigs, and was not further influenced by concomitant administration of cortisol, thereby suggesting an influence of aldosterone on GR mRNA expression. Also, when all pigs were considered, there were treatment-independent second-order polynomial regressions ($P < 0.05$) between maternal plasma cortisol concentrations and embryonic weight, allantoic size and allantoic glucose concentrations, and between plasma aldosterone concentrations and trophodermal GR mRNA expression. Such biphasic corticosteroid concentration *versus* tissue parameter curves are noteworthy, but difficult to interpret validly. They may suggest that an appropriate corticosteroid environment is necessary for optimal porcine embryonic development during this stage of gestation, but cannot overshadow the absence of treatment effects on the porcine embryonic measures evaluated.

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In many species, including swine, fetal plasma glucocorticoids such as cortisol increase as term approaches and are responsible for final maturational changes in numerous tissues (e.g. Silver, 1990; Sangild *et al.* 1993, 1994; Fowden *et al.* 1995). On the contrary, excessive exposure to glucocorticoids during gestation may cause intra-uterine growth retardation, developmental abnormalities or death, or lead to increased incidence of certain diseases during adult life (Blackburn *et al.* 1965; Reinisch *et al.* 1978; Seckl *et al.* 2000). Hence, one might speculate that a closely regulated glucocorticoid exposure is necessary throughout gestation to ensure appropriate development and survival (Klemcke *et al.* 1999). We have previously demonstrated in pregnant and cyclic pigs that intra-uterine cortisol increases 4- to 6.7-fold between days 10 and 19 of pregnancy (Klemcke *et al.* 1998). At this time (days 10–19) in conceptus (embryo plus associated extra-embryonic membranes) development, the blastocyst is undergoing quite dramatic changes (Marrable, 1971; Anderson, 1978; Anderson *et al.* 1993). Part of this development involves the allantois, which rapidly expands between days 18 and 30 owing to water accumulation (Bazer *et al.* 1981) that might in part result from Na^+,K^+-ATPase-generated water movement (Macknight & Leaf, 1977). Corticosteroids are known to regulate Na^+,K^+-ATPase in various tissues (e.g. Verrey *et al.* 1996).

Since the adrenal anlage does not appear until day 20 in swine (Whitehead, 1903), the maternal adrenal is the most likely source of this intra-uterine cortisol at

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these earlier gestational stages. Therefore, with a primary single source of cortisol (maternal adrenal) as a target for regulation, this is an optimal time interval in which to test effects of cortisol on early porcine conceptus development. We have previously shown that glucocorticoid receptor (GR) mRNA, and the cortisol-metabolizing enzyme 11β-hydroxysteroid dehydrogenase type II mRNA and activity are present in porcine placentae by day 24 of gestation, suggesting a role for cortisol during the subsequent developmental stage (Klemcke *et al.* 2003). The objective of the present study was to determine whether altered plasma concentrations of maternal cortisol prior to functioning of the embryonic adrenal are associated with altered porcine conceptus development or survival. Two corollaries to this objective were: (1) to determine the presence or absence of GR mRNA expression in conceptus tissues, because GR must be present if cortisol is to have direct effects on the conceptus; and (2) to determine whether one component of development influenced by cortisol is the allantoic fluid volume and composition.

Methods

Animals, experimental design and tissue collections

Nineteen white crossbred female pigs (gilts; *Sus scrofa*; 136 ± 3 kg, mean $\pm$ S.E.M.) were randomly assigned to one of three treatment groups: (1) vehicle ($n = 7$); (2) metyrapone [M; a cortisol and aldosterone synthesis inhibitor (Spark, 1971; Yanagibashi *et al.* 1988); 8 mg kg^{-1} body weight; $n = 6$]; or (3) metyrapone + cortisol (M + C; 100 μg kg^{-1}; $n = 6$). Pigs were naturally bred to similar breed boars after demonstrating one complete estrous cycle. On day 8 or 9, pigs were initially anaesthetized with 1.0–1.2 g sodium thiopentone and maintained under halothane anaesthesia (3–4% in 97–96% O_2). Using sterile procedures, an indwelling jugular catheter (microrenathane, 0.04′ i.d., 0.08′o.d. (1.02 mm i.d., 2.04 mm o.d.), Braintree Scientific, Braintree, MA, USA) previously coated on its internal and external surfaces with tridodecyl methyl ammonium chloride (TDMAC)-heparin (Polysciences Inc., Warrington, PA, USA) was inserted as previously described (Ford & Maurer, 1978; Klemcke *et al.* 1987). Additionally, a subcutaneous microrenathane catheter that was not pretreated with TDMAC-heparin was inserted subcutaneously (s.c.) in the inguinal region. This latter catheter was punctured with 10–15 holes using an 18 gauge needle in the last 25 cm that would lie s.c. in the inguinal region. These holes would allow for multiple ports through which the drugs could pass. Catheters were exteriorized to the dorsal cervical (jugular) or lumbar (subcutaneous) regions and attached to the skin as previously described (Ford & Maurer, 1978; Klemcke *et al.* 1987). Prophylactic injections of procaine penicillin (300 000 units ml^{-1}; Hanford Mfg. Co., Syracuse, NY, USA) were administered intramuscular (i.m.) during surgery and once more subcutaneous (s.c.) 4 days later to prevent surgery-associated infections.

Pigs were allowed to recover from this initial surgery for 5–6 days. During this time and for the remainder of the study pigs were housed individually in pens (1.77×2.13 m). An investigator would interact with the pigs 2–3 times daily, between catheterization and treatment initiation, in order to acclimate them to the subsequent treatment regimen. Beginning on day 14 of pregnancy, pigs were injected via the s.c. catheter at 07.00, 14.00 and 21.00 h daily until day 19. On days 14 and 18, ~9 ml blood samples were taken via the jugular catheter prior to injection. Doses of metyrapone used were based on those previously used in neonatal pigs injected intramuscularly (Martin *et al.* 1973; Sangild *et al.* 1993; Wu *et al.* 2000). The cortisol dose was based on previously measured plasma concentrations and the metabolic clearance rate of cortisol in pregnant pigs (Klemcke, 1995). Furthermore, a preliminary study with two pigs suggested that 8 mg kg^{-1} of metyrapone was more effective in lowering plasma cortisol concentrations than 4 mg kg^{-1}, and that 100 μg kg^{-1} cortisol was effective in reversing the effects of metyrapone (data not shown). On day 20 no additional treatments were provided, and the pigs were anaesthetized as described above. Laparotomies and complete hysterectomies were performed beginning at ~08.00 h. Once the tract was removed, its exterior was thoroughly washed with warm (37°C) sterile saline to remove maternal blood. An incision was made at the site of each conceptus, and it was gently dissected from the uterus. The allantois was cut, and the allantoic fluid was collected, weighed and placed on ice. With the aid of a dissecting microscope, the embryo was then dissected from the amnion, yolk sac and allantois, weighed and then rapidly frozen in liquid nitrogen. The allantois was blotted dry on sterile gauze, weighed and then rapidly frozen in liquid nitrogen. Within the uterus at each implantation site, very fine threads of trophoderm (trophectoderm and splanchnic mesoderm; Patten, 1948) were found after the embryo was removed. Trophoderm for three embryos was saved individually, and a 'pool' of trophoderm was also saved for each pig. These tissues were also rapidly frozen in liquid nitrogen and stored frozen at −80°C. The dams were allowed to recover from the surgery and were no longer a part of the experiment. All procedures involving use of animals were reviewed and approved by our institutional Animal Care and Use Committee.

Drug preparations and administration

Metyrapone (2-methyl-1, 2-di-3-pyridyl-1-propanone, 98% pure; Aldrich Chemical Co., Milwaukee, WI, USA) was added to a solution (1:1; v/v) of sterile glycerol and

0.15 M NaCl (glycerol-saline; pH 7.4), at a concentration of 1.2 g (10 ml)$^{-1}$. Cortisol (Sigma, St Louis, MO, USA) was also added to appropriate metyrapone preparations at a concentration of 15 mg (10 ml)$^{-1}$. Both preparations were warmed to assist in suspension, and stored in sterile bottles at 37°C. Based on the body weight of the gilt, appropriate volumes of these preparations or of glycerol-saline vehicle were injected into the subcutaneous catheter. These preparations were flushed through the catheter with heparinized saline solution.

Blood sampling

Blood samples were withdrawn into 9 ml syringes (LiHeparin, Sarstedt, Newton, NC, USA) from the jugular catheter, placed on ice, centrifuged at 1500*g*, and plasma was stored frozen at −20°C. Jugular catheters were flushed with 6–10 ml of heparinized saline after each use.

Assay procedures

Cortisol. Plasma cortisol was measured using HPLC procedures for isolation and ultraviolet detection as previously validated and reported for our laboratory (Klemcke, 1995). Briefly, to measure procedural losses, 200 ng of the synthetic steroid flumethasone (Sigma) was added in 50 μl MeOH to 2 ml plasma. After addition of 200 μl 0.75 N NaOH, samples were extracted with 4 ml of ethyl acetate. Extracts were dried under nitrogen, and samples were reconstituted in 100 μl of mobile phase (see below). Standards were prepared in a similar manner. Subsequently, 20 μl of sample or standard were injected onto a 100 × 2 mm octadecylsilane reverse phase column with 3 μm particle size (ODS-Hypersil; Keystone Scientific, Bellefonte, PA, USA) that was used in conjunction with a 3 μm ODS prefilter. The mobile phase (7% acetonitrile, 9% tetrahydrofuran, 84% water and 0.5% triethylamine, with pH adjusted to 6.5 using citric acid) was pumped at a flow rate of 0.3 ml min^{-1}. Steroids were detected at a wavelength of 242 nm. Areas under response curves were converted to mass units via use of a four-point standard curve (1.2, 2.4, 4.8 and 9.6 ng per 20 μl injection). The sensitivity of this procedure (lowest standard in the linear range of the standard curve) was 1.2 ng, and the interassay variability for seven assays was 8.6% based on a sample that was extracted and measured in all assays. The immediate precursor of cortisol, 11-deoxycortisol, was analysed using the same procedure and at the same time as cortisol. A similar four-point standard curve was used with a sensitivity of 1.2 ng (lowest standard in the linear range of the standard curve). The interassay coefficient of variation (c.v.) for 11-deoxycortisol was 13.0%. These procedures were necessary for cortisol because radioimmunoassay (RIA) procedures using antibodies from two different companies (Diagnostic Products, Los Angeles, CA, USA; Diagnostic Systems Laboratories, Houston, TX, USA), with or without extraction, would not validate properly in plasma from metyrapone-injected pigs.

Attempts were also made to use this procedure for measurement of allantoic cortisol. Allantoic fluid from embryos was combined for each pregnant pig. All volumes were adjusted to 8 ml with 0.01 M sodium phosphate containing 0.15 M NaCl and 0.1% gelatin, pH 7.4 (PBSG). These samples were extracted as described above except that two 15 ml volumes of ethyl acetate were used. However, additional peaks appeared on the HPLC chromatogram that eluted very near the cortisol peak and interfered with its accurate evaluation. Hence, allantoic values are not presented.

Aldosterone. Plasma aldosterone was measured via RIA using kits purchased from Diagnostic Products. To 1 ml of plasma ∼6700 d.p.m. of ^{3}H-aldosterone (Amersham, Arlington Heights, IL, USA) in 50 μl of PBSG was added to measure procedural losses. Plasma was then extracted with 4 ml ethyl acetate; extracts were dried under N_2 and reconstituted in 800 μl of PBSG. The average recovery was 95.7%. Aldosterone was measured in 200 μl aliquots of this reconstituted extract. The sensitivity of this RIA, based on the lowest standard in the linear range of the standard curve, was 1.9 pg. All samples were analysed in two assays with a within-assay c.v. of 2.4% and a between assay c.v. of 2.5%. Serial dilutions of pregnant pig plasma extracts had a slope ($b = -1.02$) that did not differ ($P > 0.05$) from that of the standard curve ($b = -0.92$). Accuracy estimates after adding 1.5–24 pg aldosterone to plasma averaged 108.6%, and a plot of measured *versus* expected values had a slope ($b = 1.09$) that did not differ from 1. Serial dilutions of plasma extract from a metyrapone-injected pig and to which exogenous aldosterone was added had a slope ($b = -0.93$) that did not differ from that of the standard curve ($b = -0.84$). Average accuracy of estimates after adding known amounts of aldosterone to plasma from a metyrapone-injected pig was 119%, and a plot of expected *versus* measured values had a slope ($b = 1.10$) that did not differ from 1.

Allantoic Na^+, K^+, glucose and protein. Sodium and potassium were measured in diluted aliquots of allantoic fluid using Atomic Absorption Spectrometry procedures and a Perkin Elmer Model 1100 Spectrometer (Perkin Elmer Analytical Instruments, Shelton, CT). Allantoic glucose was measured using a CIBA-Corning Glucose HK Reagents (Ciba Corning Diagnostics Corp., Oberlin, OH). Protein was measured using a modified Lowry protein procedure (Markwell *et al.* 1978). All measures were conducted in the same three randomly chosen conceptuses per pig, and each procedure was conducted in duplicate (glucose and protein) or triplicate (Na^+ and K^+). For two vehicle injected gilts, problems were encountered

accurately collecting and measuring allantoic fluid, hence allantoic fluid measures are reported for only 5 vehicle injected control gilts.

RNA isolation and Northern blot analyses. Total RNA was isolated from embryonic, allantoic and trophodermal tissues of three conceptuses per pregnant pig using RNeasy Kits (QIAGEN, Chatsworth, CA, USA). Total RNA (20 g per lane) was loaded onto denaturing Mops 1.25% agarose–formaldehyde gels and electrophoresis conducted. Subsequently RNA was transferred to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL, USA) via capillary blotting, and fixed to the membrane using UV cross-linking (UV Stratalinker 2400, Stratagene, LaJolla, CA, USA).

Porcine specific cDNA for porcine GR were generated *via* reverse transcription of total RNA isolated from female pig liver as previously described (Klemcke *et al.* 2003). For Northern analyses of GR mRNA ^{32}P-labelled cDNAs were prepared using PCR procedures, ^{32}P-dCTP (Dupont New England Nuclear, Wilmington, DE, USA) and primers specific for GR and β-actin transcripts.

Northern analyses using these labelled cDNAs were conducted essentially as previously described for erythropoietin mRNA (Klemcke *et al.* 2001). To ensure that variability among processing operations was evenly distributed across all treatments, RNA from each treatment was equally represented on each gel/membrane, and membranes were used as a blocking factor in statistical analyses. Densitometric measures were conducted with an EPI Chemi Darkroom (UVP, Inc.; Upland, CA, USA) used in conjunction with the NIH Image program. In some instances mRNA expression from the tissues of a given conceptus could not be measured validly owing to technical errors; hence, data presented represent two or three conceptuses per gilt.

Mention of trade names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Statistics

Data were analysed using the Statistical Analysis System (SAS, 1999) and program PROC MIXED contained in SAS. Plasma cortisol, aldosterone and 11-deoxycortisol concentrations were analysed using a two-way analysis of variance that modelled the effects of 'treatment' 'day' and 'treatment–day' interactions. Such an analysis statistically combines data across time periods within each day, thereby emphasizing chronic cumulative effects of treatment. For measures such as embryonic weight, allantoic weight, allantoic fluid volume, etc., a one-way ANOVA using PROC MIXED was used wherein 'pig' within 'treatment' was declared a random effect. Data were analysed for normality of distribution using PROC Univariate Normal, and for homogeneity of variance using Levene's test, and transformed to log or square-root functions where appropriate to fulfil assumptions of ANOVA. Comparison of individual treatment means were made by either *a priori* orthogonal contrasts or via the *a posteriori* Bonferroni test with adjusted probability levels to compensate for multiple comparisons. Probability levels resulting from these *a priori* and *a posteriori* comparisons are presented in the text portion of the Results section. Curvilinear relationships between maternal plasma cortisol and embryonic measures were determined and analysed using PROC MIXED and analysis of covariance techniques (ANOCVA). All data for mRNA bands of interest were also analysed using PROC MIXED and one-way ANOCVA with treatments as the main factor, and gel as a random factor. Each mRNA of interest was adjusted using ANOCVA and expression of β-actin in the same lane as a covariate to account for potential differences in lane loading and membrane transfer. Data presented are the least squares means and associated standard errors. For all statistical analyses, a probability level of ≤ 0.05 was considered significant.

Results

Plasma steroid concentrations grouped across hours and within days on days 14 (first day of the study; post-treatment concentrations at 14.00 and 21.00 h) and 18 (2 days prior to collecting embryonic data; 07.00, 14.00 and 21.00 h) are presented in Fig. 1. On day 14 plasma cortisol concentrations decreased ($P < 0.01$) in M-treated pigs and in M + C-injected pigs ($P < 0.05$) when compared with vehicle-injected controls (Fig. 1*A*). On day 18, plasma cortisol concentrations decreased ($P < 0.01$) in M-treated pigs, but not in M + C-injected pigs when compared with vehicle-injected controls (Fig. 1*A*). Furthermore, cortisol increased in M + C-injected pigs compared with metyrapone alone ($P < 0.05$; Fig. 1*A*).

Consistent with its serving as a precursor for cortisol, plasma concentrations of 11-deoxycortisol were quite low in vehicle-injected control pigs (Fig. 1*B*). Concentrations increased on days 14 and 18 in M-injected pigs compared with vehicle-injected controls on the same days ($P < 0.01$). On both days M + C injections were associated with 11-deoxycortisol concentrations that approximated those in vehicle-injected controls. Day effects were also noted ($P < 0.05$).

Compared with vehicle-treated controls, plasma aldosterone concentrations on day 14 (Fig. 1*C*) in M- and M + C-injected animals were decreased ($P \leq 0.05$) when time periods after treatment initiation are considered. On day 18, plasma aldosterone concentrations in M- and

M + C-injected pigs were decreased ($P \leq 0.05$) compared with vehicle-injected control pigs. Day effects were not noted.

Treatments had no effect on: the number of corpora lutea; number of viable embryos; percentage survival; embryonic, allantoic and allantoic fluid weights; allantoic fluid concentrations of sodium, potassium, glucose and protein; and total allantoic content of glucose and protein (Tables 1 and 2).

In two of the six pigs treated with metyrapone, neither plasma cortisol nor aldosterone were decreased by treatment at the time intervals measured, although 11-deoxycortisol in these two pigs was elevated 67- and 531-fold ($P \leq 0.01$) compared with vehicle-treated control pigs on days 14 and 18, respectively (data not shown). If cortisol concentrations from these two pigs are not included in the data, then metyrapone decreased plasma cortisol concentrations by 88% on both days 14 and 18. However, there was no clear justification for eliminating the pigs from the treatment group, since metyrapone did increase 11-deoxycortisol, and assay procedures appeared to be valid for those pigs. If data are reanalysed with these two pigs eliminated, there remains an absence of treatment effects on any variables presented in Tables 1 and 2 (data not shown). To allow inclusion of all pigs in analyses while taking into account an apparent absence of metyrapone-induced reductions in steroid concentrations, an alternative treatment-independent approach was used to examine the data. Hence, analysis of covariance procedures indicated significant treatment-independent second-order polynomial relationships between maternal plasma cortisol concentrations (for each pig the average cortisol concentration was determined in samples after treatment was initiated on day 14, at 14.00 and 21.00 h, and in samples taken on day 18, at 07.00, 14.00 and 21.00 h) and allantoic membrane weight (Fig. 2*A*), allantoic fluid weight (Fig. 2*B*), embryonic weight (Fig. 2*C*) and allantoic glucose concentrations (Fig. 2*D*). Allantoic glucose exhibited a U-shaped dose–response curve to increasing maternal cortisol concentrations (Fig. 2*D*), whereas the remainder showed inverted U-shaped relationships (Fig. 2*A–C*). It is noteworthy that these relationships were unique with cortisol, and did not occur with aldosterone (data not shown).

Glucocorticoid receptor mRNA expression was present in each conceptus tissue examined (Fig. 3). A major band was detected at ∼7.5 kb. In embryonic tissue and in allantoic tissue there were no treatment effects on this GR mRNA expression. However, in trophodermal tissue, GR mRNA expression was reduced in M- and in M + C-injected pigs when compared with controls (Fig. 3). There were no significant treatment-independent linear or polynomial relationships between maternal plasma cortisol concentrations and allantoic, embryonic or trophodermal tissue mRNA expression. There were, however, U-shaped responses to increasing maternal aldosterone concentrations, expressed as $\log_{10}$, and both embryonic ($y = 59.1 - 65.36x + 18.2x^2$; $P \leq 0.01$) and trophodermal GR mRNA ($y = 63.4 - 72.26x + 20.7x^2$; $P < 0.01$).

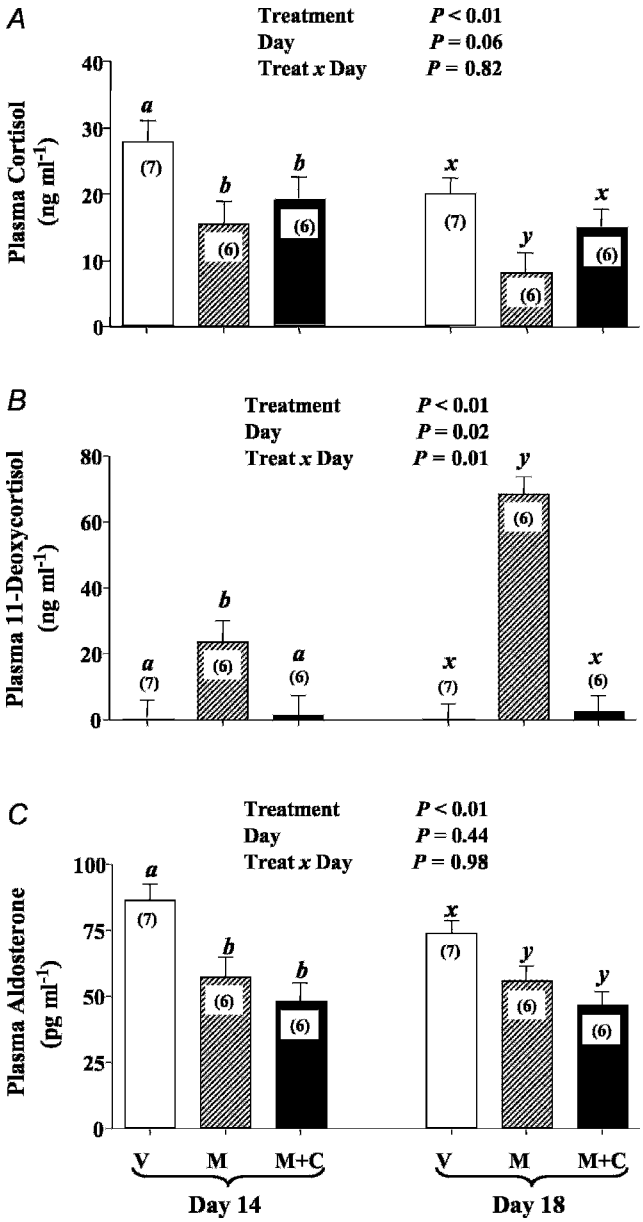

Figure 1. Plasma steroid concentrations in pigs treated with vehicle (glycerol-saline), metyrapone (8 mg (kg body weight)$^{-1}$) or metyrapone + cortisol (100 μg (kg body weight)$^{-1}$) 3 times daily on days 14–19 of pregnancy

Blood samples were taken at 07.00, 14.00 and 21,00 h on days 14 and 18. For presentation purposes to emphasize chronic, cumulative treatment effects, data were combined across time periods within a day. Each bar represents the least squares means + S.E.M. of the number of pigs in parentheses. On day 14, only data for post-treatment time periods (14.00 and 21.00 h) are presented. For each steroid on a given day, bars with different letter superscripts are significantly different. Probability levels were determined using two-way analysis of variance. V, vehicle controls; M, metyrapone; and M + C, metyrapone + cortisol.

Table 1. Effects of metyrapone and metyrapone + cortisol on parameters of porcine embryonic development and survival

Parameter	Vehicle ($n = 5$–7)	Metyrapone ($n = 6$)	Metyrapone + cortisol ($n = 6$)
No. of corpora lutea	12.6 ± 0.96	12.5 ± 1.03	12.7 ± 1.04
No. of embryos	10.4 ± 1.0	10.7 ± 1.1	10.5 ± 1.1
Survival (%)	83.2 ± 3.7	83.9 ± 3.7	82.8 ± 3.7
Average embryonic weight (mg)	38 ± 4	35 ± 4	45 ± 4
Total embryonic weight per gilt (mg)	395 ± 57	350 ± 62	458 ± 62
Average allantoic weight (mg)	269 ± 66	201 ± 72	397 ± 72
Total allantoic weight per gilt (mg)	2758 ± 622	2032 ± 672	4011 ± 672
Average allantoic fluid weight (g)	2.28 ± 0.44	1.72 ± 0.44	2.77 ± 0.44
Total allantoic fluid weight per gilt (g)	22.1 ± 5.2	16.1 ± 5.2	25.8 ± 5.2

Data represent least squares means ± S.E.M. There were no significant differences among treatments for any measure.

Table 2. Effects of metyrapone and metyrapone + cortisol on porcine allantoic fluid composition

Parameter	Vehicle ($n = 5$)	Metyrapone ($n = 6$)	Metyrapone + cortisol ($n = 6$)
Na^+ (mmol l^{-1})	145.3 ± 6.9	143.7 ± 6.3	135.7 ± 6.3
K^+ (mmol l^{-1})	15.8 ± 1.8	19.7 ± 1.6	18.1 ± 1.6
Glucose (mg ml^{-1})	1.83 ± 0.33	1.75 ± 0.30	1.37 ± 0.30
Total glucose per allantois (mg)	4.36 ± 0.74	2.44 ± 0.67	3.76 ± 0.68
Protein (mg ml^{-1})	3.02 ± 0.34	3.30 ± 0.31	2.42 ± 0.31
Total protein per allantois (mg)	7.91 ± 1.47	5.88 ± 1.34	7.91 ± 1.47

Parameters were measured in 3 conceptuses from each gilt, and the average of these values was taken as representative for conceptuses from each gilt. Data represent least squares means ± S.E.M. There were no significant differences among treatments for any measure.

Discussion

Hypotheses tested by this study were generally not supported by the data. Statistically significant reductions of cortisol and aldosterone in metyrapone-treated pigs were not associated with significant changes in conceptus size, allantoic fluid volume, allantoic fluid ionic concentrations or conceptus survival. Nor were there any changes in ionic concentration or total content of allantoic fluid. Indeed, even when only those four metyrapone-treated pigs with lowered cortisol concentrations are considered, treatment effects are still absent. Such results may well accurately reflect an absence of biological effects of cortisol on these porcine embryonic measures at this early developmental stage.

Alternatively, the results may reflect the need for larger experimental sample sizes to overcome the inherent large between-animal variability. Additionally, plasma cortisol and aldosterone concentrations were significantly reduced,

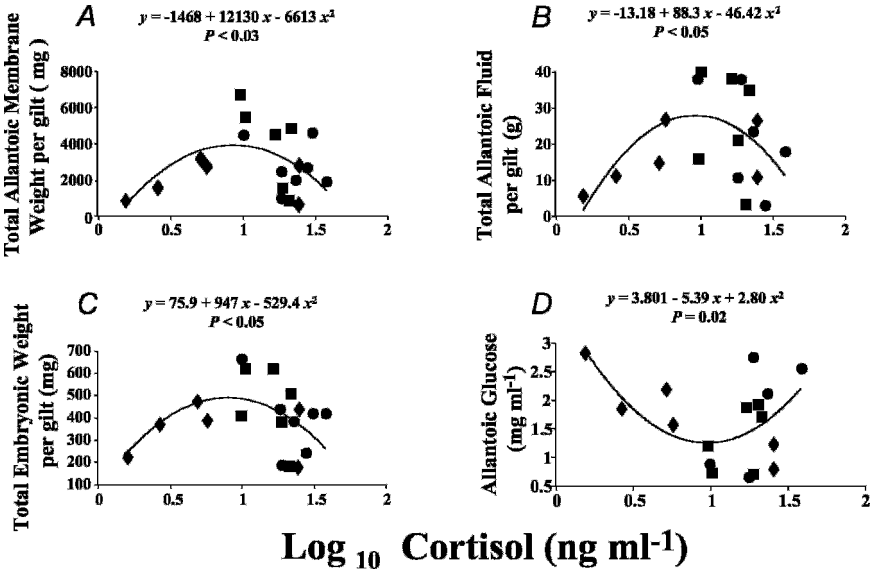

Figure 2. Treatment-independent second-order polynomial regressions between the $\log_{10}$ of maternal cortisol concentrations and various conceptus measures as determined by analysis of covariance procedures
Probability levels associated with these regressions are presented below the respective equations. The best-fit lines represented by these equations are plotted with the respective individual data points. ●, vehicle; ♦, metyrapone; and ■, metyrapone + cortisol.

but not completely eliminated, by the concentration and frequency of metyrapone used. Indeed, in humans during oral metyrapone administration (1000 mg every 2 hours for 12 hours, followed by 500 mg every 2 hours for 12 hours, Veldhuis *et al.* 2001; Liu *et al.* 2005), blood sampling at 10 min intervals for 24 h revealed reduced cortisol concentrations primarily during the morning surge of cortisol and early afternoon hours. Hence, not only does the possibility exist that the absence of diminished cortisol concentrations in two pigs may be more apparent than real because of insufficient sampling frequency, but also the remaining plasma corticosteroid concentrations in all metyrapone-treated pigs, if biologically relevant, could have been sufficient to maintain any steroid-dependent functions.

If data are evaluated for relationships between maternal plasma hormonal concentrations and size or volume, in a treatment group-independent manner, then significant polynomial relationships are revealed. Such relationships may be serendipitous. Their interpretation is obfuscated by the presence of varying concentrations of other steroids at a given steroid-of-interest concentration, and assumes that the average plasma corticosteroid concentration used in the analyses accurately reflects the steroid environment encountered by the tissues throughout the study. Nevertheless, the presence of these relationships is noteworthy, and potentially of biological relevance. For example, such relationships (inverted U) may suggest that as cortisol increases at low concentrations, tissue responses are enhanced. Subsequently, after a certain cortisol

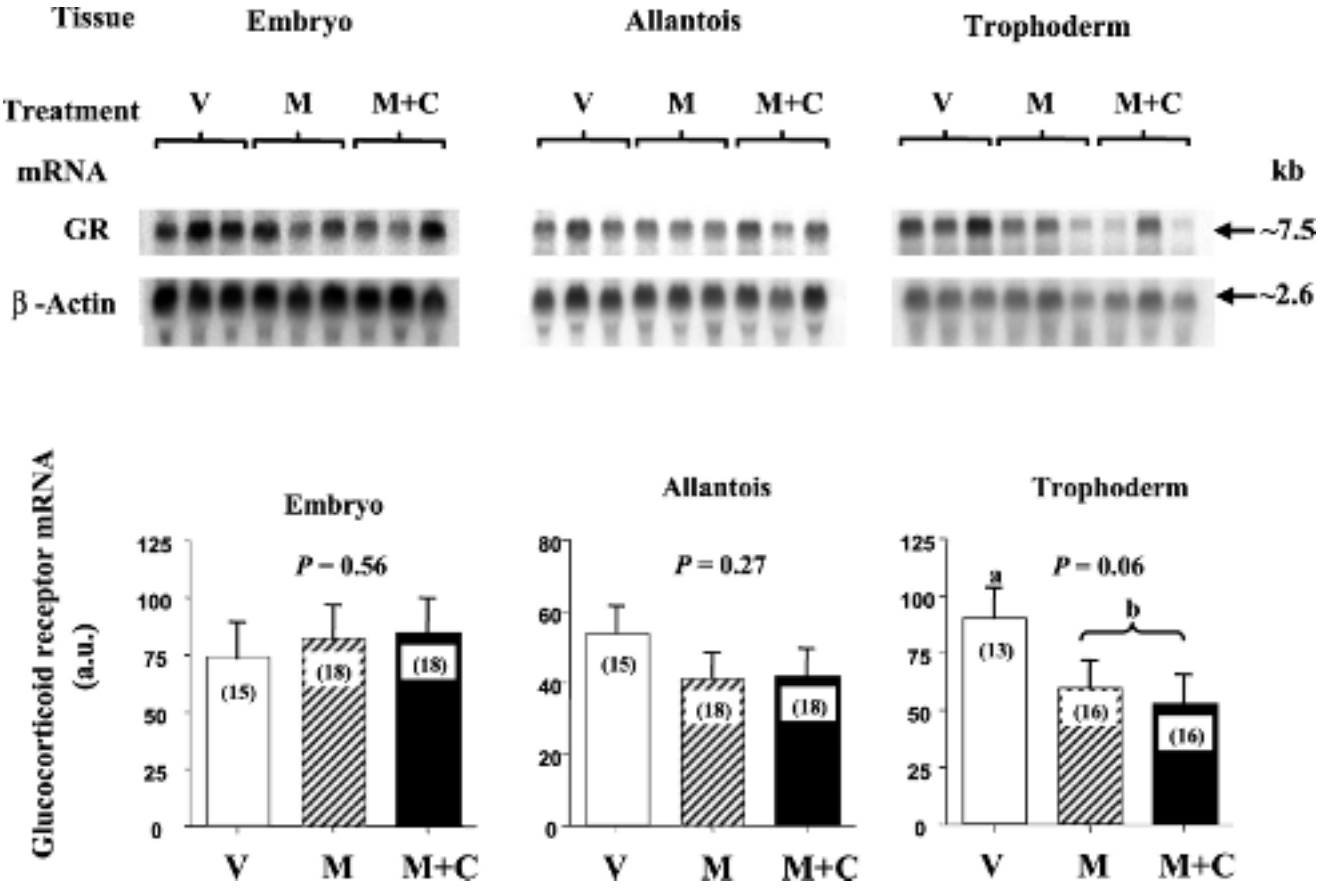

Figure 3. Effects of treatments on glucocorticoid receptor mRNA expression in various conceptus tissues
In the top panels, representative Northern blots are presented. Each lane contains 20 g of total RNA. The bottom panels provide results of densitometric analyses. Glucocorticoid receptor (GR) mRNA was adjusted using analysis of covariance and expression of β-actin in the same lane as a covariate. Each bar represents the least squares means + S.E.M. of the number of conceptuses noted within bars. These conceptus tissues originated from pregnant gilts ($n = 2$–3 per gilt) that received the various treatments (V, vehicle control, $n = 5$; M, metyrapone, $n = 6$; and M + C, metyrapone + cortisol, $n = 6$). The gilt from which the conceptuses originated was used as a random factor in the statistical analysis. Probability levels associated with analyses of variance are noted. *a versus b* ($P < 0.05$) via *a priori* orthogonal contrast of vehicle compared with both metyrapone-injected groups.

concentration has been exceeded, the tissue response becomes reduced, and ultimately the response may be inhibited. Such biphasic responses to glucocorticoids have been documented *in vitro* and *in vivo* in a number of different tissues in other species (Smith *et al.* 1972; Canalis, 1983; Quirk *et al.* 1986; Liley *et al.* 1988; Braun *et al.* 1989; Gaillard *et al.* 1991; Iannuzzi *et al.* 1993; Papachristou *et al.* 1994; Buchanan *et al.* 2001). In the present study, the range of average cortisol concentrations per pig (1.53–38.0 ng ml^{-1} or 4.2–105 nM) is 25-fold. This is considerably less than is often observed *in vitro* for biphasic responses (e.g. cortisol and effects on surfactant protein A mRNA in human fetal lung explant culture, 10–10 000 nM (Liley *et al.* 1988); corticosterone effects on glycerol-3-phosphate dehydrogenase activity in preadipocyte cell line 10^{-9}–10^{-6} M (Gaillard *et al.* 1991). However, it is a greater range than that used (4-fold differences) for *in vivo* biphasic responses to the injected synthetic glucocorticoid dexamethasone (Slotkin *et al.* 1992). Hence, it is quite conceivable that, within the range of endogenous cortisol measured, different responses could be elicited.

The glucose–cortisol relationship was U-shaped, a mirror image of that observed with embryonic size and allantoic volume. Such a relationship has also been reported for dexamethasone and neonatal rat kidney function (Slotkin *et al.* 1992), and for hydrocortisone sodium succinate effects on rat gastric mucosal prostaglandin synthesis (Avunduk *et al.* 1992). Such curves are often associated with an abatement of adverse effects at increasingly low doses; for example, the effects of X-ray dose on cancer incidence (Calabrese & Baldwin, 1999). However, the biological relevance of the glucose response to cortisol in the present study is enigmatic.

An increase in allantoic fluid volume associated with increasing, albeit low, concentrations of maternal cortisol (Fig. 2*B*) may suggest an effect of cortisol on allantoic fluid accumulation. This allantoic fluid is thought to be important in the expansion of the allantoic membranes and their eventual apposition with the trophoderm, as well as the subsequent expansion of this chorion (Patten, 1948) and its contact with the maternal uterine epithelium (Bazer, 1989). Water entering the allantois is ultimately of maternal origin, but may enter the allantois via the embryonic kidney, or directly across the allantoic membrane. The relative contribution of each site may well vary during gestation (Bazer, 1989). The embryonic porcine mesonephros is present and functioning by day 18 to redistribute water (Marrable, 1971; Bazer *et al.* 1981). However, it was suggested that at early stages (e.g. day 20) porcine allantoic fluid primarily originates via '. . .secretion by the allantoic membranes' (McCance & Dickerson, 1957). Previously, there has been evidence for progesterone, oestrogen and prolactin regulation of allantoic fluid volume and composition in pigs during early gestation (Goldstein *et al.* 1980; Bazer *et al.* 1981; McGovern *et al.* 1981; Dalton & Knight, 1983; Bazer, 1989). A potential role for aldosterone and cortisol in pigs has not been considered heretofore. In sheep, dexamethasone administration at ∼0.4 gestation (∼64 days) led to increases in allantoic fluid owing to increased fetal urine output (Wintour *et al.* 1994). Both cortisol and aldosterone have the potential for regulating this allantoic fluid volume and ionic composition via effects on Na^+, K^+ and Cl^- transport (Celsi *et al.* 1991; Verrey *et al.* 1996) and associated movement of water (Macknight & Leaf, 1977; Phillips *et al.* 1999).

Expression of glucocorticoid mRNA was evident in all conceptus tissues examined. If this mRNA is translated into active GR, then these data suggest the presence of GR in porcine conceptuses at this early gestational stage that could be capable of responding to cortisol in the uterine environment. Such data add additional credence to the relevance of the putative biphasic dose–response curves and supplement existing information concerning the ontogeny of embryonic GR in other species (Kitraki *et al.* 1997; Speirs *et al.* 2004; Hong *et al.* 2004). Indeed, evidence is presented for a metyrapone-associated decrease in trophoderm GR mRNA that was not reversed with concomitant administration of cortisol. This suggests that in this tissue aldosterone may regulate GR, although a direct effect of metyrapone cannot be excluded. The U-shaped dose–response curves between aldosterone and trophodermal and embryonic mRNA are of additional interest. However, as with the glucose–cortisol relationship, if real, the biological relevance is not easily understood. Indeed there is precedence for aldosterone modulation of GR in other tissues and species (Luttge *et al.* 1989; O'Donnell & Meaney, 1994). The present data are the first suggestive evidence for a trophic effect of aldosterone on GR mRNA expression. Although aldosterone is capable of binding with the glucocorticoid receptor, in humans the affinity is ∼900-fold less than binding to the mineralocorticoid receptor (Rupprecht *et al.* 1993). Hence, the present data also provide some indirect evidence for the presence in trophoderm of mineralocorticoid receptors.

In summary, the presence of GR mRNA in all embryonic tissues, together with the previously demonstrated presence of cortisol in the early porcine intra-uterine environment (Klemcke *et al.* 1998), suggest the opportunity for glucocorticoid modulation of early porcine embryonic development. The biphasic corticosteroid-concentration *versus* tissue-measure curves further suggest the possibility that an appropriate corticosteroid environment is necessary for optimal porcine embryonic development. Nevertheless, these latter relationships, while noteworthy, cannot overshadow the absence of treatment-associated effects on most conceptus parameters.

References

Anderson LH, Christenson LK, Christenson RK & Ford SP (1993). Investigations into the control of litter size in swine: II. Comparisons of morphological and functional embryonic diversity between Chinese and American breeds. *J Anim Sci* **71**, 1566–1571.

Anderson LL (1978). Growth, protein content and distribution of early pig embryos. *Anat Rec* **190**, 143–154.

Avunduk C, Eastwood GL, Polakowski N & Burstein S (1992). Hydrocortisone has a biphasic effect on rat gastric mucosal prostaglandin generation in vivo: inhibition at low doses, stimulation at high doses. *Prostaglandins Leukot Essent Fatty Acids* **45**, 329–332.

Bazer FW (1989). Allantoic fluid: regulation of volume and composition. *Reprod Perinatal Med* **11**, 135–155.

Bazer FW, Goldstein MH & Barron DH (1981). Water and electrolyte transport by pig chorioallantois. In *Fertilization and Embryonic Development In Vitro*, ed. Mastroianai JR & Biggers JD, pp. 299–321. Plenum Publishing, New York.

Blackburn WR, Kaplan HS & McKay DG (1965). Morphologic changes in the developing rat placenta following prednisolone administration. *Am J Obstet Gynecol* **92**, 234–246.

Braun S, Tranchant C, Vilquin JT, Labouret P, Warter JM & Poindron P (1989). Stimulating effects of prednisolone on acetylcholine receptor expression and myogenesis in primary culture of newborn rat muscle cells. *J Neurol Sci* **92**, 119–131.

Buchanan TW, Brechtel A, Sollers JJ & Lovallo WR (2001). Exogenous cortisol exerts effects on the startle reflex independent of emotional modulation. *Pharmacol Biochem Behav* **68**, 203–210.

Calabrese EJ & Baldwin LA (1999). Reevaluation of the fundamental dose-response relationship. *Bioscience* **49**, 725–732.

Canalis E (1983). Effect of glucocorticoids on type I collagen synthesis, alkaline phosphatase activity, and deoxyribonucleic acid content in cultured rat calvariae. *Endocrinology* **112**, 931–939.

Celsi G, Nishi A, Akusjarvi G & Aperia A (1991). Abundance of Na^+-K^+-ATPase mRNA is regulated by glucocorticoid hormones in infant rat kidneys. *Am J Physiol* **260**, F192–F197.

Dalton DL & Knight JW (1983). Effects of exogenous progesterone and estrone on conceptus development in swine. *J Anim Sci* **56**, 1354–1361.

Ford JJ & Maurer RR (1978). Simple technique for chronic venous catheterization of swine. *Lab Anim Sci* **28**, 615–618.

Fowden AL, Apatu RSK & Silver M (1995). The glucogenic capacity of the fetal pig: developmental regulation by cortisol. *Exp Physiol* **80**, 457–467.

Gaillard D, Wabitsch M, Pipy B & Negrel R (1991). Control of terminal differentiation of adipose precursor cells by glucocorticoids. *J Lipid Res* **32**, 569–579.

Goldstein MH, Bazer FW & Barron DH (1980). Characterization of changes in volume, osmolarity and electrolyte composition of porcine fetal fluids during gestation. *Biol Reprod* **22**, 1168–1180.

Hong SH, Nah HY, Lee YJ, Lee JW, Park JH, Kim SJ, Lee JB, Yoon HS & Kim CH (2004). Expression of estrogen receptor-alpha and -beta, glucocorticoid receptor, and progesterone receptor genes in human embryonic stem cells and embryoid bodies. *Mol Cells* **18**, 320–325.

Iannuzzi DM, Ertsey R & Ballard PL (1993). Biphasic glucocorticoid regulation of pulmonary SP-A: characterization of inhibitory process. *Am J Physiol* **264**, L236–L244.

Kitraki E, Kittas C & Stylianopoulou F (1997). Glucocorticoid receptor gene expression during rat embryogenesis. An in situ hybridization study. *Differentiation* **62**, 21–31.

Klemcke HG (1995). Placental metabolism of cortisol at mid- and late gestation in swine. *Biol Reprod* **53**, 1293–1301.

Klemcke HG, Kattesh HG, Vallet JL, Roberts MP, McGuire WJ & Christenson RK (1998). Intrauterine cortisol, aldosterone, and corticosteroid binding globulin-like activity during early porcine pregnancy and the estrous cycle. *Biol Reprod* **58**, 240–247.

Klemcke HG, McGuire WJ & Christenson RK (1999). Cortisol concentrations in early porcine embryos. *Life Sci* **64**, 1307–1312.

Klemcke HG, Nienaber JA & Hahn GL (1987). Stressor-associated alterations in porcine plasma prolactin. *Proc Soc Exp Biol Med* **186**, 333–343.

Klemcke HG, Sampath Kumar R, Yang K, Vallet JL & Christenson RK (2003). 11beta-hydroxysteroid dehydrogenase and glucocorticoid receptor messenger RNA expression in porcine placentae: effects of stage of gestation, breed, and uterine environment. *Biol Reprod* **69**, 1945–1950.

Klemcke HG, Vallet JL, Christenson RK & Pearson PL (2001). Erythropoietin mRNA expression in pig embryos. *Anim Reprod Sci* **66**, 93–108.

Liley HG, White RT, Benson BJ & Ballard PL (1988). Glucocorticoids both stimulate and inhibit production of pulmonary surfactant protein A in fetal human lung. *Proc Natl Acad Sci U S A* **85**, 9096–9100.

Liu PY, Pincus SM, Keenan DM, Roelfsema F & Veldhuis JD (2005). Joint synchrony of reciprocal hormonal signaling in human paradigms of both ACTH excess and cortisol depletion. *Am J Physiol Endocrinol Metab* **289**, E160–E165.

Luttge WG, Rupp ME & Davda MM (1989). Aldosterone-stimulated down-regulation of both type I and type II adrenocorticosteroid receptors in mouse brain is mediated via type I receptors. *Endocrinology* **125**, 817–824.

McCance RA & Dickerson JWT (1957). The composition and origin of the foetal fluids of the pig. *J Embryol Exp Morph* **5**, 43–50.

McGovern PT, Morcom CB, de Sa WF & Dukelow WR (1981). Chorionic surface area in conceptuses from sows treated with progesterone and oestrogen during early pregnancy. *J Reprod Fertil* **61**, 439–442.

Macknight AD & Leaf A (1977). Regulation of cellular volume. *Physiol Rev* **57**, 510–573.

Markwell MA, Haas SM, Bieber LL & Tolbert NE (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* **87**, 206–210.

Marrable AW (1971). *The Embryonic Pig. A Chronological Account*. Pitman Medical, London.

Martin RJ, Patt JA, Eberhart RJ & Hartsook EW (1973). The effects of metyrapone and ACTH on the development of gluconeogenesis in the neonatal pig. *Proc Soc Exp Biol Med* **144**, 603–608.

O'Donnell D & Meaney MJ (1994). Aldosterone modulates glucocorticoid receptor binding in hippocampal cell cultures via the mineralocorticoid receptor. *Brain Res* **636**, 49–54.

Papachristou DN, Liu JL & Patel YC (1994). Glucocorticoids regulate somatostatin peptide and steady state messenger ribonucleic acid levels in normal rat tissues and in a somatostatin-producing islet tumor cell line (1027B2). *Endocrinology* **134**, 2259–2266.

Patten BM (1948). *Embryology of the Pig*, 3rd edn. McGraw-Hill Book Co., New York.

Phillips JE, Wong LB & Yeates DB (1999). Bidirectional transepithelial water transport: measurement and governing mechanisms. *Biophys J* **76**, 869–877.

Quirk SJ, Gannell JE, Fullerton MJ & Funder JW (1986). Specificity and mechanism of biphasic action of glucocorticoids on α-lactalbumin production by rat mammary gland explants. *Endocrinology* **118**, 909–914.

Reinisch JM, Simon NG, Karow WG & Gandelman R (1978). Prenatal exposure to prednisone in humans and animals retards intrauterine growth. *Science* **202**, 436–438.

Rupprecht R, Reul JM, van Steensel B, Spengler D, Soder M, Berning B, Holsboer F & Damm K (1993). Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. *Eur J Pharmacol* **247**, 145–154.

Sangild PT, Diernaes L, Christiansen IJ & Skadhauge E (1993). Intestinal transport of sodium, glucose and immunoglobulin in neonatal pigs. Effect of glucocorticoids. *Exp Physiol* **78**, 485–497.

Sangild PT, Silver M, Fowden AL, Turvey A & Foltmann B (1994). Adrenocortical stimulation of stomach development in the prenatal pig. *Biol Neonate* **65**, 378–389.

SAS Online DOC, Version 8 (1999). Statistical Analysis System, Inc., Cary, NC, USA.

Seckl JR, Cleasby M & Nyirenda MJ (2000). Glucocorticoids, 11β-hydroxysteroid dehyrogenase, and fetal programming. *Kidney Int* **57**, 1412–1417.

Silver M (1990). Prenatal maturation, the timing of birth and how it may be regulated in domestic animals. *Exp Physiol* **75**, 285–307.

Slotkin TA, Seidler FJ, Kavlock RJ & Gray JA (1992). Fetal dexamethasone exposure accelerates development of renal function: relationship to dose, cell differentiation and growth inhibition. *J Dev Physiol* **17**, 55–61.

Smith RS, Sherman NA & Middleton E Jr (1972). Effect of hydrocortisone on immunoglobulin synthesis and secretion by human peripheral lymphocytes *in vitro*. *Int Arch Allergy Appl Immunol* **43**, 859–870.

Spark RF (1971). Simplified assessment of pituitary-adrenal reserve. Measurement of serum 11-deoxycortisol and cortisol after metyrapone. *Ann Intern Med* **75**, 717–723.

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. *J Endocrinol* **181**, 105–116.

Veldhuis JD, Iranmanesh A, Naftolowitz D, Tatham N, Cassidy F & Carroll BJ (2001). Corticotropin secretory dynamics in humans under low glucocorticoid feedback. *J Clin Endocrinol Metab* **86**, 5554–5563.

Verrey F, Beron J & Spindler B (1996). Corticosteroid regulation of renal Na,K-ATPase. *Miner Electrolyte Metab* **22**, 279–292.

Whitehead RH (1903). The histogenesis of the adrenal in the pig. *Am J Anat* **2**, 349–360.

Wintour EM, Alcorn D, McFarlane A, Moritz K, Potocnik SJ & Tangalakis K (1994). Effect of maternal glucocorticoid treatment on fetal fluids in sheep at 0.4 gestation. *Am J Physiol* **266**, R1174–R1181.

Wu G, Flynn NE, Knabe DA & Jaeger LA (2000). A cortisol surge mediates the enhanced polyamine synthesis in porcine enterocytes during weaning. *Am J Physiol* **279**, R554–R559.

Yanagibashi K, Shackleton CH & Hall PF (1988). Conversion of 11-deoxycorticosterone and corticosterone to aldosterone by cytochrome P-450 11 beta-/18-hydroxylase from porcine adrenal. *J Steroid Biochem* **29**, 665–675.

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