Maternal nutrient restriction in sheep: hypertension and decreased nephron number in offspring at 9 months of age

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Pregnant ewes were fed either a 50% nutrient-restricted (NR; $n = 8$) or a control 100% (C; $n = 8$) diet from day 28 to day 78 of gestation (dGA; term = 150 dGA). Lambs were born naturally, and fed to appetite throughout the study period. At 245 ± 1 days postnatal age (DPNA), offspring were instrumented for blood pressure measurements, with tissue collection at 270 DPNA. Protein expression was assessed using Western blot, glomerulus number determined via acid maceration and hormone changes by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). NR lambs had higher mean arterial pressure (MAP; 89.0 ± 6.6 *versus* 73.4 ± 1.6 mmHg; $P < 0.05$), fewer renal glomeruli (57.8 ± 23.8 *versus* 64.6 ± 19.3 × 10^4; $P < 0.05$), increased expression of angiotensin converting enzyme (ACE) in the renal cortex (942 ± 130 *versus* 464 ± 60 arbitrary pixel units (apu); $P < 0.03$), and increased angiotensin II receptor AT2 expression in the renal medulla (63.3 ± 12.1 *versus* 19.5 ± 44.2 × 10^4 apu; $P < 0.03$). All data are presented as mean ± S.E.M. The present data indicate that global maternal nutrient restriction (50%) during early to mid-gestation impairs renal nephrogenesis, increases MAP, and alters expression of AT2 and ACE without an associated change in birth weight. These data demonstrate the existence of a critical window of fetal susceptibility during early to mid-gestation that alters kidney development and blood pressure regulation in later life.

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Epidemiological evidence in concert with experimental studies has consistently demonstrated that offspring exposed to poor nutrition *in utero* are at increased risk for many adult onset diseases including renal disease (Hoy *et al.* 1998), heart disease (Roseboom *et al.* 2000), hypertension (Barker *et al.* 2000) and diabetes (Barker, 1990; Phillips *et al.* 1994). Various modes of nutritional stress such as global nutrient restriction, protein restriction, and over-nutrition have been employed to evaluate fetal adaptations and postnatal outcomes (Langley-Evans, 2001; Nishina *et al.* 2003; Wallace *et al.* 2004). Analysis of data from the Dutch hunger winter demonstrates that the period of gestation in which maternal nutrient restriction occurs has effects on birth weight and incidence of adult disease (Ravelli *et al.* 1998; Roseboom *et al.* 2000). Offspring exposed to famine during early gestation only were born at a normal birth weight but were at increased risk of heart disease (Roseboom *et al.* 2000). Researchers from a number of countries have independently shown the developing kidney is susceptible to the effects of an altered growth trajectory induced by maternal nutrient restriction, and these effects have also been related to elevated blood pressure in the offspring in the rat (Langley-Evans, 2001; Woods *et al.* 2001; Woods *et al.* 2004) and sheep (Hawkins *et al.* 2000; Gopalakrishnan *et al.* 2004).

Studies in the rat have demonstrated elevated blood pressure, reduced nephron number and altered gene expression of key components of the renin–angiotensin system (RAS) in the offspring of dams subjected to gestational protein restriction (Langley-Evans & Jackson, 1995; Woods, 1999; Langley-Evans, 2001; Woods *et al.* 2001). More recently, Woods *et al.* (2004) have demonstrated male offspring are more sensitive to the effects of moderate maternal nutrient restriction than female offspring. Importantly, only a single recent report by Gopalakrishnan *et al.* (2004) have shown hypertension in postpubescent offspring of nutrient-restricted sheep, a long gestation length animal with a long history of use in the investigation of fetal development; while a lone report has indicated increased blood pressure in 85-day-old offspring (Hawkins *et al.* 2000).

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The RAS has been consistently implicated in the renal maldevelopment associated with the progression towards a hypertensive state. Perhaps the most notable effect of *in utero* perturbation of the RAS is a reduced nephron endowment concomitant with elevated blood pressure (Woods & Rasch, 1998). Prior work has shown that effects initiated by the angiotensin II type 1 receptor (AT1) have profound impacts on renal development and subsequent morphology and physiology (Woods & Rasch, 1998); however, renal AT1 expression remains unchanged in several forms of experimental hypertension (Harrison-Bernard *et al.* 1999; Navar *et al.* 2002). While the effects of the angiotensin II type 2 receptor (AT2) disruption during development appear minimal (Hein *et al.* 1995; Ichiki *et al.* 1995), the AT2 receptor shows increased expression in several models of adult renal injury (Ruiz-Ortega *et al.* 2003). Furthermore, expression of angiotensin converting enzyme (ACE), a key enzyme in the RAS (Erdos, 1976), may have a profound impact on renal haemodynamics since ACE both converts angiotensin I to angiotensin II (Ang II; a potent vasoconstrictor) and degrades bradykinin (a vasodilator).

The aims of the present study were to characterize the effects of 50% maternal nutrient restriction in sheep during early to mid-gestation (days 28–78; term = 150 days), which we have already shown induces a 30% reduction in fetal weight in the mid-gestation ovine fetus (Vonnahme *et al.* 2003), with re-alimentation to 100% rations during the latter half of pregnancy aimed to ameliorate differences in birth weight on: (1) systemic blood pressure at rest in offspring; (2) nephron endowment, a persistent fetal maldevelopment that may contribute to increased blood pressure-affected offspring; (3) expression of intrarenal RAS proteins (AT1, AT2 and ACE) as postnatal evidence of adaptations to impaired fetal renal development; (4) intrarenal apoptosis via caspase-dependent (caspase-3 activity) and caspase-independent (apoptosis-inducing factor expression) pathways.

Methods

Animals and feeding

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. Multiparous western white-faced ewes (Rambouillet/Columbia breeding) from the University of Wyoming flock were oestrous synchronized using a controlled internal drug release (CIDR) implant (Pfizer, Auckland, New Zealand) followed 10 days later by implant removal and an intramuscular injection of $PGF_{2\alpha}$ (10 mg). On day 20 of pregnancy, ewes were weighed so that individual diets could be provided on a metabolic body weight basis ($weight^{0.75}$) as described in detail elsewhere (Vonnahme *et al.* 2003). In short, the control diet consisted of pelleted beet pulp with minerals and vitamins added to meet nutrient requirements for pregnant ewes during early gestation (National Research Council (NRC), 1985). Animals were fed a maintenance diet (∼2% of body weight) of hay during the pre- and periconceptual periods. On day 21 of gestation, all ewes were placed in individual pens and fed the control ration (100% of NRC requirements for all micro- and macronutrients). On gestational day 28 (dGA 28), ewes were randomly assigned to a control-fed group (C, $n = 20$) or a nutrient-restricted group (NR, $n = 20$, fed 50% of NRC requirements for all micro- and macro nutrients). Ewes were weighed weekly and rations adjusted for weight gain (i.e. increased amount of feed) or loss (i.e. decreased amount of feed). At dGA 79 all NR dams were re-alimented to the control ration (100% of NRC requirements), and all dams were fed these rations until dGA 104. At dGA 105 until the day of parturition, the rations fed to all pregnant ewes were increased according to NRC guidelines for late gestational ewes.

Parturition and postnatal procedures

Ewes were allowed to deliver naturally. Only male lambs from single or twin pregnancies were utilized in this study. Within 3 h of parturition, lambs were thoroughly dried; and birth weight, crown–rump length (CRL), abdominal and thoracic circumferences, biparietal diameter, humerus length and sex were determined and recorded. These measurements were also recorded at 60 days of postnatal age (DPNA). Following parturition all ewes were given free choice access to hay. At approximately 1 week and 1 month of age all lambs were immunized with 1 ml *Clostridium perfringens* types C and D plus tetanus toxoid administered intramuscularly (Colorado Serum Co. Denver, CO, USA). A *Clostridium* C and D booster was administered (1 ml, I.M.) at approximately 100 days of age (Electroid, Schering-Plough, Kenilworth, NJ, USA). Prior to two weeks of age, all lambs were tail-docked and the males castrated by placement of rubber rings according to Federation of Animal Science Societies recommendations. Lambs were given free access to a standard commercially available creep feed (Lamb Creep B30 w/Bovatec; Ranch-Way Feeds, Fort Collins, CO, USA) while still nursing. Post-weaning, lambs were transitioned to a feed concentrate (All-American Show Lamb Grower; Ranch-Way Feeds, Inc.) with partial rations of hay until the feed concentrate could be tolerated by the lambs. After weaning, lambs were group housed and fed to appetite with alfalfa hay with corn supplement once a day between 6 : 30am and 7 : 00am. Following surgery (see below) the animals were housed in metabolism crates with *ad libitum* access to alfalfa pellets and water.

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Surgical procedures

The study cohort consisted of 18 wethers. Two of these, one from each treatment group, were injured between weaning and the start of the study and were killed. At 245 ± 1 DPNA (mean ± S.E.M.) the remaining animals (C = 8 (6 twins, 2 singletons), NR = 8 (3 twins, 5 singletons)) underwent catheter implantation as follows. The animals were fasted for 24 h with water removed for the second 12 h prior to surgery. After ketamine hydrochloride sedation (1 g), induction and maintenance of general anaesthesia was performed using isoflurane (4% induction, 2% maintenance) and 100% oxygen (3 l min^{-1}) administered via a nose mask. An incision was then made in the neck, the right jugular vein and carotid artery exposed and polyethelene catheters (Tygon Microbore Tubing, Formulation S-54-HL; o.d.: 2.3 mm, i.d.: 1.3 mm) inserted to a length of 15 cm and secured to surrounding connective tissue. The skin was then closed and triple antibiotic cream (Triple Antibiotic Ointment, Taro Pharmaceuticals Inc.) placed on the incision line. The neck was then wrapped (Vetrap, 3M) such that the catheters exited along the spine between the shoulder blades. Animals were housed in individual metabolic crates, given free choice feed and water, and allowed a minimum of five days' recovery before any further procedures were attempted. Post-operative antibiotics were administered intravenously for the first 3 days of recovery (ampicillin, Bristol-Myers Squibb Company, 1 g animal^{-1} for 3 days) with feed and water consumption, faeces and urine production and animal behaviour monitored daily for the remainder of the study. An intravenous glucose tolerance test was performed at 250 DPNA. Blood pressure recordings began at 255 DPNA, 10 days after surgery as described below. Recordings were made while the animals were standing quietly in metabolism crates prior to being exposed to a CRH/AVP challenge. A hypotension stress test followed at 260 DPNA.

Blood collection and hormone analysis

Ten days after surgery, on 255 DPNA, two blood samples (6 ml each) were taken, separated by 15 min, prior to the period of blood pressure measurement and pooled. Cortisol levels were evaluated in a single radioimmunoassay (RIA) utilizing a Coat-A-Count radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA, USA). Standards, plasma and control sera (50 μl in duplicate, modification of kit protocol of 25 μl) were incubated along with radio-iodinated cortisol (Diagnostic Products Corp.; 1.0 ml) in antibody-coated polypropylene tubes (Diagnostic Products Corp.) at 37°C for 45 min. Following incubation, the supernatant was decanted isolating the antibody-bound fraction of the radiolabelled cortisol and the remaining precipitate was retained for counting. The intra-assay coefficients of variation for samples measuring 4.1 and 78.23 ng ml^{-1} were 4.14 and 3.2%, respectively. The interassay coefficients of variation were 8.66 and 8.03% for samples measuring 28.73 and 144.04 ng ml^{-1}, respectively. The sensitivity of the assay, defined as 90% bound/free, was 2.0 ng ml^{-1} plasma from two pooled baseline plasma samples according to the manufacturers' instructions (DSL-2100, Diagnostic Systems Laboratories, Inc., Webster, TX, USA); the intra-assay coefficient of variation (CV) was 9%.

ACTH was measured using a commercial chemiluminescent enzyme immunometric assay (IMMULITE ACTH, Diagnostic Products Corp.). IMMULITE ACTH is a solid phase, two-site sequential chemiluminescent immunometric assay for use with the IMMULITE Automated Analyser and designed for the quantitative measurement of ACTH in EDTA plasma. The intra-assay CVs for samples containing 14.4 and 205.1 pg ml^{-1} were 6.96 and 3.12%, respectively. The inter-assay CVs for samples measuring 52.5 and 149.5 pg ml^{-1} were 10.3 and 16.1%, respectively. The level of sensitivity defined as the concentration two standard deviations above zero was 9 pg ml^{-1}.

Data acquisition

Arterial blood pressure was recorded using Cobe pressure transducers (Argon Medical, Athens TX, USA) positioned at the level of the heart and acquired at 50 Hz to a personal computer using Windaq Pro+ software (DataQ Instruments, Akron OH, USA). Mean arterial pressure and heart rate were calculated using custom software from the pressure waveform with the animals standing. A 60-minute recording of blood pressure and heart rate was made starting at 9 : 00am on the 10th day after surgery.

Tissue collection

Animals were killed at 270 DPNA by captive bolt and exsanguination according to United States Department of Agriculture (USDA) meat processing procedures. Organs including the brain, heart, liver, pancreas, kidneys and adrenal glands were removed and weighed. The cortex and medulla from one kidney were separated prior to further processing. The other kidney was stored in saline with sodium azide (0.9% NaCl, 0.01% NaN_3) at 4°C for further analysis. Portions of tissue samples from the organs of interest were flash frozen in liquid nitrogen.

Kidney acid maceration and glomerulus counting

The method of acid maceration utilized in the present study is an adaptation of the Merlet-Benichou modification (Merlet-Benichou *et al.* 1994) of the classic

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method by Damadian and colleagues (Damadian *et al.* 1965). Following killing of the wether, kidneys were harvested, cleansed of perirenal tissues, weighed and placed in physiological saline (0.09% NaCl with 0.01% NaN_3) for storage at 4°C until further evaluation could proceed. Prior to acid maceration the kidneys were decapusulated and placed in 50% HCl at 37°C for 1 h. Following the incubation period the HCl was decanted and the kidneys were rinsed with three volumes of tap water, and stored overnight in tap water at 4°C. The kidney was then gently dilacerated and brought to a final volume 2000 ml with tap water, taking care to recover all tissue from glassware used in each step. Kidneys were coded to blind the technicians as to the experimental groups that which each sample belonged to.

The present study adapted the procedure for counting glomeruli previously described by Damadian *et al.* (1965) in the following manner. Following acid maceration and mechanical dissociation of the kidney tissue, the kidney solution was thoroughly mixed using a magnetic stir bar and stir plate. Once the solution was evenly distributed, 250 μl aliquots were placed in a rectangular counting chamber permanently fixed to a glass slide. Counting was performed with the aid of a manual hand counter and a standard light microscope (Olympus America, Melville, NY, USA) at 40× magnification. Each aliquot was counted in duplicate, and four aliquots were counted and the aliquot averages summed to obtain a glomeruli ml^{-1} value. The glomeruli ml^{-1} value was multiplied by the total volume of the kidney solution to obtain the glomerulus number (GN) for each kidney. Values for GN are the average values obtained from two independent observers. Several kidneys were chosen at random and glomerulus counts were repeated to establish a 5% CV for the procedure.

Protein extraction and Western blot analysis

Approximately 0.25 g of kidney, LV or RV tissue was homogenized in 2 ml of RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% SDS, 0.5% sodium deoxycholate, 0.1% NP-40). Samples were centrifuged at 16 000 *g* for 15 min to pellet cellular debris. Aliquots of the supernatants were placed into clean tubes and stored at −80°C. Total soluble protein in the extracts was quantified using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

Fifty micrograms of total protein was aliquoted and the final volume adjusted to 7.5 μl with water. Lithium dodecyl sulfate (LDS) sample buffer with dithiothreitol (DTT) (Invitrogen, Carlsbad CA, USA), 2.5 μl, was added and the samples were incubated at 95°C for 10 min. Samples were cooled to room temperature and pulse spun to collect the sample. Proteins were separated under denaturing reducing conditions using either precast 10%, or 4–12% gradient Tris-glycine gels (Invitrogen) in an SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at 180 constant volts. Proteins were subsequently transferred to polyvinylidene fluoride (PVDF) membranes in a Tris–glycine transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol), and gels were poststained to ensure complete protein transfer. See-Blue 2 Pre-stained Markers (Invitrogen) were used for protein size determinations.

Immunodetection of the following proteins was accomplished using commercially available polyclonal antibodies raised against AT1 (306), AT2 (H-143) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunodetection of ACE-1 (cat. no. 3500), and apoptosis-inducing factor (AIF) (cat. no. 16501) was done with commercially available mono- or polyclonal antibodies obtained from Chemicon International (Temecula, CA, USA). Immunodetection of proteins was done in the following manner: membranes were blocked for 1 h in protein blocking solution (Blotto in TBS, Pierce; Rockford, IL, USA), then incubated in the primary antibody for 1 h (AT1, AT2, all at 1 : 500; AIF at 1 : 1000; ACE at 1 : 5000) in Blotto TBS blocking buffer (Pierce). All dilutions were determined empirically to achieve minimal background. Following the primary incubation, the membrane was washed 4 × 5 min each using Pierce Western wash (Pierce). The membranes were then incubated in the appropriate secondary antibody: either goat antirabbit IgG-conjugated horseradish peroxidase (Santa Cruz Biotechnology) diluted to 1 : 1000, or goat antimouse IgG HRP at 1 : 5000 (Calbiochem cat. no. 401215; San Diego, CA, USA). Following incubation in the secondary antibody, membranes were washed 4 × 5 min in Pierce Western wash (Pierce). Blots were detected using a chemiluminescent detection system (Supersignal West Pico substrate, Pierce). Membranes were incubated in chemiluminescent substrate for 5 min, and exposed to film (Kodak, New Haven, CT, USA) from 0.5 to 20 min as necessary to detect a signal. Images were optically scanned (Hewlitt-Packard 5200C with HP PrecisionScan software v2.02) and digitized, and the antigen signal was quantified by pixel density (Un Scan It gel v5.1; Silk Scientific, Orem, UT, USA). Quantification was performed only after linearity was established between the amount of protein and film exposure time. Membranes were reprobed for detection of the β-actin band (42 kDa) using rabbit antihuman β-actin antibody as a housekeeping control.

Caspase-3 activity assay

Caspase-3 activity was determined in an assay adapted from Ren *et al.* (2002). Snap frozen renal tissue was homogenized in ice-cold assay buffer (50 mM Hepes, 1 mM DTT, 0.1 mM EDTA, 0.1% (3-[3-cholamidopropyl-dimethyl-ammonio)]-1-propane sulfonate

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(CHAPS), 0.1% NP-40, 100 mM NaCl, pH 7.4) and pelleted at 4°C to remove cellular debris. Protein quantification was performed using the Bio-Rad DC assay. The assay for caspase-3 activity was carried out in a 96-well plate. Each well contained 20 μl of cell lysate, 70 ml of assay buffer and 10 μl of 1 mM caspase-3 colorimetric substrate Ac-DEVD-pNA (CalBiochem). The sample plate was incubated at 37°C for 2 h, during which time the caspase in the sample was allowed to cleave the chromophore p-NA from the substrate molecule. Absorbance readings were obtained at 405 nm with the caspase-3 activity being directly proportional to the colormetric reaction. Caspase-3 activity was then normalized per microgram of protein in each sample.

Statistical analysis

All data are presented as mean ± S.E.M. and significance was accepted when $P < 0.05$. Statistical comparisons could not be properly made between singleton and twinned offspring because of the small sample of C singletons ($n = 2$). Comparisons were made between C and NR offspring, using independent Student's t tests, with a Welch's correction applied when appropriate (GraphPad Prism 4.0, San Diego, CA, USA). Regression analysis and correlation coefficients were calculated for either C or NR animals separately or all animals grouped as a whole (SigmaPlot 2001 Chicago, IL, USA).

Results

All data are presented at mean ± standard error of the mean (S.E.M.), except where indicated. In all analyses the sample size for both C and NR treatment groups was eight except where noted.

Biometry: morphometrics (birth and 9 months of age)

NR lambs were born the same size as C lambs (Table 1). While kidney weight was greater in the NR compared to the C lambs, this difference was not quite statistically significant (Table 1, $P = 0.06$). No relationship was observed between weight at birth and 9-month body weight in the wethers in the present study ($P = 0.36$).

Hormone analyses

Blood samples collected during the period of blood pressure measurement revealed that blood pH, gases and haematocrit (HCT) were within expected ranges for all animals and not different between the control and NR groups (Table 1). Plasma cortisol and ACTH concentrations were not different between the groups (Table 1).

Table 1. Biometric measurements at birth and nine months of age, endocrinology and blood chemistry and cardiovascular measurements at nine months of age in the offspring of pregnant ewes fed control (C) and nutrient-restricted (NR) diets during early to mid-gestation

	C	NR	*P*
Biometry at birth	($n = 10$)	($n = 10$)	
Weight (kg)	5.81 ± 0.22	5.95 ± 0.25	n.s.
CRL (cm)	53.83 ± 0.69	55.4 ± 1.10	n.s.
Biometry at 9 months	($n = 8$)	($n = 8$)	
Kidney weight (g)	129.8 ± 4.2	120.3 ± 3.1	0.06
Endocrinology at 9 months	($n = 8$)	($n = 8$)	
ACTH (pg ml^{-1})	12.2 ± 1.5	16.8 ± 2.6	n.s.
Cortisol (ng ml^{-1})	6.9 ± 1.4	10.1 ± 2.6	n.s.
Blood chemistry at 9 months	($n = 8$)	($n = 8$)	
pH	7.38 ± 0.02	7.40 ± 0.04	n.s.
P_{O_2} (mmHg)	92.2 ± 1.3	93.5 ± 2.1	n.s.
P_{CO_2} (mmHg)	32.0 ± 1.8	31.3 ± 1.3	n.s.
Hct (%)	37 ± 1	37 ± 1	n.s.
Cardiovascular parameters at 9 months	($n = 8$)	($n = 8$)	
MAP (mmHg)	73.4 ± 1.6	89.0 ± 6.6	<0.05
HR (beats min^{-1})	72.2 ± 2.7	74.5 ± 4.7	n.s.

CRL, crown–rump length; Hct, haematocrit; HR, heart rate; MAP, mean arterial pressure; n.s., not significant.

Blood pressure and heart rate

Mean arterial pressure (MAP) was approximately 17 mmHg higher in the offspring exposed to nutrient restriction during development than in animals whose mothers were fed the C diet (Table 1). There was no difference in heart rate (HR) between the dietary groups (Table 1).

Glomerulus number and kidney weigh

Absolute GN was lower in the NR kidneys than in the C kidneys (578 362 ± 23 792 *versus* 646 284 ± 19 309; $P < 0.05$). Kidney weight relative to body weight was also unchanged between the NR and C lambs (2.3 ± 0.1 g kg^{-1} *versus* 2.2 ± 0.1 g kg^{-1}; $P < 0.05$). When GN was evaluated as a function of blood pressure, a significant negative relationship was observed among the NR lambs ($r = –0.85$; $P < 0.01$; $y = –5.82x + 416.3$, Fig. 1). This negative relationship between GN and mean arterial pressure was not observed in the C lambs ($r = 0.38$; $P = 0.35$; $y = –3.23x + 95.0$; Fig. 1). Regression analysis was performed on the data for plasma cortisol, GN and MAP. This analysis did not reveal any significant relationship between those variables ($P > 0.2$).

Renal protein expression

Expression of the Ang II receptors AT1 (103 ± 20 *versus* 137 ± 13 apu) and AT2 (1178 ± 186 *versus*

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1375 ± 116 apu) did not differ in the kidney cortex between the C and NR wethers. ACE expression was increased two-fold in the cortex of the NR wethers when compared to the C wethers (942 ± 130 *versus* 464 ± 60 apu, $P < 0.05$; Fig. 2).

Expression of the AT1 receptor (862.6 ± 116.4 *versus* 1165.8 ± 195.9 apu), and ACE (2078 ± 263 *versus* 1790 ± 216 apu) in the kidney medulla were unchanged between the C and NR wethers. However, AT2 abundance was increased in the renal medulla of the NR lambs when compared to the C lambs (195367 ± 44156 apu *versus* 63337 ± 12164 apu, $P < 0.03$; Fig. 3).

Evaluation of apoptosis in the kidney

Levels of AIF expression were not different in the renal cortex (610 ± 77 *versus* 623 ± 41 apu, $P > 0.05$) or medulla (204761 ± 11147 *versus* 205423 ± 21466 apu) between the C and NR lambs. Likewise, caspase-3 activity was not different in the renal cortex (29 ± 2 *versus* 29 ± 3 U mg^{-1}) or renal medulla (44 ± 6 *versus* 46 ± 6 U mg^{-1} of protein) between dietary groups (C *versus* NR). However, activity of caspase-3 was found to be greater in the renal medulla when compared to the renal cortex in both the C (44 ± 6 *versus* 29 ± 2 U mg^{-1}; $P < 0.05$) and the NR (46 ± 6 *versus* 29 ± 3 U mg^{-1}; $P < 0.05$).

Discussion

The present study reports several important findings with regard to the developmental origins of adult diseases in a large animal model. Foremost, the present endeavour demonstrates increased MAP in young male offspring of ewes that were nutrient restricted during a discrete period (dGA 28–78) of gestation. The observed MAP increase coincides with a reduced nephron endowment, and alterations in expression of renin–angiotensin system (RAS) proteins in the kidney. The present endeavour therefore characterizes a large animal model of programmed hypertension and provides direction for further research in this area by demonstrating complicity of the kidney in the reported hypertension. For many of the endpoints presented there is no difference between twins and singletons, but because of their more compromised intrauterine situation we suggest that twins represent a quantitatively more challenged example of the effects of maternal nutrient restriction. Taken together, these observations lend further support to the thrifty phenotype hypothesis of metabolic dysfunction and support hypotheses for a renal contribution to developmentally programmed hypertension.

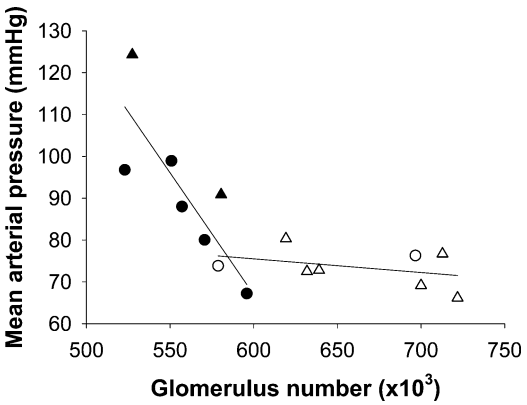

Figure 1. Mean arterial pressure (MAP) *versus* glomerulus number (GN) of control (C, $n = 8$, open symbols) and nutrient-restricted (NR, $n = 8$, filled symbols) lambs at 9 months of age

NR lambs show a strong negative relationship between MAP and GN ($r = -0.85$; $P < 0.01$; $y = -5.82x + 416.3$). This negative relationship between MAP and GN was not observed in the C lambs ($r = 0.38$; $P = 0.35$; $y = -3.23x + 95.0$). Singles and twins are shown as circles and triangles, respectively.

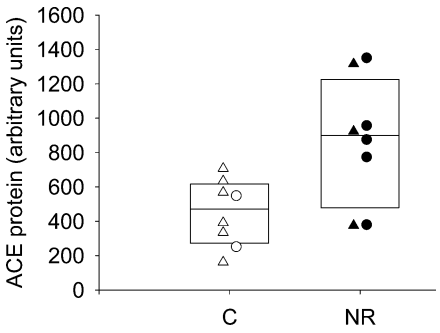

Figure 2. ACE expression was increased ($P < 0.01$) in the renal cortex of the nutrient-restricted lambs (NR, $n = 8$, filled symbols) in contrast to the controls (C, $n = 8$, open symbols) and lambs at 9 months of age

Singles and twins are shown with circles and triangles, respectively. Box plots represent the 75th and 25th percentiles and the median.

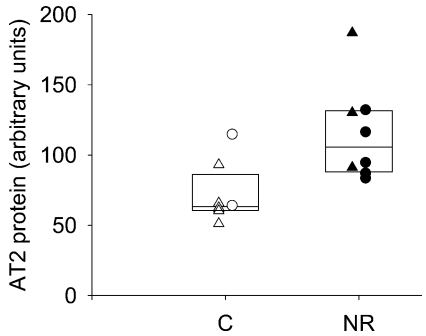

Figure 3. AT2 expression was increased ($P < 0.05$) in the renal medulla of the nutrient-restricted lambs (NR, $n = 8$, filled symbols) in contrast to the controls (C, $n = 8$, open symbols) and lambs at 9 months of age

Singles and twins are shown with circles and triangles, respectively. Box plots represent the 75th and 25th percentiles and the median.

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The present report demonstrates elevated blood pressure concomitant with nephron deficit in an ovine model of global nutrient restriction during early to mid-gestation. A compromised nephron endowment, first advanced by Brenner *et al.* (1988), as an avenue to increased blood pressure is thought by many investigators to be a central mechanism that results in developmentally programmed hypertension, and is present in several of the experimental models utilized in this area of research (Langley-Evans *et al.* 1999; Woods *et al.* 2001; Ortiz *et al.* 2003). The present data are consistent with the hypothesis advanced by Brenner *et al.* (1988) that proposed that nephron number and blood pressure are inversely related. As depicted in Fig. 1, a strong negative correlation between MAP and nephron number is evident only in the nutrient-restricted group of offspring. It is important to note that twinning alone may predispose fetal sheep to a deficit in nephron number (Mitchell *et al.* 2004), although a report to the contrary has recently been published (Brennan *et al.* 2005). In addition, a moderate increase in blood pressure occurs in offspring that have been exposed to nutrient deprivation during gestation, either as a result of twinning or as a result of dehydration-induced anorexia (Desai *et al.* 2003). The inclusion of both twins and singleton offspring in the present report therefore presents a treatment response distribution that highlights the susceptibility of the twin conceptus to *in utero* nutritional deficit. Our data set does not allow for the independent analysis of the effects of twinning and nutrient deprivation. However, the greater proportion of twins in our study fell in the control fed group, indicating that if there is any bias in the present data due to twinning it would act to minimize the differences reported between treatment groups.

Previous authors have shown protein restriction in pregnant rats impairs nephrogenesis and subsequently raises blood pressure in the offspring (Langley-Evans *et al.* 1999; Woods *et al.* 2001; McMullen *et al.* 2004). These studies also demonstrate the existence of a critical window of gestation whereby a nutritional insult impairs nephrogenesis. The metanephric kidney begins to develop near day 27 of gestation (Moritz & Wintour, 1999). This time period corresponds to the initiation of the nutrient restriction period in the present study, and when viewed in concert with the present data points to a critical window in renal development that has permanent effects on nephrogenesis. The present data are in agreement with a previous report showing hypertension and reduced nephron number in offspring of pregnant ewes administered glucocorticoids at day 28 of gestation in the pregnant ewe (Wintour *et al.* 2003). While the literature relating to rodents is replete with studies demonstrating hypertension in offspring of nutrient-restricted dams (Langley-Evans *et al.* 1999; Woods *et al.* 2001; McMullen *et al.* 2004); very little has been reported on the matter in the literature relating to sheep.

Elevated blood pressure has been reported in 85-day-old offspring of mildly undernourished ewes (Hawkins *et al.* 2000). Increased blood pressure has also recently been reported in 3-year-old progeny of pregnant ewes undernourished in a manner similar to that utilized in the present study (Gopalakrishnan *et al.* 2004). However, no data were reported from the kidneys of these animals. It is important to note that in the study by Gopalakrishnan and colleagues, elevation in blood pressure was documented only immediately before, but not following feeding (Gopalakrishnan *et al.* 2004). These authors also report a decreased sensitivity of the baroreflex in three year old animals. We describe increased MAP without an increase in heart rate in offspring at 9 months of age, which is indicative of resetting of the baroreflex set point to a higher systemic pressure without a change in sensitivity. Differences between our results and those of Gopalakrishnan *et al.* (2004) probably reflect the difference in age of the animals studied and the progressive nature of the hypertensive state that has been noted in the hypertension literature for some time (Guo & Abboud, 1984; Chapleau *et al.* 1988). Because a full interrogation of the baroreceptor reflex is lacking in the present data set we feel further discussion is beyond the scope of this manuscript.

The observed ∼20% reduction in glomerulus number is in agreement with a recent report by Ortiz *et al.* (2003). which used the same glomerulus counting technique and demonstrated a similar decrease in glomerulus number and increased blood pressure in male offspring of glucocorticoid-treated rat dams. In contrast, Langley-Evans *et al.* (2003) noted that amino acid supplementation during maternal low protein diets (during gestation) restores glomerulus number, but only glycine supplementation attenuates the increased blood pressure. It therefore appears that, at least in the low protein model in the rat, the hypertension found in offspring may not be driven by impaired glomerulogenesis but may merely coincide with reduced nephron number. It must be recognized that work by Woods clearly showed that compromising nephron number (via unilateral nephrectomy) during fetal life results in increased blood pressure in the adult rat (Woods, 1999). The impact of amino acid supplementation on offspring blood pressure in a unilateral nephrectomy model would certainly shed further light on these arguments. Recent work by Mitchell *et al.* (2004) further demonstrates that the timing of fetal insult has a profound effect on nephron endowment since intrauterine growth restriction (IUGR) induced during late gestation in the sheep does not compromise nephron endowment, while significantly compromised nephrogenesis occurs when the IUGR

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occurs earlier in development. In contrast, a recent study by Brennan and colleagues clearly shows no difference in ovine fetal nephron number between twin and singleton pregnancies (Brennan *et al.* 2005), indicating that the impact of twinning on fetal kidney development remains equivocal. The present report augments the current body of knowledge by characterizing an association between nephron number and intrauterine programmed hypertension in a large animal model.

We report dysregulation of the intrarenal RAS. ACE expression in the renal cortex of the NR offspring was elevated in comparison with the C offspring. The overexpression/overactivity of renal ACE has recently been recognized as a hallmark in the progression of renal dysfunction (Vio & Jeanneret, 2003). The present observation of increased ACE expression is in agreement with observations from several models of diseased kidneys (Vio & Jeanneret, 2003), as well as human diabetic nephropathy and proteinuric membranous nephropathy (Mezzano *et al.* 2003*a*,*b*). The mechanism by which ACE elevation may act to contribute to increased blood pressure or the progression of renal disease is unclear. Regional ischaemia at the cortico–medullary interface has been proposed to be involved (Fine *et al.* 2000), as well as a disturbance of the balance between Ang II and bradykinin in the kidney (Vio & Jeanneret, 2003). A recent report by Manning and Vehaskari demonstrates administration of the ACE inhibitor captopril postweaning attenuates hypertension in low protein (LP) programmed hypertensive rats (Manning & Vehaskari, 2005). These data support the hypothesis that dysregulation of the RAS is involved in programmed hypertension in the sheep. Further work using this model will have to be undertaken to evaluate renal physiology and perhaps clarify the role of increased intrarenal ACE in these animals.

The AT1 receptor has been long recognized as the primary mediator of the traditional effects of Ang II, including the pressor effect on vascular smooth muscle and aldosterone release from the adrenal. Previous work has long implicated the RAS and dysfunction of the RAS as a contributing factor to the aetiology of hypertension as exemplified by the use of AT1 receptor antagonists and ACE inhibitors in the treatment of both hypertension and heart disease. The present observation demonstrates no alteration in the expression of AT1 protein in the kidney. This is in agreement with the other observations from hypertension models such as Ang II-induced hypertension (Harrison-Bernard *et al.* 2002), which demonstrates that under the influence of excess Ang II, AT1 is not increased in the kidney. In contrast to the present observations, two recent reports in the rat indicate AT1 expression is increased in the kidneys of young hypertensive offspring born to mothers fed low protein diets during gestation (Sahajpal & Ashton, 2003; Vehaskari *et al.* 2004). The discrepancy between the present data and these recent observations in the rat may reflect differences in the nutritional stressors to the animal. Recent work by Nishina *et al.* (2003) has shown differences in vasodilatory capacity in sheep fetuses that were fed diets that were either globally nutrient restricted (NR = 30%) or protein restricted. The results of the present study suggest perturbations of the intrarenal RAS contribute to hypertension without change in the expression of the AT1 receptor.

The function of the Ang II AT2 receptor has not been clarified completely. Once thought to exist primarily during fetal life, postnatal re-expression of the AT2 receptor has been consistently demonstrated in pathological adult conditions in the kidney (Ruiz-Ortega *et al.* 2003). While the precise function of the AT2 receptor in these pathologies remains nebulous, several actions such as AT1 antagonism (Horiuchi *et al.* 1997), nitric oxide release, apoptosis (Tejera *et al.* 2004; Horiuchi *et al.* 1997), and blunting pressure-natriuresis (Liu *et al.* 1999) have been ascribed to AT2 in non-pathological conditions. The present data show increased renal medullary AT2 expression, and agree with a recent report by Vehaskari *et al.* which evaluated kidneys from hypertensive rat offspring from protein-restricted dams and showed increased AT2 protein in whole kidney homogenate (Vehaskari *et al.* 2004). The upregulation of AT2 expression reported in the present study is similar to reports of several models of kidney damage (Ruiz-Ortega *et al.* 2003) and of human diabetic nephropathy (Mezzano *et al.* 2003*a*). Prior work has shown the AT2 receptor to be involved in the regulation of pressure-natriuresis in rats (Lo *et al.* 1995). Because abnormal pressure-natriuresis is associated with chronic hypertension (Guyton, 1990), the observed increase in medullary AT2 may indicate a maladaptive phenotype in which blunted pressure-natriuresis contributes to elevated blood pressure. Alternatively, it could be viewed as an adaptive response to ameliorate the effects of renal impairment, a scenario that has been proposed in recent work by Vazquez *et al.* (2005). When viewed together with the lack of change in caspase-3 activity and AIF expression, the current results indicate the increased AT2 is not promoting increased apoptosis. This may not be a discrepancy as much as a reflection of the young age and early onset of increased blood pressure of the animals in the present study in comparison to the older animals in other reports (Tejera *et al.* 2004). The increase in AT2 observed in the present study agrees with observations in other models of kidney pathology, and further support hypotheses proposing an intimate involvement of the RAS in renal hypertension.

The present study reports higher levels of caspase-3 activity in the renal medulla when compared to the renal cortex in both the C and NR kidneys. Little has been reported in the literature regarding comparisons of caspase-3 activity or relative amounts of apoptosis between

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the renal cortex and medulla. Prior work has shown cultured medullary cells show increased apoptosis and caspase-3 activity in response to elevated concentrations of NaCl and urea (Michea *et al.* 2000; Copp *et al.* 2005). Thus the present observations may simply reflect increased apoptosis and caspase-3 activity along the medullary concentration gradient. Further studies will have to be performed to evaluate this observation further and to contrast *in vitro* experiments with *in vivo* observations.

Limitations

There are several concerns that represent potential limitations with respect to the interpretation of the present data. The effects of maternal NR on milk production during the lactation period were not incorporated into the study design. A cross-fostering paradigm as is often performed in rodent models is beyond the scope of this model. Consequently the impact of maternal NR during early to mid-gestation on *post partum* milk production remains unclear. Another concern in the current study is the imbalance of twin and singleton pregnancies between the C and NR groups. We found the impact of the nutritional paradigm during pregnancy on the offspring's blood pressure and glomerulus number emphasizes the susceptibility of the twin conceptus to perturbation during gestation, since the effect of fetus number on blood pressure and glomerulus number was less in the C group that had the greater number of twins. Since the impact of nutrient restriction could putatively be greater on twins than on singletons, it should follow that the differences between the C and NR lambs would have been minimized, since the larger number of twins occurred in the C group. We therefore do not consider the distribution of twins to have biased the outcome of this study.

Summary

In summary, the present study demonstrates increased MAP, decreased glomerulus number, increased expression of ACE in the renal cortex, increased medullary AT2, and metabolic dysregulation in male offspring of ewes that were nutrient restricted 50% between early and mid-gestation. The present endeavour characterizes a large animal model of programmed hypertension and provides direction for further research in this area by demonstrating complicity of the kidney in the reported hypertension. For many of the endpoints presented, there is no difference between twins and singletons, suggesting that twins are not *per se* different from singletons, but because of their more compromised intrauterine situation they may represent a quantitatively more challenged example of the effects of maternal nutrient restriction. Taken together, these observations lend further support to the thrifty phenotype hypothesis of metabolic dysfunction and support hypotheses for a renal origin of developmentally programmed hypertension.

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