C48

Comparison of two clones of the BeWo choriocarcinoma cell line

H.N. Thomas, C.J. Ashworth, K.R. Page† and H.J. McArdle*

*Animal Biology Division, SAC, Craibstone Estate, Bucksburn, Aberdeen, †Biomedical Sciences, University of Aberdeen, Aberdeen and *Rowett Research Institute, Bucksburn, Aberdeen, UK*

BeWo cells derived from a choriocarcinoma have been extensively used as a model of placental trophoblast. There are several clones of the BeWo cell line. Authentication and characterisation of these cloned cell lines is important to ensure their identity and purity prior to use in research. In order to be classified as cytotrophoblasts, cells need to fulfil certain criteria including the expression of cytokeratin-7 and secretion of human chorionic gonadotrophin (hCG). BeWo cells were obtained from ATCC (hereafter referred to as the ATCC clone) and from A.L. Schwartz, St Louis Children's Hospital, St Louis, Missouri, USA (hereafter referred to as the Schwartz clone). Cells in culture were maintained in Ham's F12 and DMEM, supplemented with 10% fetal calf serum and 2% penicillin/streptomycin. Aliquots of both clones were sent to the European Collection of Cell Cultures for DNA profiling which indicated 75 and 80% homology between the original cell line and the ATCC and Schwartz clones, respectively. Cells were seeded onto glass coverslips and immunocytochemistry was used to visualise cytokeratin-7 and vimentin. Collagen-coated aluminium oxide filters were seeded with 2×10^5 cells per filter and after 4 days cells were exposed to forskolin for 48 h. Media samples were collected and assayed for hCG. Polycarbonate filters were seeded and cells grown for 10 days. The transepithelial electrical resistance (TEER) was measured in order to determine if cells formed a polarised monolayer. Cells from both clones stained with the anti-cytokeratin-7 antibody. The ATCC clone but not the Schwartz clone showed reactivity with the anti-vimentin antibody. hCG was detectable in media samples from ATCC BeWo at (mean ± S.E.M.) 341 ± 200 international units per litre ($U\,l^{-1}$) and Schwartz BeWo at 437 ± 184 $U\,l^{-1}$ ($n = 6$ for each clone) and increased following exposure to forskolin to 6363 ± 1289 $U\,l^{-1}$ ($n = 3$) in ATCC BeWo and 9746 ± 47 $U\,l^{-1}$ ($n = 3$) in Schwartz BeWo. The Schwartz clone formed a confluent monolayer on the polycarbonate filters and generated a TEER of 42.6 ± 16.6 $\Omega\ cm^2$ ($n = 18$). The ATCC clone did not generate a TEER. These results demonstrate that the ATCC and Schwartz clones differ in characteristics such as monolayer formation and sensitivity to forskolin but they express trophoblast specific markers and undergo biochemical differentiation after exposure to forskolin. Both clones are derived from the parent BeWo line and can be used as trophoblast models.

We thank Dr Alan L. Schwartz (St Louis Children's Hospital, St Louis, Missouri). This work is funded by SEERAD flexible fund and Framework V QLK-1999-00337.

C49

Purinergic receptor activation by agonist stimulation of ^{86}Rb efflux from human placental cytotrophoblast cells in culture

V.H.J. Roberts*, S.L. Greenwood* and L.H. Clarson†

**Academic Unit of Child Health, University of Manchester, St Mary's Hospital, Manchester M13 0JH and †Division of Development, Growth and Function, Rowett Research Institute, Greenburn Road, Aberdeen AB21 9SB, UK*

We have previously demonstrated that extracellular ATP elevates intracellular calcium ($[Ca^{2+}]_i$) and stimulates calcium-activated K^+ efflux from human placental cytotrophoblast cells in culture (Clarson *et al.* 2002). ATP can elevate $[Ca^{2+}]_i$ via P2X or P2Y purinergic receptor activation. Human placental tissue and trophoblast cells express, at the messenger RNA level, P2Y receptors ($P2Y_1$–$P2Y_{11}$) cloned from human tissue, and of the P2X receptors, $P2X_1$, $P2X_2$, $P2X_4$ and $P2X_7$ (Roberts *et al.* 2001; Roberts & Clarson, 2002). In order to determine which of the purinergic receptors have a functional role in the syncytiotrophoblast, the transporting epithelium of the human placenta, this study has examined selective purinergic agonist stimulation of ^{86}Rb (K^+) efflux from cultured cytotrophoblast cells.

Cytotrophoblast cells were isolated from human term placenta and maintained in culture for 66 h, where they provide a model of the syncytiotrophoblast in placenta. Cells were loaded for 2 h with ^{86}Rb, washed, and ^{86}Rb efflux measured at 1 min intervals for 10 min. Eight selective agonists were applied at a concentration of 100 μM from 5 to 10 min of the efflux experiment.

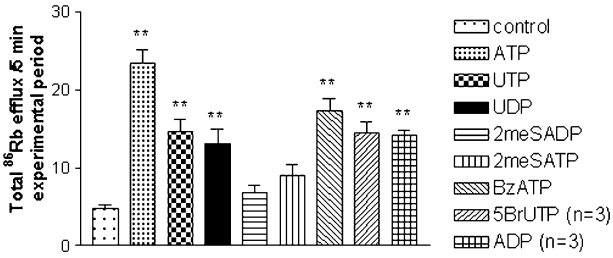

Figure 1. Total ^{86}Rb efflux following agonist stimulation. Values are means ± S.E.M. ($n = 4$ placentas). $P < 0.01$ *vs.* control; ANOVA with Dunnett's *post* test.

Of the agonists studied ATP (a non-selective agonist), UTP ($P2Y_2$ and $P2Y_4$ specific), UDP and 5BrUTP ($P2Y_6$ specific agonists), BzATP ($P2X_7$ selective agonist) and ADP elevated ^{86}Rb efflux above control. 2MeADP and 2MeSATP (agonists for $P2Y_1$, $P2Y_{11}$, $P2X_1$, $P2X_2$ and $P2X_4$) had no effect at this concentration.

Based on published agonist selectivity (Burnstock, 1997) and receptor mRNA expression in placenta, the data reported here are consistent with $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2X_7$ receptors having a role in Ca^{2+}-activated K^+ efflux from the syncytiotrophoblast. These receptors may therefore be important in regulation of placental transport function.

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Clarson LH *et al.* (2002). *Am J Physiol* **282**, R1077–1085.

Roberts VHJ *et al.* (2001). *Placenta* **22**, P56.

Roberts VHJ & Clarson LH (2002). *J Physiol* **539.P**, 125*P*.

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All procedures accord with current local guidelines.

C50

Evidence for store-operated Ca^{2+} entry (SOCE) in human term placental villous fragments

L.H. Clarson*† and T. Powell‡

**Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, †Academic Unit of Child Health, University of Manchester, St Mary's Hospital, Hathersage Road, Manchester M13 0JH, UK and ‡Perinatal Center, Department of Physiology and Pharmacology, Gothenburg University, Gothenburg, Sweden*

Maintaining $[Ca^{2+}]_i$ in the syncytiotrophoblast is necessary for normal placental function and fetal growth. $[Ca^{2+}]_i$ homeostasis is a balance between Ca^{2+} entry and release of Ca^{2+} from intracellular stores (Berridge *et al.* 1998). In non-excitable tissue a major pathway for Ca^{2+} entry is SOCE (Putney, 1997) and this pathway has been implicated in human placenta (Robidoux *et al.* 2000). In this study we have directly examined stimulation of SOCE in the syncytiotrophoblast of term villous fragments following depletion of intracellular stores with thapsigargin (Tg) in a Ca^{2+}-free buffer. The collection and processing of placental tissue was approved by the Committee for Ethical Research at Gothenburg University, Sweden. Term villous fragments were loaded with fura-2 and superfused with control Tyrode buffer (mM: 135 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5.6 glucose, 10 Hepes; pH 7.4 with NaOH). $[Ca^{2+}]_i$ was determined by the 340/380 nm ratio. Fragments were exposed to 1 μM Tg in Ca^{2+}-free Tyrode buffer (zero Ca^{2+} + 1 mM EGTA) followed by control buffer to stimulate SOCE. The effect of 150 μM $GdCl_3$, 200 μM $NiCl_2$ and 200 μM $CoCl_2$ on SOCE was examined by adding blockers to Ca^{2+}-free buffer for 1 min followed by control buffer. Superfusion with control buffer following application of Tg in Ca^{2+}-free buffer caused a rapid increase in fluorescence ratio in 19 out of 22 fragments, suggesting a rapid increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ was reduced significantly by 150 μM $GdCl_3$, 200 μM $NiCl_2$ and 200 μM $CoCl_2$ (see Fig. 1).

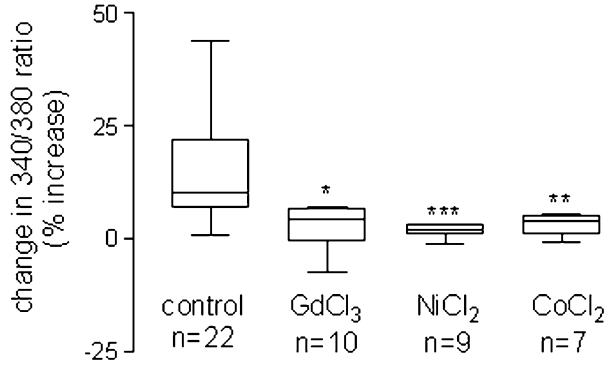

Figure 1. Effect of 150 μM $GdCl_3$, 200 μM $NiCl_2$ and 200 μM $CoCl_2$ on store depletion-stimulated increase in $[Ca^{2+}]_i$. Data are medians and range. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* control. Kruskal-Wallis with Dunn's multiple comparisons test.

These data show that the syncytiotrophoblast of human term placenta exhibits a store-operated Ca^{2+} entry pathway, which is sensitive to $GdCl_3$, $NiCl_2$ and $CoCl_2$. This pathway may be a key mechanism for maintaining $[Ca^{2+}]_i$ in syncytiotrophoblast, particularly following agonist stimulation to release Ca^{2+} from intracellular stores.

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All procedures accord with current local guidelines and the Declaration of Helsinki.

C51

Na^+/H^+ exchanger isoforms in the regulation of intracellular pH of the human placental syncytiotrophoblast

P.F. Speake, S.L. Greenwood, J.D. Glazier and C.P. Sibley

Academic Unit of Child Health, University of Manchester, Manchester M13 0JH, UK

The Na^+/H^+ exchanger (NHE) isoforms NHE1, NHE2 and NHE3 are expressed on both maternal facing, microvillous plasma membrane (MVM) and fetal facing, basal plasma membrane (BM) of the human placental syncytiotrophoblast (Pepe *et al.* 2001). We used fragments of placental tissue to investigate the functional activity of NHE isoforms in the control of intracellular pH in the syncytiotrophoblast.

Fragments of term placenta were incubated in Tyrode buffer (containing (mM): NaCl 135, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1, Mops 10 and glucose 5) with 1 μM BCECF (Molecular Probes) for 30 min at 37 °C, washed in buffer without BCECF and then incubated for 2 × 15 min at 37 °C with fresh Tyrode solution. Fragments were plated onto coverslips and BCECF excited with 440 and 490 nm wavelengths using a monochromator (Cairn Research Limited). Emitted light was detected using a CoolSnap camera (Roper Scientific) and the data acquired using MetaFluor software (Universal Imaging Corporation). NHE activity was assessed as the rate of Na^+-induced recovery of cytosolic pH (pH_i) following an acid load, imposed by prepulsing with 20 mM NH_4Cl, expressed as pH units min^{-1}, mean ± S.E.M., n = number of placentas.

Control rate of recovery from an acid load was 0.18 ± 0.03 pH units min^{-1} (n = 18) and in Na^+-free solutions (Na^+ substituted with choline 135 mM) was −0.04 ± 0.03 (n = 6). In tissue incubated in Na^+-containing Tyrode solution, with amiloride (500 μM), a non-specific inhibitor of NHE transport (Mahnensmith *et al.* 1985) or HOE 694 (100 μM inhibits NHE1; Counillon *et al.* 1993), the rate of recovery from an acid load was significantly reduced to 0.01 ± 0.01 (n = 5) and −0.04 ± 0.03 pH units min^{-1} (n = 6), respectively ($P < 0.05$, ANOVA followed by Bonfferoni multiple comparisons test). S3226 at 1 μM (inhibits NHE3; Schwark *et al.* 1998) had no effect on the rate of recovery, 0.12 ± 0.04 pH units min^{-1} (n = 9).

In conclusion, isolated placental fragments exhibited a Na^+-dependent recovery from an acid load. Both amiloride and HOE694 blunted such recovery, but S3226 had no effect. These data support evidence from membrane vesicles (Speake *et al.* 2002) showing that NHE3 is not functionally active in the term syncytiotrophoblast under basal conditions.

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This work was supported by The Wellcome Trust. HOE 694 and S3226 were kindly donated by Dr Jurgen Punter, Aventis Pharma.

All procedures accord with current local guidelines.

C52

The role of alternative transcripts in differential regulation in intestine and placenta of the zinc transporter hZTL1/ZnT5

Rachel M. Russi and Dianne Ford

School of Cell and Molecular Biosciences, University of Newcastle upon Tyne, King's Road, Newcastle upon Tyne NE1 7RU, UK

We have previously reported that the zinc transporter hZTL1 is regulated at the mRNA level by zinc in human intestinal Caco-2 cells but not in the placental cell-line JAR (Cragg *et al.* 2002). Comparison of the cDNA sequences of hZTL1 and ZnT5 (Kambe *et al.* 2002) reveals that they are splice variants of the same gene and that the assay used previously to examine gene regulation is non-specific for the different transcripts. ZnT5 has additional exons upstream of the first exon of hZTL1, therefore we hypothesise that the two transcripts are expressed from alternative, tissue-specific promoters of which only the promoter active in intestine is zinc responsive. Consistent with this hypothesis, we present evidence that the genomic region immediately upstream of the first exon of the hZTL1 transcript is inactive as a promoter in JAR cells and that an exon unique to the ZnT5 transcript is expressed in JAR but not Caco-2 cells.

A 2899 bp region of the putative hZTL1 promoter region, including the 5′-most end of the cDNA, was subcloned into pBlue TOPO upstream of the *E. coli* β-galacotosidase reporter gene to give the plasmid pC2899. The plasmid pC2899 was transfected into JAR cells for transient expression. The plasmid pcDNA3.1/*lacZ*, including the *E. coli* β-galactosidase gene expressed from the strong CMV promoter, was used as a positive control and pBlue TOPO without insert was included as a negative control. β-Galactoside activity was measured in cell lysates prepared 48 h post-transfection. Data are means ± S.E.M. in arbitrary units; $n = 6$; statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. β-Galactosidase activity expressed from pC2899 was not different from that of the negative control (1.58 ± 0.02 compared with 3.04 ± 0.34); however, activity of the positive control (39.0 ± 3.74) was significantly greater than both the negative control and pC2899 ($P < 0.001$). Addition of 100 μM $ZnCl_2$ 24 h post-transfection did not induce promoter activity. Analysis of RNA from Caco-2 and JAR cells by RT-PCR followed by sequencing, using primers specific for a region unique to the ZnT5 transcript, revealed expression only in JAR cells.

These data are consistent with expression of ZnT5 in JAR cells from a promoter region other than the region of genomic DNA immediately 5′ of the first exon of hZTL1. We suggest that Caco-2 cells express hZTL1 and that the promoter for hZTL1 expression is distinct from the ZnT5 promoter and, unlike the ZnT5 promoter, is transcriptionally activated by zinc.

Cragg RA *et al.* (2002). *J Biol Chem* **277**, 22789–22797.

Kambe T *et al.* (2002). *J Biol Chem* **277**, 19049–19055.

This work was funded by BBSRC grant 13/D11912 and by a BBSRC studentship.

C53

Characterisation of long-term cat placental explant cultures for transport studies: uptake of taurine by system β

E.E. Champion*, S.J. Bailey‡, J.D. Glazier*, C.J.P. Jones†, S.J. Mann‡, J.M. Rawlings‡, C.P. Sibley* and S.L. Greenwood*

**Academic Unit of Child Health and †Obstetrics and Gynaecology, University of Manchester, St Mary's Hospital, Manchester M13 0JH and ‡Waltham Centre for Pet Nutrition, Leics LE14 4RT, UK*

Dietary taurine is essential for cats, and deficiency during pregnancy may lead to abortion, growth retardation or impaired neurological function of kittens. A cat placental fragment model has identified Na^+ and Cl^--dependent taurine transport by system β (Champion *et al.* 2001). Here we describe long-term culture of cat placental explants as a model to study chronic regulation of amino acid transport in this species. Explant viability was assessed by examining explant morphology, endocrine function and by characterising taurine uptake on day 7.

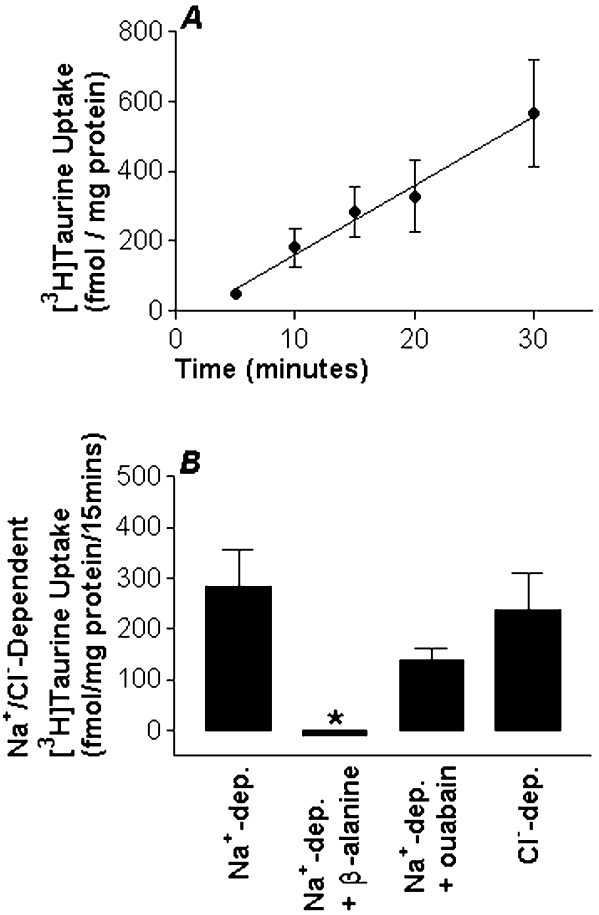

Figure 1. *A*, Na^+-dependent [^{3}H]taurine uptake into day 7 explants was linear over 5–30 min, $r^2 = 0.46$ $P < 0.001$, least squares linear regression. *B*, over 15 min, 10 mM β-alanine (system β substrate) completely inhibited Na^+-dependent [^{3}H]taurine uptake ($P < 0.05$, ANOVA with Bonferroni post test), whereas 3 mM ouabain (Na^+/K^+/ATPase inhibitor) achieved 50 % inhibition. Replacing all chloride with gluconate gave a Cl^--dependent component that was 60.23 ± 4.89 % of overall uptake. Values: means ± S.E.M.; $n = 5$ placentas.

Cat placentas were collected after natural, normal litter production, with full consideration of cat welfare. Explants

(~10 mg wet weight) were cultured in supplemented CMRL-1066 medium for 11 days. Medium was assayed for $PGF_{2\alpha}$ as a measure of endocrine function and lactate dehydrogenase (LDH) as a measure of cellular integrity. On days 2, 5, 7 and 11 explants were fixed in 2.5 % (v/v) glutaraldehyde and embedded in epoxy resin. Semi-thin sections (0.5 μm) were stained with 1 % toluidine blue in 1 % borax. On day 7 [^{3}H]taurine uptake was measured ± Na^+ using methods described previously (Champion *et al.* 2001). Na^+-dependent taurine uptake, attributable to system β, was calculated as the difference between uptake in the presence and absence of Na^+ (Fig. 1).

$PGF_{2\alpha}$ release in pmol (mg protein)$^{-1}$ h^{-1} was 158.7 ± 46.6 at day 2 and increased to stabilise at a mean of 303.2 ± 15.1 over days 3–11 (n = 5; means ± S.E.M.). LDH activity decreased from 264.7 to 61.9 U l^{-1} (mg protein)$^{-1}$ at days 2 and 11, respectively (n = 1). Morphological examination on days 2 and 5 revealed explants of typical lamellar structure. As time progressed, new growth in the lamellar region formed atypical structures whilst degeneration and disruption of the original lamellae architecture occurred.

Taurine uptake into explants at day 7 exhibited characteristics typical of system β. Endocrine function was preserved to day 11 and explant structure to day 5. This *in vitro* culture system may be useful for the study of chronic regulation of amino acid transport by the cat placenta.

Champion EE *et al.* (2001). *Placenta* **22**, P135.

All procedures accord with current UK legislation.

C54

Expression of angiotensin II type 2 receptor in human placenta

J. Zainol, G. Robinson and F. Broughton Pipkin

School of Human Development, Faculty of Medicine and Health Sciences, University of Nottingham, Nottingham NG7 2UH, UK

Angiotensin II (Ang II), the potent agonist of the renin-angiotensin system (RAS), initiates its actions via specific receptors. Two subtypes of Ang II receptors have been cloned in the human, and these are designated AT_1R and AT_2R. When activated both these transmembrane receptors initiate the stimulation of different signalling pathways and produce contrasting effects, as the action of AT_1R antagonises the effect of AT_2R and vice versa (De Gasparo *et al.* 2000). Although its exact role is uncertain, the existence of a placental RAS is well recognised (Nielsen *et al.* 2000). In cows, AT_1R is predominantly expressed in the uterus and AT_2R in the fetal compartment, whereas in the human, AT_2R is predominantly expressed in the uterus and AT_1R in the placenta. This reflects the suggestion that its distribution is tissue and species specific (Nielsen *et al.* 1996).

To determine the expression of AT_2R subtype in human placenta, three polyclonal antibodies raised against three different epitopes of the receptor protein were used. Two of these antibodies are directed against sites close to N-terminal of the receptor protein and the other to a site close to the C-terminal. With ethical approval, placenta from early (first trimester, n = 6), second trimester (n = 5) and term placenta (n = 8) were collected, fixed in formalin and embedded in paraffin wax. The Dako Envision Plus System and the avidin-biotin complex methods were then used to investigate the immunolocalisation of the receptor.

With all three antibodies, immunoreactivity for AT_2R was present in the syncytiotrophoblast and the cytotrophoblast in all three periods of placental growth. Expression of the receptor in perivascular areas was only evident using the antibody closest to the N-terminal and only in the first trimester samples. Overall, the distribution of AT_2R is similar to that reported for AT_1R in the human placenta (Cooper *et al.* 1999).

We have shown for the first time using immunohistochemistry with three different antibodies acting on three different epitopes of the receptor protein that human placenta expresses AT_2R. The different expression of AT_2R with different antibodies may reflect some unknown characteristic of this receptor.

In conclusion, these studies add support to the hypothesis that Ang II has a physiological role in human placenta through stimulation of AT_1R and AT_2R. However, which receptor Ang II activates and what factors determine which receptor is activated remain to be elucidated.

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Nielsen AH *et al.* (2000). *Placenta* **21**, 468–477.

All procedures accord with current UK legislation and the Declaration of Helsinki.

C55

Effect of angiotensin II type 1 receptor blockade on vascular morphology and the pressor response to cortisol in fetal sheep

A.J. Forhead, H. Whiting, F.B.P. Wooding and A.L. Fowden

Department of Physiology, University of Cambridge, Cambridge CB2 3EG, UK

Glucocorticoids increase blood pressure *in utero* although the mechanisms responsible are unclear. This study investigated the role of angiotensin II (AII) in the hypertensive actions of cortisol in fetal sheep during late gestation.

Under general anaesthesia (1.5 % halothane in O_2/N_2O), vascular catheters were implanted into 27 sheep fetuses. From 124 ± 1 days of gestation (term 145 ± 2 days), the fetuses were infused I.V. for 5 days with either: (1) saline (0.9 % NaCl at 2.5 ml day^{-1}, n = 6), (2) cortisol (3–5 mg kg^{-1} day^{-1}, n = 7), (3) AII type 1 (AT_1) receptor antagonist (GR138950, 1–3 mg kg^{-1} day^{-1} in saline, GRS, n = 7), or (4) cortisol and GR138950 (GRC, n = 7). For 2 days before and during the infusion, blood samples, arterial blood pressure and pressor responses to exogenous AII (100 ng kg^{-1}) were obtained daily from the fetuses. All ewes and fetuses were killed according to UK legislation on the fifth day of infusion, and fetal aortae and femoral and carotid arteries were fixed in 10 % formaldehyde. Blood pressure, less amniotic pressure, was measured using pressure transducers and standard recording equipment, and plasma cortisol was measured by RIA. Tunica media area, smooth muscle cell (SMC) nuclei density and proliferation were assessed using histochemical staining (α-SMC actin, haematoxylin and proliferating cell nuclear antigen, respectively). All data were analysed by either one-way ANOVA or two-way ANOVA with repeated measures followed by the Student-Newman-Keuls test.

On all days of infusion, plasma cortisol was greater in both groups of cortisol-infused fetuses than in the respective control fetuses ($P < 0.05$), and GR138950 prevented the pressor response to exogenous AII. Over 5 days of infusion, blood pressure rose by a maximum of 7.6 ± 1.4 mmHg (mean ± S.E.M., $P < 0.05$) in

cortisol, but not saline-infused, fetuses; GR138950 caused similar decreases in blood pressure in both GRS and GRC groups ($P < 0.05$). In GRS-treated fetuses, the fall in blood pressure was significant from the first day of infusion while in GRC-treated fetuses, the decrement was not significant until the second day ($P < 0.05$). There were no differences between the groups in tunica media area, or the number and density of total and dividing SMC in any of the vessels studied.

Therefore, in the sheep fetus, 5 days of AT_1 receptor antagonism suppresses the cortisol-induced rise in blood pressure. These results suggest that cortisol may increase blood pressure within 24 h of administration by a mechanism that is independent of the fetal renin-angiotensin system. Thereafter, AII, via the AT_1 receptor, may mediate, in part, the hypertensive effects of cortisol *in utero*, although this appears not to involve changes in arterial SMC number.

This work was supported by The Royal Society.

All procedures accord with current UK legislation.

C56

Reduced growth of the lung in fetuses from ewes exposed to acute nutrient restriction in mid-gestation

S. McMullen and D.C. Wathes

Royal Veterinary College, Hawkshead Lane, Hatfield, Herts AL9 7TA, UK

Compromised fetal growth has been associated with increased risks of mortality and morbidity at birth and in later life. Maternal diet is known to have a profound effect on fetal growth. Extensive sheep farming may lead to periods of acute undernutrition, e.g. after snowfall. In humans, ill health may reduce food intake temporarily. This study assessed the effects of acute nutrient restriction in mid-gestation on parameters of fetal growth.

All procedures were performed under the UK Animals (Scientific Procedures) Act, 1986. Welsh Mountain ewes of body condition score 2.0–2.5 were fed a complete pelleted diet providing 100 % of their maintenance requirements. The ewes were bedded on wheat straw, to provide minimum nutritional value, with free access to water. At day 83 of gestation, ewes were allocated to fed or nutrient restricted groups (NR). The concentrate ration of the NR ewes was reduced from days 83 to 85 and withdrawn completely between days 85 and 90. At day 90, half the ewes (NR: $n = 7$, fed: $n = 8$) were humanely slaughtered. The remainder (NR: $n = 9$, fed: $n = 9$) were fed their maintenance diet until slaughter at day 135 (term ~147 days). Upon removal, the fetus was given a fatal intracardial injection of sodium pentobarbitone. At each time point, data were analysed using Student's unpaired *t* test with a level of significance of $P < 0.05$; data are presented as means ± S.E.M.

Fetal weight was unaffected by the mid-gestation nutrient restriction at both time points. At day 90, there was a tendency towards decreased weight of the fetal lung in the NR group, both as actual weight (fed, 27.1 ± 1.63; NR, 22.9 ± 0.93 g; $P < 0.06$) and as a percentage of fetal body weight (fed, 4.9 ± 0.25; NR, 4.3 ± 0.19%; $P < 0.1$). This was associated with a significantly decreased thoracic girth (fed, 17.4 ± 0.26; NR, 16.5 ± 0.34 cm; $P < 0.05$) and a reduced volume of uterine fluid (fed, 724 ± 49.6; NR, 530 ± 66.9 ml; $P < 0.05$). At day 135, the difference in actual lung weights between the groups was significant (fed, 116.4 ± 6.01; NR, 100.0 ± 3.18 g; $P < 0.05$).

Reduction in amniotic fluid volume imposes exaggerated trunk flexion on the fetus, narrowing the thoracic cavity and displacing the diaphragm upwards (Harding *et al.* 1991); this may limit the expansion of the lungs by lung fluid. Reduced amniotic volume is associated with fetal lung hypoplasia, which may lead to respiratory insufficiency at birth and is present in 14–20 % of neonatal autopsies (Sherer *et al.* 1990). In lambs, intra-uterine growth retardation is associated with impairments in respiratory function during early postnatal life (Joyce *et al.* 2001). The association of reduced fluid volume and thoracic girth with decreased lung weight in this study suggests that this chain of events occurred in response to the mid-gestation nutrient restriction, with potentially adverse consequences for the neonate.

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Joyce BJ *et al.* (2001). *Pediat Res* **50**, 641–649.

Sherer DM *et al.* (1990). *Obstet Gynecol Surv* **45**, 792–803.

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All procedures accord with current UK legislation.

C57

Relationship between hepatic fatty acid profile and δ-6-desaturase gene expression in different sized pig fetuses at three stages of gestation

C.J. McNeil, A.M. Finch, K.R. Page†, S.D. Clarke‡, C.J. Ashworth* and H.J. McArdle.

*Rowett Research Institute, Bucksburn, Aberdeen, †Biomedical Sciences, University of Aberdeen, Aberdeen, ‡Pennington Biomedical Research Center, Baton Rouge, Louisiana, USA and *Animal Biology Division, SAC, Craibstone Estate, Bucksburn, Aberdeen, UK*

δ-6-Desaturase (D6D) activity limits the desaturation of essential fatty acids (FA) during the synthesis of long chain polyunsaturated fatty acids (LC-PUFA). These LC-PUFA include vital n-6 and n-3 FA such as arachidonic acid (AA) and docosahexanoic acid (DHA). D6D is downregulated by glucocorticoids such as cortisol that are found at higher concentrations in the plasma and allantoic fluid of growth-retarded pig fetuses in late gestation (Ashworth *et al.* 2001). It is possible that some developmental problems of low birthweight neonates are caused by altered prenatal FA supply. In this study we investigated liver D6D gene expression and FA profile in growth-retarded and average-sized porcine fetuses at early, mid- and late gestation.

Large White × Landrace sows were exsanguinated under deep anaesthesia (8 % v/v halothane) on days 45 ($n = 6$), 65 ($n = 6$) and 100 ($n = 6$) of gestation in accordance with UK legislation. Day 65 and 100 fetuses were killed by an intracardiac injection of sodium pentobarbitone. RNA and lipid were extracted from the liver of an average sized ('normal') and the smallest ('runt') fetus from each litter. Gene expression of D6D was measured using Northern blot analysis and normalised to a maternal control. At day 100 D6D expression (2.96 ± 0.26) was significantly higher ($P < 0.05$, using ANOVA) than at days 45 (2.08 ± 0.14) and 65 (2.08 ± 0.19). Runt D6D expression was not significantly different from that of the normal at any stage of gestation. Fetal liver lipids were extracted, separated into phospho-neutral and glycolipid fractions and the FA profiles then determined by gas chromatography. Fetal size and stage of gestation had no significant effect on the proportion of FA as LC-PUFA in any lipid fraction.

In conclusion, D6D gene expression was increased at day 100 of gestation. This was not accompanied by greater proportion of FA as LC-PUFA and was, therefore, likely to be part of a general up-regulation of FA metabolism. No evidence for any downregulation of D6D gene expression was detected in runts. Furthermore, products of D6D (e.g. AA and DHA) were not found in reduced proportions in liver lipid. Therefore, the rate of desaturation by D6D was unlikely to have limited LC-PUFA supply in this model of intra-uterine growth retardation.

Ashworth CJ *et al.* (2001). *Reprod* suppl. **58**, 233–246.

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All procedures accord with current UK legislation.

C58

A cardiovascular and endocrine study of singleton and twin fetuses

D.S. Gardner*, A.L. Fowden† and D.A. Giussani†

**School of Human Development, Academic Division of Child Health, Queen's Medical Centre, Nottingham NG7 2UH and †Department of Physiology, University of Cambridge, Cambridge CB2 3EG, UK*

Perinatal complications, morbidity and mortality are some 3- to 6-fold higher in twin relative to singleton fetuses, even after adjustment for gestational age (Cheung *et al.* 2000). While some studies have examined differences in the hypothalamic-pituitary-adrenal (HPA) axis between singleton and twin fetuses (Schwartz & Rose, 1998; Edwards & McMillen, 2002), no study has yet investigated basal and stimulated cardiovascular and other endocrine physiology in single *vs.* twin fetuses during late gestation. The present study has examined fetal cardiovascular (blood pressure, heart rate, femoral vascular resistance) and endocrine (ACTH, cortisol, catecholamines, vasopressin: AVP) variables in single and twin fetuses during baseline and during exposure to an episode of acute hypoxaemia.

Twenty sheep fetuses were chronically instrumented under general anaesthesia (1.5% halothane in O_2/N_2O) with vascular catheters and a flow probe around the fetal femoral artery. Of these, $n = 10$ were singleton and $n = 10$ were twin fetuses, in which only one fetus from each twin pregnancy was instrumented. At 0.9 gestation all fetuses were exposed to a single episode of acute hypoxaemia by reducing maternal F_{i,O_2} for 1 h. Fetal carotid blood samples were taken at appropriate intervals before, during and after the 1 h episode of acute hypoxaemia for analyses of blood gases, metabolites and concentrations of ACTH, cortisol, AVP (RIA) and catecholamines (HPLC), with assays validated for ovine plasma. All data were analysed by either one-way ANOVA or two-way ANOVA with repeated measures followed by Tukey's test.

Basal blood gas and metabolic status was similar in singleton and twin fetuses and was appropriate for fetuses at 130 ± 3 dGA (pH 7.34 ± 0.01; P_{a,O_2}, 22 ± 1 mmHg; blood glucose, 0.96 ± 0.10 mmol l^{-1}). Basal heart rate was similar but mean arterial blood pressure (ABP) and femoral blood flow (FBF) tended to be lower ($P = 0.07$) in twin relative to singleton fetuses (HR, 166 ± 4 *vs.* 165 ± 5 beats min^{-1}; ABP, 44.5 ± 2.3 *vs.* 51.4 ± 2.9 mmHg; FBF, 32.2 ± 3.3 *vs.* 38.5 ± 2.6 ml min^{-1}). Basal ACTH (39.0 ± 4.7 *vs.* 30.7 ± 2.6 pg ml^{-1}), adrenaline (98 ± 23 *vs.* 83 ± 13 pg ml^{-1}) and AVP (2.4 ± 0.6 *vs.* 3.6 ± 0.6 pg ml^{-1}) concentrations were similar, but basal cortisol was lower (17.2 ± 1.4 *vs.* 26.9 ± 3.3 ng ml^{-1}) and noradrenaline higher (720 ± 167 *vs.* 359 ± 39 pg ml^{-1}) in twin, relative to singleton, fetuses. In addition, the cardiovascular and plasma ACTH and catecholamine responses to acute hypoxaemia were similar, but there were trends for the increase in cortisol to be blunted, and that of AVP to be exacerbated, during hypoxaemia in twins relative to singleton fetuses, respectively. All ewes and fetuses were humanely killed at the end of all experiments by giving an overdose of barbiturates. At post-mortem twins were of significantly lower body weight than single fetuses (2.28 ± 0.14 *vs.* 2.86 ± 0.14 kg).

The data indicate that certain aspects of the physiological differences between singleton and twin fetuses may be related to body mass, such as basal blood pressure and femoral blood flow, while others reflect genuine alterations in basal and stimulated function (pituitary-adrenal axis) due to multiple pregnancy.

Cheung YB *et al.* (2000). *Am J Epidemiol* **152**, 1107–1116.

Edwards LJ & McMillen IC (2002). *Biol Reprod* **66**, 1562–1569.

Schwartz J & Rose JC (1998). *Am J Physiol* **274**, R1–8.

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All procedures accord with current UK legislation.

C59

Cardiovascular regulation in chronically hypoxic alligator embryos

Jordi Altimiras*, James W. Hicks† and Dane A. Crossley II†

**Department of Biology, Linköpings Universitet, Sweden and †Department of Ecology and Evolutionary Biology, University of California, Irvine, USA*

Chronic hypoxia (10 kPa O_2) stunts overall embryonic growth in alligator embryos (*Alligator mississippiensis*). At 90 % of a 72 day incubation length (30 °C) hypoxic embryos averaged 25.8 ± 1.7 g ($n = 8$), which was significantly lower than 38.7 ± 1.3 g ($n = 18$) of normoxic controls. All data are presented as means ± S.E.M. Wilcoxon and Mann-Whitney *U* tests were used to test for statistical significance of the data.

However, cardiac growth is maintained, which results in a concurrent increase in the heart-to-body mass index in hypoxic embryos (0.58 ± 0.03 *vs.* 0.48 ± 0.01 %). Maintenance of cardiac growth during chronic hypoxia while other organ systems are growth restricted has also been observed in other species such as the domestic fowl (Metcalfe *et al.* 1981).

Chronic hypoxia also had important functional consequences. Measurement of heart rate and arterial blood pressure via catheterization of a tertiary chorioallantoic artery showed that hypoxic embryos at 90 % incubation had significantly lower resting heart rates (69.2 ± 3.6 *vs.* 86.3 ± 2.4 min^{-1} in controls) and lower resting blood pressures (1.39 ± 0.14 *vs.* 2.29 ± 0.18 kPa in controls).

The subsequent sequential injection of antagonists of cholinergic (atropine, 3 mg kg^{-1}), β-adrenergic (propranolol, 3 mg kg^{-1}) and α-adrenergic receptors (phentolamine, 1 mg kg^{-1}) quantified the role of these receptors on the resting cardiovascular status of the embryo. In control embryos (top panels in Fig. 1), atropine and phentolamine induced a significant hypotension and no change in heart rate, while propranolol triggered a significant hypertensive response coupled to a marked bradycardia. This is similar to the response of embryonic chickens (Crossley & Altimiras, 2000; Crossley *et al.* 2002). Although starting at lower blood pressures and heart rates, chronically hypoxic embryos

display the same absolute responses (bottom panels in Fig. 1). All embryos were killed with an overdose of xylocaine.

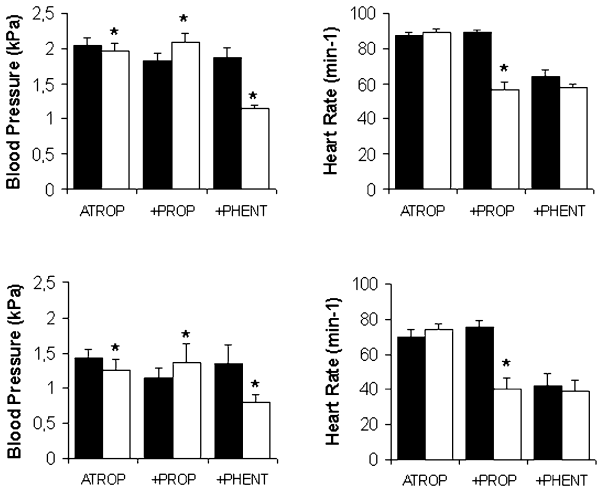

Figure 1. Blood pressure and heart rate before (■) and after (□) the sequential administration of atropine (3 mg kg^{-1}), propranolol (3 mg kg^{-1}) and phentolamine (1 mg kg^{-1}) in 90 % alligator embryos. Top graphs correspond to normoxic conditions, bottom graphs to chronic hypoxic incubation (10 kPa O_2). Data are means ± S.E.M. * Significant differences ($P < 0.05$).

We conclude that cholinergic and adrenergic responses are not altered under chronic hypoxia despite the lowered heart rates and blood pressures displayed by embryos chronically exposed to hypoxia.

Crossley DA II & Altimiras J (2000). *Am J Physiol* **279**, R1091–1098.
Crossley DA II *et al.* (2002). *Am J Physiol* (in the Press).
Metcalfe J *et al.* (1981). *Resp Physiol* **46**, 81–88.

This study was carried out in accordance with USA National guidelines for animal research (IACUC Protocol Number 2000-2180, University of California at Irvine).

All procedures accord with current National and local guidelines.

C60

Effects of maternal hypercholanaemia and ursodeoxycholic acid treatment on rat placenta-maternal liver tandem excretory pathway for fetal cholephilic substances

M.A. Serrano, R.I.R. Macias, O. Briz, M. Vallejo and J.J.G. Marin

University of Salamanca, Spain

Owing to the immaturity of the hepatobiliary system the placenta–maternal liver tandem must carry out the elimination of fetal bilirubin and bile acids that are produced during intrauterine life. This excretory route is impaired by maternal hypercholanaemia. The aim of the present study was to characterize this alteration and to investigate the effect of ursodeoxycholic acid treatment (UDCA; intragastric, 60 μg (100 b.w.)$^{-1}$ day^{-1}) in pregnant rats, in which hypercholanaemia was induced by obstructive cholestasis for the last week of pregnancy (OCP) under anaesthesia. Obstruction was released on day 21 and bile was drained to reach steady state in bile acid output before carrying out the experiments under pentobarbital anaesthesia (approved by the Ethical Committee of the University of Salamanca).

Real-time quantitative RT-PCR indicated that the level of placental lactogen II mRNA, a trophoblastic marker, was reduced in OCP but normalized in OCP+UDCA.

The trophoblastic expression of organic anion transporter polypeptides (Oatp1, Oatp2 and Oatp4) and multidrug resistance associated proteins (Mrp1, Mrp2 and Mrp3) was enhanced at similar levels in both OCP and OCP+UDCA although, in general, the overall expression in the whole placenta was OCP+UDCA > OCP ⩾ control. However, kinetic analysis of ATP-dependent [^{14}C] glycocholate transport by apical membrane vesicles isolated from rat trophoblast revealed that transport efficiency (V_{max}/K_m) was control ≈ OCP+UDCA > OCP. Electron microscopy studies revealed that OCP induced loss of trophoblastic tissue together with morphological alterations that included the disappearance of plasma membrane microvilli. The functional tissue able to carry out transplacental exchange was evaluated by the diffusion of antipyrin that was reduced in OCP and partially restored by UDCA. The ability to secrete [^{14}C] glycocholate into bile after infusion into the umbilical artery of one '*in situ*' perfused placenta or through the maternal jugular vein was dramatically reduced in OCP due to impairments in both placental transfer and liver secretion. Our results indicate that maternal hypercholanaemia induced morphological and functional changes that were in part prevented by UDCA, which had beneficial effects on both components of the placenta–maternal liver tandem excretory pathway. At the placental level where the effects were stronger, these include preservation of the amount and structure of the trophoblast and enhanced expression and function of carrier proteins involved in placental transfer of bile acids and other cholephilic organic anions. This may account for the fact that maternal and fetal body weight, together with the number of fetuses per pregnancy, were reduced by OCP and restored by UDCA treatment.

All procedures accord with current National and local guidelines.

C61

Impact of maternal nutrient restriction in early to mid-gestation on insulin-like growth factor-2 (IGF-2) receptor mRNA abundance in the 110 day gestation ovine placenta

G. Gopalakrishnan*, S.M. Rhind†, C.E. Kyle†, J. Bispham*, A.N. Brooks‡, M.T. Rae†, T. Stephenson* and M.E. Symonds*

**School of Human Development, University Hospital, Nottingham NG7 2UH, †Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen AB15 8QH and ‡Astrazeneca, Alderley Park, Cheshire SK10 4TJ, UK*

Maternal nutrient restriction during early to mid-gestation, the period of rapid placental growth (30–80 days) in the sheep, is associated with altered fetal and placental development (Heasman *et al.* 1998). Consequently the resulting offspring may be at increased risk of diseases, in adulthood, including hypertension and obesity. The insulin-like growth factors (IGF) are important regulators of growth pre- (IGF-2) and postnatally (IGF-1). In particular IGF-2 hormone levels regulate fetal growth with down-regulation of its receptor being linked to fetal overgrowth (Young *et al.* 2001). This study aimed to determine the extent to which maternal nutrient restriction at specific stages of early and mid-gestation might result in altered placental IGF-2 receptor mRNA abundance.

Thirty-one Scottish Blackface singleton-bearing ewes of similar liveweight and body condition were individually housed from day of mating. Ewes were then randomly assigned to one of five

nutritional groups ($n = 4$–8 per group). Group 1, control animals, were fed 100 % (8 MJ day^{-1}) of metabolisable energy (ME) requirements to maintain liveweight from day of mating to 110 days gestation. Groups 2, 3 and 4 were fed 50 % (4 MJ day^{-1}) of ME requirements, from 0–30, 31–65 and 66–110 days gestation, respectively, and 100 % at all other times. Group 5 received 50 % of ME requirements from mating to 110 days gestation. At 110 days gestation, all ewes were humanely killed with a barbiturate overdose (Euthatal; 500 mg ml^{-1}, 30 ml, I.V.), to enable placental and fetal sampling. All procedures were carried out according to UK legislation. The samples collected were frozen at −70 °C until later analysis. Total RNA was extracted from the placentomes and IGF-2 receptor mRNA abundance was examined by RT-PCR, using oligonucleotide primers specific to the IGF-2 receptor (forward 5′-ACCGGCAC-TTCAACTACACC-3′ and reverse 5′-ACTCAGAATGA-CGGCTTCGT-3′). Results are expressed as mean values and standard errors in arbitrary units (a.u.) as a ratio of an 18S rRNA internal control. Statistically significant differences between groups were assessed using a Kruskal-Wallis H and Mann-Whitney U tests ($P < 0.05$).

There was no difference in fetal and placental weights between groups. At 110 days gestation placental IGF-2 receptor mRNA abundance was greater for ewes nutrient restricted between 31 and 65 days compared with the control group 1 (Group 1, 0.14 ± 0.02; Group 3, 0.19 ± 0.01 a.u.). There was no significant difference in IGF-2 receptor mRNA abundance between the other groups nutrient restricted during all other gestational periods.

Maternal nutrient restriction specifically during 31–65 days gestation up-regulates IGF-2 receptor abundance in the ovine placenta, to limit growth by the fetus. This coincides with the period of maximal placental growth and suggests that adaptive responses within the placenta may be dependent on the stage of nutritional manipulation.

Heasman L *et al.* (1998). *Pediatr Res* **44**, 546–551.
Young LE *et al.* (2001). *Nature Genetics* **27**, 153–154.

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All procedures accord with current UK legislation.

C62

Maternal nutrient restriction during early to mid-pregnancy and the programming of mitochondrial cytochrome *c* and voltage-dependent anion channel in the liver and lung of the ovine fetus

D.P. Yakubu, J. Dandrea, A. Mostyn, M.E. Symonds and T. Stephenson

Academic Division of Child Health, School of Human Development, University Hospital, Nottingham NG7 2UH, UK

Mitochondria play a central role in energy metabolism. This process is regulated in part by specific mitochondrial proteins. These include cytochrome *c*, located in the mitochondrial intermembrane space and the voltage-dependent anion channel (VDAC) located in the outer mitochondrial membrane (Kirk & Strange, 1998), which are involved in energy conversion, provision and apoptosis. Maternal nutrition during pregnancy plays an important role in determining mitochondrial protein abundance in the offspring (Budge *et al.* 2003). The extent to which such effects may be programmed *in utero* remain to be established. In the present study we examined the influence of maternal nutrient restriction over the period of placental growth on the abundance of these proteins in the fetal liver and lung.

Eighteen Welsh Mountain ewes of similar body weight and fat distribution were individually housed from 28 days of gestation. Six ewes were nutrient restricted (NR); these consumed 3.5 MJ of metabolisable energy (ME) per day (60 % of ME requirements for maintenance and growth of the conceptus) until 80 days gestation, with six controls (C) consuming 6.8–7.5 MJ day^{-1}. After 80 days gestation, until near to term (term = 147 days), all animals were fed to appetite and consumed 8–10.9 MJ day^{-1}. Tissues were sampled from 4–5 singleton fetuses from NR and C ewes at either mid- (80 days) and late (140 days) gestation after humane euthanasia (barbiturate overdose, 100 mg kg^{-1} pentobarbital sodium: Euthatal). Mitochondria were prepared and analysed by immunoblotting. Results (in arbitrary units) are presented as means ± S.E.M. Differences between nutritional groups were analysed using a Mann-Whitney U test.

Table 1. Influence of maternal nutrient restriction during early to mid-pregnancy on the abundance of VDAC and cytochrome *c* in the liver

		VDAC	Cytochrome *c*
		Mean ± S.E.M.	Mean ± S.E.M.
80 days	NR	61 ± 3.9	52 ± 4.3
	C	51 ± 3.1	34 ± 4.0*
140 days	NR	79 ± 15.2	29 ± 1.8
	C	78 ± 12.9	33 ± 3.8

Significant differences between groups: *$P < 0.05$.

Table 2. Influence of maternal nutrient restriction during early to mid-pregnancy on the abundance of VDAC and cytochrome *c* in the lung

		VDAC	Cytochrome *c*
		Mean ± S.E.M.	Mean ± S.E.M.
80 days	NR	34 ± 2.1	6 ± 0.7
	C	31 ± 1.9	10 ± 1.8*
140 days	NR	98 ± 11.6	25 ± 5.3
	C	65 ± 5.6	11 ± 1.3*

Significant differences between groups: *$P < 0.05$.

Body and organ weights were similar between groups at either sampling age. In livers from NR fetuses cytochrome *c* abundance was significantly greater than C at 80 but not 140 days gestation. VDAC abundance between groups was similar but levels increased with gestational age. In the lung, however, cytochrome *c* abundance was lower in NR animals at 80 days gestation, but greater than C near to term when a similar trend was observed for VDAC.

In conclusion, maternal nutrient restriction in early fetal life has differential effects on mitochondrial protein abundance, which are tissue specific and only appear to result in persistent effects in the fetal lung.

Budge H *et al.* (2003). *Pediatr Res* (in the Press).
Kirk K & Strange K (1998). *Ann Rev Physiol* **60**, 719–739.

D.P. Yakubu is supported by a University of Nottingham International Office Scholarship.

All procedures accord with current UK legislation.

C63

Effect of maternal nutrient restriction during early to mid-gestation on hepatic insulin-like growth factor (IGF) mRNA abundance in juvenile sheep

M.A. Hyatt, G. Gopalakrishnan, J. Bispham, T. Stephenson, D. Walker and M.E. Symonds

Academic Division of Child Health, School of Human Development, University Hospital, University of Nottingham, Nottingham NG7 2UH, UK

Maternal nutrient restriction during the period of rapid placental growth (30–80 days) followed by adequate feeding up to term results in normal sized fetuses with a larger placenta in which the normal relationship between plasma insulin-like growth factor (IGF)-I and body conformation is lost (Heasman *et al.* 2000). IGFII and -I are synthesised primarily by the liver and are essential for normal fetal and postnatal growth and development, respectively, but the extent to which they may be nutritionally programmed after birth has not been examined. The aim of the present study was to determine whether maternal nutrient restriction during early to mid-gestation can result in altered hepatic IGF mRNA abundance in the resulting offspring.

Twelve singleton-bearing Welsh Mountain ewes of similar age, weight, and body condition score were entered into the study and individually housed from 28 days gestation. Six ewes were fed a nutrient restricted (NR) diet (3.5 MJ day^{-1}) until 80 days gestation, whilst the remaining ewes were fed a control (C) diet 6.8–7.5 MJ day^{-1}. After 80 days gestation, until term (147 days), all ewes received (6.8–7.5 MJ day^{-1}), sufficient to fully meet their metabolisable energy (ME) requirements to produce a 4.5 kg lamb at term. Lambs were born spontaneously, and at 6 months of age, all animals were humanely euthanased (100 mg kg^{-1} pentobarbital sodium: Euthatal, I.V.), to enable liver tissue sampling. All procedures were carried out according to UK legislation. Tissue samples collected were stored at −70 °C until molecular analysis. Total RNA was extracted from liver tissue, reverse transcribed and abundance of IGF-1, and IGF-2 mRNA was measured by RT-PCR using oligonucleotide primers specific to ovine IGF-1 (forward 5′-CCCATCTCCCTGGATTTCTT-3′ and reverse 5′-ACATCTCCAGAATCCTCAGA-3′) and IGF-2 (forward 5′-TCACAGCAGGAAAGTCTATG-3′ and reverse 5′-GGCACAGTAAGTCTCCAGCA-3′). Results are given as means ± S.E.M. in arbitrary units as a ratio of 18S rRNA and are expressed as a percentage of a reference sample present on all gels. Statistical differences between nutritional groups were analysed using a Mann-Whitney *U* test.

There were no significant differences in growth, body or liver weights between C and NR offspring up to 6 months of age. The abundance of IGF-II (C 51 ± 8 a.u; NR 112 ± 21 a.u ($P < 0.05$)) but not IGF-I (C 115 ± 36 a.u; NR 145 ± 30 a.u) mRNA was higher in livers of NR offspring.

The increased hepatic IGF-II mRNA in NR compared with C offspring may represent an adaptation to nutrient restoration after 80 days gestation. It did not, however, contribute to any immediate change in juvenile growth but may be important in regulating endocrine adaptations during subsequent periods of nutrient deprivation.

Heasman L *et al.* (2000). *Reprod Fertil Dev* **12**, 1–6.

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All procedures accord with current UK legislation.

C64

Influence of genotype on the discrepancy between UCP2 mRNA and protein expression in piglet subcutaneous adipose tissue (SCAT)

A. Mostyn, J.C. Litten, K.S. Perkins, M.C. Alves-Guerra*, C. Pecqueur*, B. Miroux*, M.E. Symonds† and L. Clarke

*Animal Research Group, Imperial College at Wye, Wye, Ashford, Kent TN25 5AH, †Academic Division of Child Health, School of Human Development, University Hospital, Nottingham NG7 2UH and *CEREMOD, 9 rue Jules Hetzel, 92190 Meudon, France*

Neonatal mortality is greater in the leaner commercial (C) porcine genotypes compared with the ancient Meishan (M) breed, which has a higher percentage of body fat. Piglets do not express UCP1, the mitochondrial protein primarily responsible for increasing heat production in neonatal mammals, although they do express UCP2 mRNA in SCAT (Damon *et al.* 2000). C piglets express higher levels of UCP2 mRNA in SCAT than M (Mostyn *et al.* 2002). However, differences in UCP2 mRNA do not always correlate with protein changes because of an upstream open reading frame in the UCP2 gene (Pecqueur *et al.* 2001). The present study aimed to determine whether the ontogeny of UCP2 protein abundance differed between C and M genotypes.

Piglets from 15 C and 15 M litters were ranked according to birth weight and the three median piglets were assigned to be randomly sampled on days 0, 4, 7, 14 or 21 of neonatal life. Piglets were weighed and colonic temperature measured on these days and a venous blood sample taken. SCAT was also sampled from each piglet following euthanasia with an overdose of barbiturate (100 mg kg^{-1} pentobarbital sodium: Euthatal). UCP2 mRNA was measured as described previously (Mostyn *et al.* 2002) and UCP2 protein abundance determined by immunoblotting using a fully validated UCP2 antibody (Pecqueur *et al.* 2001). Results, in arbitrary units (means ± S.E.M.), are expressed as a percentage of a reference sample present on all gels. Significant differences between breeds at each sampling age were assessed by GLM.

As shown in Table 1, UCP2 protein abundance was higher in M piglets at all ages except on the first day of birth. This difference was greatest on days 4 and 7, despite C piglets having higher UCP2 mRNA expression. UCP2 protein abundance was not correlated with UCP2 mRNA in either breed.

Table 1

Postnatal age	Commercial		Meishan	
	mRNA	Protein	mRNA	Protein
0	7.6 ± 3.5	10.9 ± 1.5	5.17 ± 4.2	7.7 ± 1.9
4	17.8 ± 5.3	25.3 ± 4.9*	6.0 ± 1.6	65.4 ± 8.1
7	12.2 ± 3.9	35.3 ± 5.6*	2.8 ± 0.2	133.0 ± 31.5
14	6.4 ± 2.4	43.1 ± 23.5	7.6 ± 1.3	116.0 ± 19.0
21	7.6 ± 3.1	n/a	8.4 ± 3.3	62.6 ± 23.6

* Significance at the $P < 0.05$ level between breeds.

In conclusion, we confirm that changes in UCP2 protein can occur in the absence of a parallel change in mRNA, indicating that a post-transcriptional factor is critical in regulating expression of UCP2. Identification of the mechanism promoting this response in M piglets may subsequently enable neonatal survival to be enhanced in C breeds.

Damon M *et al.* (2000). *Gene* **246**, 133–141.

Mostyn A *et al.* (2002). *J Endo* (in the Press).

Pecqueur C *et al.* (2001). *JBC* **276**, 8705–8712.

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All procedures accord with current UK legislation.

C65

Postnatal ontogeny of insulin-like growth factor (IGF) and prolactin receptor (PRL-R) in ovine perirenal adipose tissue

J. Bispham*, L. Clarke†, M.E. Symonds* and T. Stephenson*

**Academic Division of Child Health, School of Human Development, University Hospital, Nottingham NG7 2UH and †Huxley School, Imperial College at Wye, University of London, Ashford TH25 5AH, UK*

Prolactin (PRL) and insulin-like growth factor (IGF)-I acting through class I cytokine receptors (R) regulate fetal growth and development and may also control adipose tissue deposition. After birth rapid growth of adipose tissue occurs as the newborn establishes independent feeding (Clarke *et al.* 1997). Plasma prolactin and IGF-I concentrations peak around the time of birth, coincident with the initiation of non-shivering thermogenesis in brown adipose tissue. Then over the first week of life abundance of the brown adipose tissue-specific uncoupling protein 1 decreases deposition and white adipose tissue is promoted. The following study aimed to determine whether the abundance of mRNA species for IGF and prolactin receptor (PRL-R) increase in adipose tissue over the first week of neonatal life.

Twelve twin-bearing Bluefaced Leicester × Swaledale ewes of known mating date and of similar body weight and parity were entered into the study. All ewes were allowed to give birth normally at term and were randomly assigned to one of the lamb sampling times (i.e. within 1 h of birth, 2, 4 and 7 days post-lambing ($n = 3$ per group)). The lambs were humanely killed by intravenous overdose of sodium pentobarbitone to allow perirenal adipose tissue sampling. All samples were weighed and snap-frozen in liquid nitrogen before being stored at −80 °C until analysis. Total RNA was extracted. All work performed was carried out in accordance with both national and local guidelines. The expression of mRNA species for IGF-I and both the long and short forms of PRL-R were examined by RT-PCR using specific oligonucleotide primers: IGF-I (Genbank M31735: forward 5′-CCC-ATC-TCC-CTG-GAT-TTC-TT-3′ and reverse 5′-ACA-TCT-CCA-GCC-TCC-TCA-GA-3′ product 401 bp), long form of PRL-R (Genbank AF041257: forward 5′-CCA-GAT-ACC-TAA-TGA-CTT-CCC-3′ and reverse 5′-TCT-TCG-GAC-TTG-CCC-TTC-TCC-3′ product 200 bp), short form of PRL-R (Genbank AF041977: forward 5′-CCA-GAT-ACC-TAA-TGA-CTT-CCC-3′ and reverse 5′-GCC-CTT-CTA-TTA-AAA-CAC-AGA-3′ product 229 bp). Results, in arbitrary units (a.u.; mean ± S.E.M.) are a ratio of an 18S rRNA internal control. Differences between ages were analysed using Kruskal-Wallis and Mann-Whitney *U* tests.

During the first week of life adipose tissue weight steadily increased (0.1 days: 20 ± 2.8 g, 2 days: 26 ± 3.7 g, 4 days: 41 ± 3.9 g, 7 days: 70 ± 9.1 g; $P = 0.01$).

IGF I mRNA abundance increased up to 2 days before reaching a plateau (0.1 days: 62.1 ± 12.7 a.u., 2 days: 117.7 ± 2.1 a.u., 4 days: 104.1 ± 2.2 a.u., 7 days: 117.1 ± 10.7 a.u.; $P = 0.05$), whereas IGF-II abundance remained unchanged.

In contrast, abundance of mRNA for both the long and short forms of PRL-R peaked at 4 days of age and then declined (e.g. short form: 0.1 days: 3.9 ± 1.4 a.u., 2 days: 4.6 ± 0.9 a.u., 4 days: 8.5 ± 2.7 a.u., 7 days: 5.3 ± 1.3 a.u.).

The peak in mRNA abundance for IGF-I and PRL-R between 2 and 4 days after birth may be important in enhancing adipose tissue up to 1 week after birth.

Clarke L *et al.* (1997). *Exp Physiol* **82**, 1015–1027.

All procedures accord with current UK legislation.

PC44

The effect of blockers of Ca^{2+}-permeable channels on luteinising hormone releasing hormone (LHRH)-stimulated human chorionic gonadotrophin (hCG) secretion by villous fragments from human term placenta

K. Cole* and L.H. Clarson*†

**Academic Unit of Child Health, University of Manchester, St Mary's Hospital, Hathersage Road, Manchester M13 0JH and †Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, UK*

Secretion of hCG from human placenta is regulated by agonists such as LHRH via an increase in $[Ca^{2+}]_i$ (Mathialagan & Rao, 1989). It has previously been proposed that LHRH raises $[Ca^{2+}]_i$ in human placenta by stimulation of L-type voltage-dependent Ca^{2+} channels (Sharma & Rao, 1997); however, there is little direct evidence for these channels in this tissue (Cronier *et al.* 1999). Rather, the role of voltage-independent Ca^{2+} channels for raising $[Ca^{2+}]_i$ in human placenta is becoming increasingly apparent (Clarson *et al.* 2002; Moreau *et al.* 2002). Therefore, in this study we have examined the effect of various blockers of Ca^{2+}-permeable channels on LHRH-stimulated hCG secretion from human term placental villous fragments.

Fragments were dissected from term placentas, washed in control Tyrode buffer (mM: 135 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5.6 glucose, 10 Hepes; pH 7.4 with NaOH) and then incubated for 1 h (six fragments per vial) with 10 μM LHRH in control Tyrode buffer in the presence and absence of three channel blockers. The blockers examined were: 50 μM SKF96365 (blocks store-operated Ca^{2+} channels), 150 μM $GdCl_3$ (a blocker of Ca^{2+} entry pathways including non-selective cation channels) and 1 μM nifedipine (blocks L-type Ca^{2+} channels). Following incubation, hCG secretion was assessed by assay and placental fragment blotted wet weight was determined. Data were calculated as mIU ml^{-1} (mg wet weight)$^{-1}$.

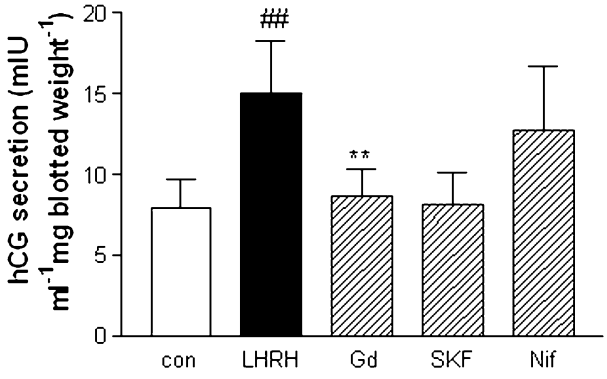

Figure 1. Graph showing LHRH-stimulated hCG secretion from human term placenta. Values are means ± S.E.M., $n = 6$. ##$P < 0.01$ *vs.* control; **$P < 0.01$ *vs.* LHRH (ANOVA + *t* test with Bonferroni correction). con, control; LHRH, 10 μM LHRH; Gd, 150 μM $GdCl_3$; SKF, 50 μM SKF96365; Nif,

1 μM nifedipine (all blockers were in the presence of 10 μM LHRH).

LHRH (10 μM) significantly increased hCG secretion. This was significantly reduced in the presence of 150 μM $GdCl_3$. SKF96365 (50 μM) reduced LHRH-stimulated secretion, which was significant at the 10% level. Nifedipine (1 μM) had no effect on LHRH-stimulated hCG secretion (see Fig. 1). These data suggest that the role of L-type Ca^{2+} channels in LHRH-stimulated hCG secretion from human term placenta is inconclusive. It does, however, appear that voltage-independent Ca^{2+}-permeable channels, such as non-selective cation and store-operated Ca^{2+} channels, are of greater importance for entry of Ca_o^{2+} following LHRH stimulation and thus in hCG secretion from human term placenta.

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All procedures accord with current local guidelines.

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$VEGF_{165}$ and $VEGF_{165}$b expression in normal placenta and pre-eclamptic placenta

Sarah J. Hudson*, Alyson J. Hunter†, Steven J. Harper*, David O. Bates* and Lucy F. Donaldson*

*Departments of *Physiology and †Obstetrics & Gynecology, University of Bristol, Bristol, UK*

Vascular endothelial growth factor (VEGF) plays a vital role in the development and maintenance of placental function throughout pregnancy and is implicated in complications of pregnancy, affecting the placenta. Pre-eclampsia (PE) is a leading cause of maternal morbidity and mortality, affecting 5–10% of first pregnancies worldwide. VEGF has been shown to be significantly elevated in the serum of pregnant women before the onset of pre-eclampsia (Hunter *et al.* 2000). This study looks at two VEGF isoforms: $VEGF_{165}$, and a recently discovered isoform, $VEGF_{165}$b, (Bates *et al.* 2002), which is thought to be anti-angiogenic.

Placental biopsies were obtained post-birth or from Caesarean section from uncomplicated pregnancies ($n = 19$) and pregnancies complicated by PE ($n = 14$). PE was determined as in Hunter *et al.* (2000). Ethical approval was granted by The North Bristol NHS Trust Ethics Committee and written consent obtained from all volunteers.

Total RNA was extracted from 100–200 mg of tissue homogenised in Trizol reagent (Invitrogen Life Technologies), using the method of Chomczynski & Sacchi (1997). 10 μg of mRNA was reverse transcribed using 250U Moloney murine leukaemia virus RT (Abgene) and 0.5 μg Oligo dT primer (Invitrogen Custom Primers) at 37 °C for 1.5 h in 20 μl volume.

cDNA was amplified using primers to specifically detect each isoform and a pan-specific primer that detects all isoforms of VEGF. 1 μg of cDNA was used per 20 μl reaction, with 1.5 mM $MgCl_2$, 1 μM forward and reverse primer, 200 μM dNTPs and 0.5 U Thermoprime plus DNA Polymerase (Abgene). PCR protocols were optimised: $VEGF_{165}$, 65 °C annealing for 40 cycles producing an amplicon of 200 bp; $VEGF_{165}$b, 69 °C annealing for 35 cycles producing an amplicon of 220 bp. 1 ng of each isoforms' cloned cDNA was included as a control.

Data were analysed using Fisher's exact test. No significant difference ($P = 1.00$), was found between the two groups of placental samples in the expression of $VEGF_{165}$ (7/14 PE and 9/19 normal positive); or $VEGF_{165}$b (6/14 PE and 4/19 normal positive). However, $VEGF_{165}$ was expressed at a significantly higher frequency than $VEGF_{165}$b in normal patients. All samples were positive for at least one isoform of VEGF using pan-specific primers. In conclusion, we have shown in this study that $VEGF_{165}$ and $VEGF_{165}$b are not seen in increased frequency in placenta affected by pre-eclampsia than in placenta from uncomplicated pregnancy, but that neither isoform is present in up to half of normal placentae.

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All procedures accord with current local guidelines.

PC46

Investigation of the involvement of *rho*-associated kinase (ROK) in agonist-induced contractions of rat and human uterine smooth muscle

D.F. Soydemir, Y.-H. Lee*, M. Riley, P.N. Baker and M.J. Taggart

*Maternal & Fetal Health Research Centre, University of Manchester, Manchester, UK and *College of Medicine, Yonsei University, Seoul, Korea*

Delivery of the fetus and placenta at term requires precise regulation of the mechanisms underlying uterine contractility. Recent evidence indicates that agonist-induced Ca^{2+}-sensitisation of smooth contractility, including the uterus, involves activation of a rho-associated kinase (ROK) signalling cascade (Lee *et al.* 2001). In addition, thromboxane receptor stimulation increased ROK activity in cultured human myometrial cells (Moore *et al.* 2002). Therefore, we have analysed the effects of pharmacological inhibition of ROK on *in vitro* thromboxane-stimulated uterine contractility. This was also compared with the effect of ROK inhibition on oxytocin-stimulated contractions of myometria isolated from (a) near-term (gestation day 19–21) pregnant rats (killed by cervical dislocation following stunning in accordance with national guidelines;) or (b) non-labouring term humans (following written informed consent according to local ethics committee guidelines; gestation 37–41 weeks). Small myometrial strips were dissected and mounted for contractile activation on standard organ baths in HCO_3^--buffered physiological saline solution (37 °C, 95% air and 5% CO_2). Addition of the ROK inhibitor HA1077 (10 μM) significantly reduced the amplitude and duration of contractions to 10 μM of the thromboxane mimetic U46619 ($P < 0.05$, Wilcoxon non-parametric test); peak contractile amplitude was reduced by 26 ± 6.0% of control (mean ± S.E.M., $n = 8$). For oxytocin-stimulated contractions (0.1 μM), addition of another ROK inhibitor, Y-27632 (10 μM), also significantly reduced contractile duration and amplitude; peak contractile amplitude in myometria of humans was reduced by 31 ± 3.7% ($n = 8$) similar to the findings of others (Kupittayanant *et al.* 2001) and decreased by 44 ± 7.1% ($n = 6$) in rat myometria. Western blotting analysis of homogenised tissue indicated strong expression of ROKα isoform in myometria from both term rats and humans. The data indicate

that pharmacological inhibitors of ROK partly reduce *in vitro* agonist-induced contractile force of intact myometria of pregnant rats and humans. Further investigation is required to determine the importance of ROK activation in (i) uterine contractions during labour at term and (ii) the manifestation of preterm labour that may be linked to enhanced thromboxane receptor stimulation.

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All procedures accord with current UK legislation.