

Renal growth retardation following angiotensin II type 1 (AT₁) receptor antagonism is associated with increased AT₂ receptor protein in fetal sheep

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Abstract

The actions of angiotensin II on type 1 (AT₁) and type 2 (AT₂) receptor subtypes are important for normal kidney development before birth. This study investigated the effect of AT₁ receptor antagonism on renal growth and growth regulators in fetal sheep during late gestation. From 125 days of gestation (term 145 ± 2 days), chronically catheterised sheep fetuses were infused intravenously for 5 days with either an AT₁-specific receptor antagonist (GR138950, 2–4 mg/kg per day, *n* = 5) or saline (0.9% NaCl, *n* = 5). Blockade of the AT₁ receptor decreased arterial blood oxygenation and pH and increased blood pCO₂, haemoglobin and lactate, and plasma cortisol and IGF-II. Blood glucose and plasma thyroid hormones and IGF-I were unchanged between the treatment groups. On the 5th day of infusion, the kidneys of the GR-treated fetuses were lighter

than those of the control fetuses, both in absolute and relative terms, and were smaller in transverse cross-sectional width and cortical thickness. In the GR-infused fetuses, renal AT₂ receptor protein concentration and glomerular density were significantly greater than in the saline-infused fetuses. Blockade of the AT₁ receptor had no effect on relative cortical thickness, fractional or mean glomerular volumes, or renal protein levels of the AT₁ receptor, IGF type 1 receptor, insulin receptor or protein kinase C ζ. Therefore, in the ovine fetus, AT₁ receptor antagonism causes increased renal protein expression of the AT₂ receptor subtype, which, combined with inhibition of AT₁ receptor activity, may be partly responsible for growth retardation of the developing kidney.

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Introduction

The renin–angiotensin system is present and active in the mammalian fetus from relatively early in gestation (Lumbers 1995). Angiotensin II (AII) acts on two major receptor subtypes: the AII type 1 (AT₁) receptor mediates the main actions of AII on the cardiovascular and renal systems before and after birth, while the AII type 2 (AT₂) receptor is primarily and widely expressed in fetal tissues and, although its function is less clear, it appears to be involved in the control of tissue growth and differentiation (de Gasparo *et al.* 2000). In particular, the renin–angiotensin system has an important role in the regulation of growth and development of the fetal and postnatal kidney (Guron & Friberg 2000, Matsusaka *et al.* 2002, Chen *et al.* 2004). Both of the major AII receptor subtypes show spatial and temporal patterns of expression in association with nephrogenesis (Kakuchi *et al.* 1995, Shanmugam & Sandberg 1996, Butkus *et al.* 1997). In mice, mutation of the genes for angiotensinogen, angiotensin-converting enzyme (ACE) and the AT₁ receptor causes

a variety of renal abnormalities, including delayed glomerular maturation, tubulointerstitial inflammation, papillary atrophy, fibrosis and vascular thickening (Guron & Friberg 2000, Matsusaka *et al.* 2002, Chen *et al.* 2004). In addition, normal branching of the ureteric bud is impaired in the metanephroi cultured from mouse embryos with mutation of the AT₂ receptor gene (Song *et al.* 2010). Furthermore, treatment of newborn rats with ACE inhibitors or AT₁-specific receptor antagonists, such as losartan, leads to persistent defects in both the structure and concentrating ability of the kidney (Friberg *et al.* 1994, Tufro-McReddie *et al.* 1995, Woods & Rasch 1998). Indeed, the adverse effect of inhibitors of the renin–angiotensin system on the development of the fetal kidney and other tissues has led to the contraindication of these drugs during pregnancy (Alwan *et al.* 2005).

Previous studies have demonstrated that direct administration of the AT₁-specific receptor antagonist, GR138950, to the sheep fetus during late gestation causes hypotension, hypoxaemia and hypercapnia (Forhead *et al.* 2000, Forhead & Fowden 2004). However, the consequences of AT₁ receptor

blockade for the growth of the developing ovine kidney, and the regulatory pathways by which AT₁ receptor antagonists influence renal development, are unknown. Some of the mechanisms of action of GR138950 may be direct, via the effects of loss of function of the AT₁ receptor on renal development, and others may involve secondary changes in oxygenation, blood flow and other endocrine systems in the fetus. In addition, the extent to which AT₁ receptor blockade influences renal expression of the AT₁ and AT₂ receptor subtypes is unknown in the developing kidney. Changes in AT₂ receptor expression are of particular interest as the unblocked receptor subtype is exposed to high concentrations of AII following loss of negative feedback on renin release during AT₁-specific receptor antagonism (Forhead *et al.* 1997, 2000, de Gasparo *et al.* 2000).

Therefore, the aim of this study is to investigate the effects of an AT₁ receptor antagonist, GR138950, on renal structure, and the concentrations of hormones and tissue proteins involved in the control of growth and development, in the sheep fetus during late gestation. The study hypothesised that a 5-day period of AT₁-specific receptor blockade during late gestation would cause growth retardation of the fetal kidney, and that this would be associated with changes in circulating concentrations of insulin-like growth factors (IGFs), and in the expression of receptors for hormones known to be important for normal growth of the developing kidney, such as AII, IGFs and insulin.

Materials and Methods

Animals

Ten singleton Welsh Mountain sheep fetuses of known gestational age were used in this study; four of the fetuses were male and six were female fetuses. The ewes were multi-parous and were of similar body condition score. The ewes were fed with 200 g/day concentrates and hay and water *ad libitum* and had access to a salt-lick block. For 18–24 h before surgery, all food was withheld from the ewes but water remained available. The surgical and experimental procedures were performed under licence according to the UK Animals (Scientific Procedures) Act 1986 and were approved by the animal ethics committee at the University of Cambridge.

Surgical procedures

At 121 ± 3 days of gestation (term, 145 ± 2 days) and under halothane anaesthesia (1.5% in O₂–N₂O) with positive pressure ventilation, catheters were inserted into the femoral artery of the ewe, and the femoral artery and both femoral veins of the fetus, using surgical techniques described previously (Comline & Silver 1972). Procaine penicillin (Depocillin, Mycofarm, Cambridge, UK) was administered intramuscularly to the ewes on the day of catheterisation and for 3 days afterwards. The fetuses were treated with 100 mg

ampicillin (Penbritin, Beecham Animal Health, Brentford, UK) intravenously at the time of surgery. The catheters were flushed daily with heparinised saline solution (100 IU/ml heparin in 0.9% saline) from the day after surgery. All ewes and fetuses were studied after at least 3 post-operative days.

Experimental procedures

At 125 ± 1 days of gestation, one group of five fetuses were infused intravenously for 5 days with saline (0.9% NaCl) and the other group of five fetuses were infused with the AT₁ receptor antagonist, GR138950 (GR, Glaxo-Wellcome Research and Development, Ware, UK; 2–4 mg/kg per day in 0.9% saline, *n* = 5). Both treatments were administered at a rate of 2.5 ml/day using portable infusion pumps. In the saline-treated group, there were two female and three male fetuses while in the GR138950-treated group, there were four female and one male fetuses. GR138950 is a bromobenzofuran trifluoromethanesulphonamide and a potent, long-lasting and specific non-peptide AT₁ receptor antagonist (Hilditch *et al.* 1995). Previous studies have shown that this dose of GR138950 abolishes the blood pressure response to exogenous AII (100 ng/kg estimated fetal bodyweight in 0.9% saline intravenously; Hypertensin, Ciba-Geigy Pharmaceuticals, Horsham, UK) on all days of the infusion (Forhead & Fowden 2004). Arterial blood samples (4 ml) were taken daily between 0800 and 0900 h from the fetuses from 2 days before the start of the infusion and on each of the 5 days of treatment. All samples of blood were placed into EDTA-containing tubes and centrifuged for 5 min at 1000 g and 4 °C, after which the plasma was stored at –20 °C until analysis. On the 5th day of infusion, at 130 ± 1 days of gestation, the animals were killed by administration of sodium pentobarbitone to both the ewe and fetus (200 mg/kg *i.v.*). All tissue collections were carried out at between 0900 and 1100 h. Fetal bodyweight and crown–rump length were measured, and the position of the catheters was checked. Both the kidneys of the fetus were dissected and weighed; one was cut into three pieces and placed into 10% formaldehyde fixative for up to 24 h, and the other was divided into regions of cortex and medulla and frozen in liquid nitrogen.

Biochemical analyses

Immediately after collection, all arterial blood samples were analysed for pH and partial pressures of oxygen and carbon dioxide (pO₂ and pCO₂) using an ABL330 Radiometer analyser corrected for fetal body temperature, and for haemoglobin content and O₂ saturation using an OSM2 Haemoximeter (Radiometer, Copenhagen, Denmark). Blood glucose and lactate concentrations were determined using an autoanalyser (2300 Statplus, Yellow Springs, OH, USA). Blood O₂ content (mmol/l) was calculated as haemoglobin content (g/dl) × O₂ saturation (%) × 0.00062, assuming the insignificance of O₂ dissolved in plasma. Total plasma cortisol concentration was measured by RIA

(Robinson *et al.* 1983) where the lower limit of detection was 1.0–1.5 ng/ml and the inter-assay coefficient of variation was 12%. Total plasma triiodothyronine (T₃) and thyroxine (T₄) concentrations were measured by RIA using a commercial kit (Fowden & Silver 1995; ICN Biomedicals, Thame, UK). The lower limits of detection were 0.07 ng/ml for T₃ and 7.6 ng/ml for T₄, and the inter-assay coefficients of variation were 10% for both assays.

Plasma concentrations of IGF-I and IGF-II were measured in duplicate based on the method described previously (Gluckman *et al.* 1983). Interference by binding proteins was minimised by the acid-ethanol cryoprecipitation method validated for ruminants (Breier *et al.* 1991). The efficiency of the acid-ethanol extraction was $91 \pm 1\%$ for both IGF-I and IGF-II as calculated using 10 plasma samples with labelled IGF-I and IGF-II added. Highly purified human IGF-I and IGF-II (IGF-I: cat no. 30-A188, Fitzgerald, Concord, MA, USA; IGF-II: cat no. 031-30, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) were iodinated using the chloramine T method (Greenwood *et al.* 1963). The labelled hormones were purified with a pre-albuminated Sephadex G25 column and re-purified on a pre-albuminated 9 mm × 300 mm Sephadex G100 column (Pharmacia). The iodinated hormones were eluted with 0.1% gelatin in 0.01 M PBS at pH 6.2. The assay buffer contained 0.02% protamine sulphate, 0.47% NaH₂PO₄·2H₂O, 0.37% EDTA, 0.25% BSA, 0.02% NaN₃ at pH 7.5 and was used in all dilutions. After extraction, the first antibody (IGF-I: rabbit antiserum to hIGF-I, #AFP4892898, NIDDK, Torrance, CA, USA, 1:15 000; IGF-II: rabbit antiserum to hIGF-II, PAC1, GroPep, Adelaide, SA, Australia, 1:5000) was added to 300 µl of diluted samples (1 in 10) and incubated overnight at 4 °C. Afterwards, 100 µl tracer (~12 000 d.p.m.) was added and incubated overnight at 4 °C, followed by an addition of 100 µl of a 1:5 mixture of secondary antibody (donkey anti-rabbit, DARS11, University of Western Australia, 1:20) and normal rabbit serum (1:500). After incubating overnight at 4 °C, 1 ml of 6% polyethylene glycol (PEG 6000, BDH, Poole, UK) in assay buffer was added; the samples were centrifuged at 1500 g for 30 min at 4 °C, and the supernatant was aspirated. The activity of the precipitate was determined on a gamma counter (Packard Cobra-II, Auto Gamma, Meriden, CT, USA). Both assays were validated for use in sheep by checking for parallelism using a serial dilution of pooled samples of ovine plasma. All samples were processed in a single assay; the limit of detections were 0.08 ng/ml for IGF-I and 4.0 ng/ml for IGF-II, and the inter-assay coefficients of variation were 5.3 and 4.3% for IGF-I and IGF-II respectively.

Renal protein levels of the IGF type 1 receptor, insulin receptor, PKCζ, and AII receptor subtypes, AT₁ and AT₂, were determined by western blotting. Samples of renal cortex were homogenised on ice in a lysis buffer of 50 mM HEPES pH 8: this contained 150 mM NaCl, 1% Triton X-100, 1 mM Na₃VO₄, 30 mM sodium fluoride, 10 mM sodium pyrophosphate and 10 mM EDTA. The buffer also included a protease inhibitor cocktail (Calbiochem Novabiochem

Biosciences, Nottingham, UK), which contained 0.5 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride, 25 µM bestatin, 10 µM leupeptin, 5 µM pepstatin A, 1.5 µM E-64 and 0.4 µM aprotinin. After the centrifugation of the homogenate at 1000 g and 4 °C, the supernatant was removed for analysis. A bicinchoninic acid kit (Sigma) was used to measure protein concentration in each sample. Each tissue lysate was standardised to a final concentration of 2 mg/ml by dilution in Laemmli's sample buffer, which contained 62.5 mM Tris, pH 6.8, 150 mM dithiothreitol, 10% glycerol, 2% SDS and 0.02% bromophenol blue. The protein samples were boiled for 5 min, and 20 µg were loaded on 10% SDS polyacrylamide gels for electrophoretic separation. The separated proteins were transferred using a buffer containing 20 mM Tris-base, 192 mM glycine, 0.1% SDS and 20% methanol onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA), according to the manufacturer's protocol.

The antibodies used in this study were AT₁ receptor, AT₂ receptor, IGF type 1 receptor, insulin receptor-β subunit and PKCζ (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Following blockade with 5% non-fat dry milk powder in Tween-20-Tris-buffered saline for 1 h at room temperature, membranes were incubated with the primary antibody at a 1:1000 (AT₁ receptor) or 1:5000 (AT₂ receptor) dilution overnight at 4 °C, or at a dilution of 1:200 (IGF type 1 receptor, insulin receptor) or 1:500 (PKCζ) for 1 h at room temperature in 5% non-fat dry milk powder in Tween-20-Tris-buffered saline. Blots were rinsed, washed and then incubated with a HRP-conjugated secondary antibody to rabbit diluted to 1:10 000 in 5% non-fat dry milk powder in Tween-20-Tris-buffered saline for 1 h at room temperature (Amersham Biosciences). Membranes were analysed with Ponceau S to control for protein loading. Antibody binding was detected using an enhanced chemiluminescence kit (Amersham Biosciences). Proteins were revealed and quantified using Image J software (National Institutes of Health, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>). Where appropriate, values are expressed as a percentage of the control lysate (100%) for each experiment. Ratios of protein expression were arcsine transformed prior to statistical analysis. All immunoblots showed a single band of expected size, except that probed with the AT₁ receptor antibody. In this case, the immunoblot showed four bands of expected size with the major band at 67 kDa as reported previously in fetal sheep (Segar *et al.* 1997); this band was quantified individually and in combination with the other bands.

Morphological analyses

The middle third of each kidney was dissected in transverse cross section at the renal pelvis and embedded in paraffin wax. For light microscopy, 7 µm sections were cut and stained with haematoxylin and eosin. Photographs of each kidney were taken for the measurement of total kidney width, capsule-pyramid length and cortical thickness. Total kidney width was

determined as the widest point across the kidney in transverse cross section; capsule–pyramid length was measured as the distance from the renal capsule to the tip of the medullary pyramid; and cortical thickness was measured as distance from the capsule to the medulla at ten different positions around the kidney. Further indices of kidney size were derived from these measurements, including the cortex–medulla ratio where cortical thickness was expressed as a percentage of both capsule–pyramid length and half of the total kidney width.

The study was carried in sheep fetuses at a gestational age near the completion of nephrogenesis. Previous studies in fetal sheep have demonstrated that there are only mature glomeruli present in the developing kidney from 120 days of gestation (Gimonet *et al.* 1998). In this study at 130 days of gestation, all the glomeruli were mature and were identified with a clearly defined capsule and capillary structure. No comma or S-shaped bodies were observed in the sections. Unfortunately, it was not possible to estimate total nephron number in the kidney samples prepared. However, fractional glomerular volume (the fraction of renal cortex occupied by glomeruli) was determined stereologically in ten regions of cortex with an online microscope imaging system (Gundersen *et al.* 1988). Using a grid with 36 points, the number of points in contact with a glomerulus was counted and expressed as a percentage of the total number of points. The total number of points on the grid was optimised to ensure that no more than one point came in contact with a single glomerulus. Glomerular density was determined from the number of glomeruli observed exclusively in two sections taken 35 µm apart. The sampling volume was 0.21 mm³. Mean glomerular volume was calculated as the fractional glomerular volume divided by glomerular density. All analyses were performed blind to the treatment group.

Statistical analysis

All data are presented as mean ± S.E.M. Significant differences in renal measurements between the treatment groups were determined by unpaired *t*-test. Significant differences between values obtained before and during the infusion were analysed by two-way ANOVA for repeated measures followed by Tukey's test. Relationships between the variables were assessed by linear regression. In all cases, *P* < 0.05 was considered significant.

Results

Arterial blood gases and metabolite concentrations

A 5-day period of AT₁ receptor antagonism caused significant changes in blood gas and metabolite status in the fetus (Table 1). Compared to baseline values, significant decrements in arterial blood pO₂, O₂ saturation, O₂ content and pH were observed from the 2nd to 5th days of GR infusion (*P* < 0.05, Table 1). On the last 3 days of infusion, all these

Table 1 Mean (± S.E.M.) arterial blood levels of pO₂, O₂ saturation, O₂ content, haemoglobin, pCO₂, pH and lactate in fetuses infused for 5 days with either saline or GR (*n* = 5 in each group)

Day of treatment	-1	0	1	2	3	4	5	Treatment	Day	Interaction
pO ₂ (mmHg)										
Saline	19.5 ± 1.3	20.0 ± 0.7	17.4 ± 1.2	19.6 ± 0.2	19.2 ± 0.9	18.0 ± 0.9	19.6 ± 1.5	NS	<i>P</i> < 0.001	<i>P</i> < 0.001
GR	22.0 ± 2.8 ^a	21.4 ± 2.8 ^{ab}	20.8 ± 2.7 ^{ab,b*}	17.8 ± 2.5 ^{b,c}	16.6 ± 2.4 ^{c,d*}	13.2 ± 2.4 ^{d*}	14.0 ± 2.6 ^{c,d*}			
O ₂ saturation (%)										
Saline	60.7 ± 4.2	60.9 ± 3.3	55.1 ± 4.5	59.7 ± 2.4	61.5 ± 2.2	59.5 ± 2.4	62.7 ± 3.6	<i>P</i> < 0.005	<i>P</i> < 0.001	<i>P</i> < 0.001
GR	58.9 ± 0.7 ^a	58.1 ± 1.8 ^a	52.8 ± 4.1 ^{ab}	43.9 ± 3.7 ^{b,c,*}	38.2 ± 4.0 ^{c,d*}	28.1 ± 5.1 ^{d,*}	30.7 ± 5.6 ^{d,*}			
O ₂ content (mmol/l)										
Saline	0.35 ± 0.03	0.36 ± 0.02	0.33 ± 0.02	0.36 ± 0.02	0.36 ± 0.02	0.36 ± 0.01	0.37 ± 0.03	<i>P</i> < 0.005	<i>P</i> < 0.001	<i>P</i> < 0.001
GR	0.35 ± 0.02 ^a	0.35 ± 0.02 ^a	0.31 ± 0.03 ^{ab}	0.26 ± 0.02 ^{b,c,*}	0.24 ± 0.02 ^{b,c,*}	0.19 ± 0.03 ^{c,*}	0.21 ± 0.04 ^{c,*}			
Haemoglobin (g/dl)										
Saline	9.3 ± 0.2	9.7 ± 0.3	9.7 ± 0.3	9.6 ± 0.4	9.5 ± 0.2	9.8 ± 0.7	9.3 ± 0.4	NS	<i>P</i> < 0.005	<i>P</i> < 0.001
GR	9.7 ± 0.5 ^a	9.8 ± 0.6 ^a	9.5 ± 0.5 ^a	9.6 ± 0.5 ^a	10.2 ± 0.5 ^{ab}	10.8 ± 0.4 ^{b,c}	11.1 ± 0.4 ^{c,*}			
pCO ₂ (mmHg)										
Saline	48.3 ± 1.8	51.0 ± 1.6	49.2 ± 2.8	51.4 ± 2.0	49.0 ± 1.5	49.5 ± 3.2	50.4 ± 1.6	<i>P</i> < 0.005	<i>P</i> < 0.001	<i>P</i> < 0.001
GR	51.6 ± 1.3 ^a	51.2 ± 1.2 ^a	54.0 ± 1.8 ^{ab}	54.8 ± 0.8 ^{ab,c}	58.8 ± 1.5 ^{b,c,d,*}	59.4 ± 2.2 ^{c,d,*}	61.6 ± 2.0 ^{d,*}			
pH										
Saline	7.38 ± 0.01	7.36 ± 0.01	7.34 ± 0.01	7.35 ± 0.01	7.37 ± 0.002	7.37 ± 0.02	7.37 ± 0.01	NS (<i>P</i> = 0.08)	<i>P</i> < 0.001	<i>P</i> < 0.001
GR	7.37 ± 0.01 ^{ab}	7.37 ± 0.01 ^a	7.35 ± 0.01 ^{ac}	7.34 ± 0.01 ^{b,c,d}	7.33 ± 0.01 ^{c,d,*}	7.33 ± 0.01 ^{c,d,*}	7.31 ± 0.01 ^{d,*}			
Lactate (mmol/l)										
Saline	1.13 ± 0.13	1.13 ± 0.18	1.03 ± 0.08	1.03 ± 0.08	1.06 ± 0.10	1.11 ± 0.12	1.12 ± 0.11	<i>P</i> < 0.005	<i>P</i> < 0.001	<i>P</i> < 0.001
GR	1.11 ± 0.09 ^a	1.11 ± 0.13 ^a	1.12 ± 0.09 ^a	1.40 ± 0.11 ^{ab}	1.65 ± 0.25 ^{bc*}	2.47 ± 0.28 ^{c,*}	1.61 ± 0.09 ^{bc*}			

NS, not significant. *Significantly different from the saline group, *P* < 0.05. For each treatment group, values within a row with different letters are significantly different from each other, *P* < 0.05.

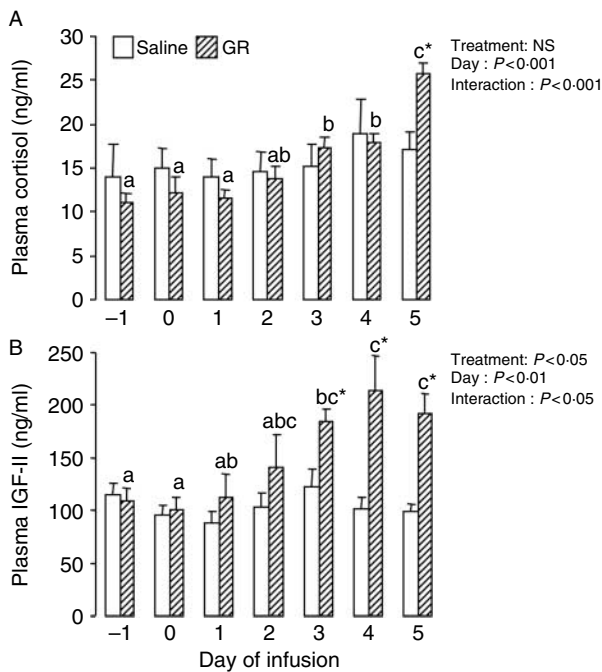


Figure 1 Mean (\pm S.E.M.) plasma concentrations of (A) cortisol and (B) IGF-II in fetuses infused for 5 days with either saline or GR ($n=5$ in each group). *Significant difference between the treatment groups, $P<0.05$. Within each treatment group, columns with different letters are significantly different from each other, $P<0.05$. NS, not significant.

blood variables were significantly lower in the GR-treated compared to saline-treated fetuses ($P<0.05$, Table 1). In the fetuses treated with GR, significant increments in arterial blood haemoglobin, pCO_2 and lactate were seen over the period of infusion ($P<0.05$, Table 1) and all of these variables were significantly greater than those observed in the saline-treated fetuses on the last 2 or 3 days of infusion ($P<0.05$, Table 1). During the period of saline or GR infusion, there was no significant change from baseline in blood glucose concentration (saline: 0.84 ± 0.06 , GR: 0.96 ± 0.05 mmol/l, $n=5$ in each group). In addition, no significant difference in blood glucose was seen between the two groups of fetuses at any time point during the study.

Plasma hormone concentrations

Compared to baseline, plasma cortisol and IGF-II levels were significantly higher on the 3rd, 4th and 5th days of GR infusion ($P<0.05$, Fig. 1). On the 5th day of infusion, plasma cortisol concentration was significantly greater in the GR-treated fetuses compared to those infused with saline ($P<0.05$, Fig. 1A). Plasma IGF-II concentration was significantly greater in the GR-treated fetuses, compared to saline-treated fetuses, on the 3rd, 4th and 5th days of infusion ($P<0.05$, Fig. 1B). There was no significant change from baseline in plasma concentrations of T_3 (saline: 0.17 ± 0.03 ,

GR: 0.20 ± 0.05 ng/ml), T_4 (saline: 88.6 ± 7.4 , GR: 153.0 ± 26.3 ng/ml) or IGF-I (saline: 20.8 ± 3.6 , GR: 19.9 ± 3.0 ng/ml) during the period of saline or GR infusion ($n=5$ in each group). Furthermore, no significant differences in plasma T_3 , T_4 or IGF-I were observed between the two treatment groups at any time point during the study.

Fetal body and kidney weights

There were no significant differences in bodyweight or crown-rump length between the two groups of fetuses (Table 2). Absolute kidney weights were significantly less in the GR-treated fetuses than in the fetuses infused with saline ($P<0.005$, Fig. 2A). When expressed as a percentage of bodyweight, relative kidney weight in the GR-treated fetuses remained lower than that in the saline-treated fetuses ($P<0.05$, Fig. 2A).

Renal morphology and protein expression

Compared to the saline-infused group, the fetuses treated with GR had reduced total kidney width and cortical thickness ($P<0.05$ in both cases, Fig. 2B). There was no effect of GR infusion on capsule-pyramid length or cortical thickness expressed as either a percentage of total kidney width or capsule-medulla length (Table 2). There were no effects of AT_1 receptor antagonism on fractional or mean glomerular volumes (Table 2). Glomerular density in the kidneys of the GR-infused fetuses was significantly higher than in the fetuses treated with saline ($P<0.05$, Fig. 2C). When observations from all fetuses were considered, a significant inverse relationship was identified between glomerular density and cortical thickness (glomerular density = $820.3 - (2656.9 \times \text{cortical thickness})$, $r=0.72$, $P<0.05$).

Table 2 Mean (\pm S.E.M.) values of bodyweight, crown-rump length, capsule-pyramid length, relative cortical thickness (as a percentage of total kidney width or capsule-pyramid length), fractional and mean glomerular volumes, and renal protein expression of insulin-like growth factor (IGF) type 1 receptor, insulin receptor and PKC ζ in fetuses infused for 5 days with either saline or GR

	Saline ($n=5$)	GR ($n=5$)
Bodyweight (g)	3044 \pm 134	2732 \pm 246
Crown-rump length (cm)	43.6 \pm 1.3	43.2 \pm 1.8
Capsule-pyramid length (cm)	0.91 \pm 0.05	0.81 \pm 0.04
Relative cortical thickness		
Percentage of total kidney width	27.3 \pm 1.3	24.8 \pm 0.5
Percentage of capsule-pyramid length	23.0 \pm 1.5	21.0 \pm 1.4
Fractional glomerular volume (%)	6.03 \pm 0.57	7.34 \pm 0.60
Mean glomerular volume ($\times 10^{-4}$ mm ³)	2.29 \pm 0.26	1.99 \pm 0.19
IGF type 1 receptor (AU)	1.00 \pm 0.48	0.89 \pm 0.28
Insulin receptor (AU)	1.00 \pm 0.46	1.26 \pm 0.42
PKC ζ (AU)	1.00 \pm 0.10	1.08 \pm 0.07

AU, Arbitrary units.

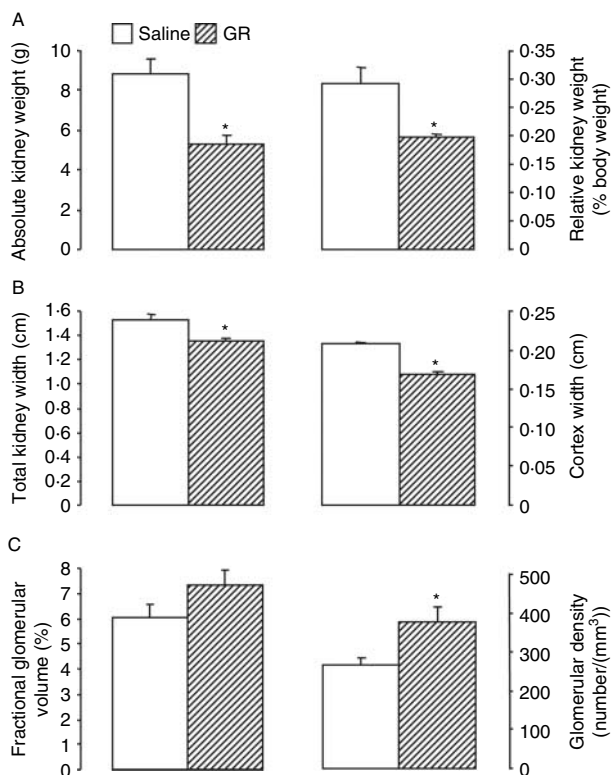


Figure 2 Mean (\pm S.E.M.) measurements of (A) absolute and relative kidney weights, (B) total kidney and cortex width and (C) fractional glomerular volume and glomerular density in fetuses infused for 5 days with either saline or GR ($n=5$ in each group). *Significant difference between the treatment groups, $P<0.05$.

On the 5th day of treatment, renal AT_1 receptor protein level did not differ significantly between the two treatment groups, both when analysed as a single major band at 67 kDa or in combination with the three other minor bands (Fig. 3). However, protein expression of the AT_2 receptor was significantly greater in the fetuses infused with GR compared to those infused with saline ($P<0.05$, Fig. 3). There were no significant differences in the protein expression of the IGF type 1 receptor, insulin receptor or PKC ζ between the fetuses treated with GR or saline (Table 2).

Discussion

In this study, a 5-day infusion of an AT_1 -specific receptor antagonist, GR138950, to the sheep fetus caused a significant reduction in the weight of the kidneys, both in absolute terms and as a percentage of bodyweight. The kidneys of the GR-treated fetuses had smaller transverse width and cortical thickness, with no change in the ratio of cortex to medulla, which indicated a proportionate decrease in the dimensions of the kidney. Renal growth retardation induced by GR was

associated with an increase in glomerular density and no change in either fractional or mean glomerular volumes. In this study, an inverse relationship was observed between glomerular density and cortex thickness, which suggested that although the renal cortex of the GR-treated fetus was thinner than that of the control fetus, it contained normal-sized glomeruli, in terms of mean volume, that were more closely associated with each other. Fractional glomerular volume is an estimation of total filtration capacity, taking into consideration both glomerular number and volume. Nephron number was not determined in this study, although nephrogenesis in the sheep fetus is near completion at 130 days of gestation (Gimonet *et al.* 1998). These findings confirm those observed in rats where administration of the AT_1 -specific receptor antagonist, losartan, in the first 3 weeks of postnatal life causes a decrease in the absolute and relative size of the kidney (Tuffro-McReddie *et al.* 1994).

This study is the first to demonstrate that the reduction in kidney size caused by AT_1 receptor blockade *in utero* is associated with increased renal protein expression of the AT_2 receptor. The AT_2 receptor is widely expressed in fetal tissues and, in particular, contributes to the regulation of cell proliferation, differentiation and apoptosis (Nakajima *et al.* 1995, Yamada *et al.* 1996, Nishimura *et al.* 1999, Johren *et al.* 2004). At the gestational age investigated in this study, the AT_2 receptor is localised to the macula densa and interstitial cells of the cortex and medulla in the developing ovine kidney (Butkus *et al.* 1997, Gimonet *et al.* 1998). Therefore, in the GR-treated fetuses, the reduction in kidney size without any change in fractional or mean glomerular volume may have resulted from the anti-proliferative effects of AT_2 receptor stimulation on renal interstitial cells. Indeed, AII has been shown to suppress cell division induced by basic fibroblast growth factor, via the AT_2 receptor, in cultured renomedullary interstitial cells obtained from rat embryos (Maric *et al.* 1998). In the GR-treated fetuses, the activity of the AT_2 receptor in the kidney is likely to increase not only by upregulation of receptor protein content but also by binding of elevated circulating levels of AII. Plasma AII concentration is raised during AT_1 receptor antagonism due to a decrease in

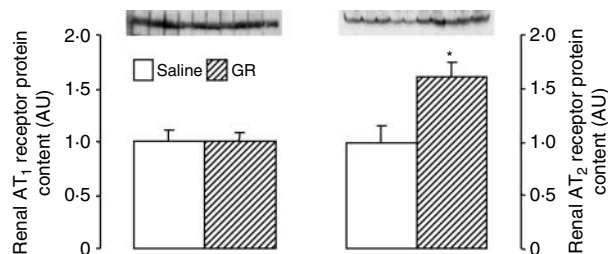


Figure 3 Mean (\pm S.E.M.) renal AT_1 and AT_2 protein expression in fetuses infused for 5 days with either saline or GR ($n=5$ in each group). *Significant difference between the treatment groups, $P<0.05$. AU, Arbitrary units.

feedback inhibition of renin production by AII via AT₁ receptors (Forhead *et al.* 1997, 2000, de Gasparo *et al.* 2000).

There are several mechanisms that may be responsible for the increase in renal AT₂ receptor protein level observed during AT₁ receptor antagonism *in utero*. First, high circulating AII concentration may upregulate AT₂ receptor number. AII has been shown to influence AT₂ receptor expression in a manner dependent on the cell type and the presence of functional AT₁ receptors. In cultured bovine adrenal cells that express both AII receptor subtypes, AII decreases AT₂ receptor mRNA abundance via the actions on the AT₁ receptor (Ouali *et al.* 1997). Indeed, in fetal sheep at a similar gestational age to those in this study, both AT₁ and AT₂ receptor mRNA levels in the kidney decrease in response to AII infusion for 24 h (Robillard *et al.* 1995). However, in R3T3 cells, a mouse fibroblast cell line known to express only AT₂ receptors, AII increases AT₂ receptor protein but not gene expression (Li *et al.* 1999). Therefore, in this study, AII may have a direct stimulatory effect on the translation of renal AT₂ receptors in the absence of functional AT₁ receptors. Second, the rise in circulating IGF-II level observed following AT₁ receptor antagonism *in utero* may influence tissue AT₂ receptor content. Previously, IGF-II has been shown to increase AT₂ receptor mRNA abundance and binding via the IGF-1 receptor in cultured vascular smooth muscle cells obtained from adult rat aorta (Kambayashi *et al.* 1996). Third, the increase in renal AT₂ receptor protein seen in the GR-treated fetuses may have occurred secondary to the decrease in oxygenation and tissue growth induced by AT₁ receptor antagonism. A transient increase in pulmonary AT₂ receptor mRNA abundance and binding is observed in adult rats during 2 weeks of chronic normobaric hypoxia (Chassagne *et al.* 2000), although little is known about the effects of hypoxaemia on AT₂ receptor expression in the fetal or postnatal kidney. In addition, AT₂ receptor expression in cultured cell lines coordinates with changes in the cell cycle and apoptosis, such that receptor mRNA abundance and binding increase when the cells are confluent and decrease when the cells are in the proliferative phase (Camp & Dudley 1995, Yamada *et al.* 1996). Finally, AT₁ receptor blockade *in utero* may delay the normal developmental reduction in renal AT₂ receptor protein expression. In fetal ovine kidneys, AT₂ receptor gene expression decreases during late gestation to an undetectable level by term (Butkus *et al.* 1997, Gimonet *et al.* 1998). This developmental decline in AT₂ receptor expression may depend on AT₁ receptor activity, although AII receptor subtype levels were not determined at the gestational age at the start of the infusion in this study.

Renal growth retardation in the GR138950-treated fetuses did not appear to be associated with suppression of IGF activity. In contrast, circulating IGF-I level was unchanged and plasma IGF-II increased during AT₁ receptor antagonism *in utero*. Renal levels of IGFs, IGF-binding proteins and the IGF type 2 receptor were not determined in this study, but there were no changes in the expression of the insulin or IGF type 1 receptor in the fetal ovine kidney. In adult human

subjects, AT₁-specific receptor antagonism with losartan has been shown previously to increase circulating free IGF-I concentration (Zandbergen *et al.* 2006). Furthermore, in adult rats, i.v. AII infusion for 7 days decreases plasma IGF-I, and this effect is prevented by pretreatment with losartan (Brink *et al.* 1996). The reduction in circulating IGF-I induced by AII is associated with tissue-specific effects on IGF synthesis, including suppression of IGF-I mRNA abundance in the liver and skeletal muscle (Brink *et al.* 2001). In this study, IGF-II is the major circulating IGF in the ovine fetus at 125–130 days of gestation and appears to be more responsive than IGF-I to the stimulatory effects of AT₁ receptor antagonism. Alternatively, the hypoxaemia induced by AT₁ receptor blockade may have suppressed IGF-I synthesis to a greater degree than that of IGF-II. Indeed, in fetal sheep and rodents, circulating IGF-I is reduced and IGF-II is unaffected by a variety of experimental models of undernutrition and/or hypoxaemia (Fowden 2003).

The decrease in kidney weight observed in the ovine fetus following AT₁ receptor blockade may be due to effects on components of the kidney other than the glomeruli, such as tubular and interstitial cell structures, and the volume of blood and tubular fluid. Previous studies have shown that AII stimulates hypertrophy in proximal tubular cells cultured from mouse kidneys (Wolf & Neilson 1990). Furthermore, in fetal sheep, losartan reduces glomerular filtration rate and urine flow, despite an increase in renal blood flow and decrease in renal vascular resistance (Stevenson *et al.* 1996). These responses may be due to the direct effects of AT₁ receptor antagonism on glomerular and vascular, especially efferent arteriolar, function in the developing ovine kidney and/or secondary to the haemodynamic effects of GR138950 on fetal blood pressure and oxygenation seen in this and previous studies (Forhead *et al.* 2000, Forhead & Fowden 2004).

Therefore, AT₁ receptor antagonists, such as GR138950, may retard renal growth *in utero* both by suppression of AT₁ receptor activity and by augmentation of AT₂ receptor protein expression and activity. These findings provide further mechanisms by which AT₁ receptor antagonism is detrimental to the growth and development of the fetal kidney, and support the contraindication of inhibitors of the renin–angiotensin system during pregnancy (Alwan *et al.* 2005). The effects of different types of inhibitors of the renin–angiotensin system on the structural development of the kidney and other tissues appear to be similar (Alwan *et al.* 2005). However, the presence of AT₂ receptors in fetal tissues means that intrauterine exposure to AT₁-specific receptor antagonists may have other consequences for normal tissue growth and development, and long-term function, compared with blockers of AII production, such as ACE inhibitors. In addition, AT₁ receptor antagonists may have a differential effect on the fetal kidney in early gestation when AT₂ receptor protein expression is at a greater and more widespread level than later in gestation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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