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Effect of cortisol on fetal ovine vascular angiotensin II receptors and contractility

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Abstract

The renin angiotensin system is important in the regulation of fetal blood pressure. This study investigated the expression of angiotensin AT_1 and AT_2 receptors in the ovine fetal heart, aorta and umbilical artery, and how these receptors are affected by cortisol. Cortisol infusion into the fetus has previously been shown to cause an increase in fetal blood pressure. We hypothesised that this effect of cortisol is mediated by upregulation of the angiotensin AT_1 receptor. Binding studies performed on tissues with intact endothelium demonstrated both receptor subtypes in the fetal aorta and right ventricle, although the latter contained mainly angiotensin AT_2 receptors. In contrast, only angiotensin AT_1 receptors were found in the umbilical artery. Cortisol infusion into fetuses (3 mg/day for 3–5 days) caused a physiological increase in plasma cortisol levels to 29 ± 4 nM. This was associated with an increase in systolic pressure (57.8 ± 1.7 vs. 52.2 ± 1.5 mm Hg, $P < 0.05$), but cortisol had no effect on the density or affinity of angiotensin receptors, nor on the in vitro contractile responses of carotid and umbilical arterial rings to 5-μM angiotensin II. In conclusion, this study has demonstrated differential expression of angiotensin AT_1 and AT_2 receptors in the different regions of the ovine fetal cardiovascular system and that the angiotensin AT_1 receptor is functional. The lack of any effect of low doses of cortisol on these receptors and on the contractility of isolated fetal vessels to angiotensin II suggests cortisol acts by other mechanisms to raise fetal arterial pressure. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Fetal, sheep; Angiotensin; AT_1 receptor; AT_2 receptor; Cardiovascular; Cortisol

1. Introduction

The renin angiotensin system, through angiotensin II, plays an important role in the regulation of blood pressure. Angiotensin II interacts with two major subtypes of cell-surface receptors, type 1 (AT_1) and type 2 (AT_2). Nearly all the known actions of angiotensin II, including its vasoconstrictor effect, are mediated via the angiotensin AT_1 receptor (Griendling et al., 1996). The functional role of the angiotensin AT_2 receptor is yet to be fully elucidated. Quantitative autoradiographic studies have demonstrated a widespread expression of angiotensin AT_2 receptors in fetal tissues that declines dramatically after birth (Grady et al., 1991; Tsutsumi and Saavedra, 1991) suggesting that angiotensin AT_2 receptors play a role in growth and development. However, mice lacking the angiotensin AT_2 receptor develop normally (Hein et al., 1995; Ichiki et al., 1995). Others propose that angiotensin II acting at the angiotensin AT_2 receptor may actually oppose its pressor and growth effects mediated via the angiotensin AT_1 receptor (Scheuer and Perrone, 1993; Ichiki et al., 1995; Hein et al., 1995; Stoll et al., 1995; Munzenmaier and Greene, 1996; McMullen et al., 1999). This inhibitory role of the angiotensin AT_2 receptor may be unique to the vasculature. Studies investigating angiotensin-induced prostaglandin synthesis and contraction of the non-vascular smooth muscle of the myometrium found no evidence for a similar function of the angiotensin AT_2 receptor in this tissue (Cox et al., 1993, 1996).

We are interested in the role of the fetal renin angiotensin system in cardiovascular function and development. Infusions of angiotensin II into fetal sheep cause a rise in arterial pressure (Stevenson and Lumbers, 1995),

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and angiotensin-converting enzyme inhibitors reduce fetal arterial pressure (Lumbers et al., 1992, 1993). Furthermore, administration of losartan, the angiotensin AT_1 receptor antagonist, decreases mean fetal arterial pressure and markedly attenuates the pressor response to angiotensin II. This indicates that the fetal renin angiotensin system contributes to the maintenance of blood pressure and its effects are mediated via the angiotensin AT_1 receptor (Stevenson et al., 1996).

One aim of the present study was to investigate the expression of the angiotensin AT_1 and AT_2 receptors in the ovine fetal cardiovascular system. We also wanted to determine if fetal angiotensin AT_1 receptors were functional by testing the ability of fetal systemic vessels to elicit contractile responses to angiotensin II in vitro. Since beginning these studies, another group has published results showing that the ovine umbilical artery contains only angiotensin AT_1 receptors while fetal systemic arteries, including the aorta, carotid and femoral arteries, express only angiotensin AT_2 receptors (Kaiser et al., 1998; Cox and Rosenfeld, 1999). These observations would suggest that the pressor effects of angiotensin II and the hypotensive actions of losartan described above, might solely be due to actions of these drugs in the placental circulation, although actions of angiotensin II within the fetal central nervous system cannot be excluded (Ismay et al., 1979).

A second aim of this study was to determine the effects of cortisol on the density and distribution of angiotensin AT_1 and AT_2 receptors in the ovine fetal cardiovascular system. Tangalakis et al. (1992) found that infusion of a physiological dose of cortisol into fetal sheep caused an increase in fetal arterial pressure and an increased pressor response to angiotensin II. Sato et al. (1994) showed that glucocorticoid treatment increased angiotensin AT_1 receptor expression in the vascular smooth muscle cells of the adult rat. Thus, we hypothesized that the rise in fetal arterial pressure and increased sensitivity to angiotensin II occurring with cortisol infusion would be due to upregulation of the angiotensin AT_1 receptor. In the present study, we expected cortisol infusion to increase cardiovascular angiotensin AT_1 receptor expression and that this would be accompanied by an increased contractile response of arterial vessels to angiotensin II in vitro.

2. Materials and methods

2.1. Materials

[Sar^1,$I1e^8$]angiotensin II (Auspep, Australia); angiotensin II (Hypertensin, Ciba, a gift from Ciba-Geigy, Basle, Switzerland); bovine serum albumin (Sigma, St. Louis, MO, USA); bradykinin (Sigma); bupivacaine (Marcaine, Astra Pharmaceuticals, North Ryde, NSW, Australia); halothane (Flurothane, ICI, Macclesfield, Cheshire, UK); hydrocortisone sodium succinate (Solucortef, UpJohn, Rydalmere, NSW, Australia); lactoperoxidase (Sigma); losartan (DuP 753, a gift from DuPont Merck Pharmaceutical, Wilmington, DE, USA); noradrenaline (Levophed, Sanofi, Winthrop, Ermington, NSW, Australia); PD 123319, (*S*)1-[[4-(dimethylamino)-3-methylphenyl]-methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo(4,5-c) pyridine-6-carboxylic acid, ditrifluoroacetate, monohydrate (Parke-Davis, Ann Arbor, MI, USA); phosphoramidon (Sigma); procaine penicillin/dihydrostreptomycin sulphate (Hydropen, Bomac Laboratories, Asquith, NSW, Australia); serotonin (Sigma); sodium pentobarbitone (Lethabarb, Virbac, Peakhurst, NSW, Australia); sodium thiopentone (Pentothal, Abbott Australasia, Kurnell, NSW, Australia).

2.2. Animal experiments

The Animal Care and Ethics Committee of the University of New South Wales approved these experiments. Twenty crossbred Merino ewes and their 24 fetuses (10 cortisol treated, 10 saline treated/non-infused and 4 non-catheterised twins) were studied. Ewes were housed individually in metabolic cages and ate freely from 1200-g chaff, 100-g oats, 6-g salt and 6-l of water daily.

Ewes were anaesthetized i.v. with 1-g sodium thiopentone and anaesthesia was maintained with 2–3% halothane in oxygen. Catheters were inserted into a fetal femoral artery and tarsal vein and a maternal femoral artery and vein using methods described by Lumbers and Stevens (1983). An amniotic catheter was stitched to the fetal back. Antibiotics (3 ml, 600-mg procaine penicillin + 750-mg dihydrostreptomycin sulphate) were administered i.m. to the ewe at the end of surgery and to the fetus via the amniotic cavity at the end of surgery and for 2 days post-operatively. Maternal suture lines were infiltrated with 0.5% bupivacaine. Sheep were allowed to recover for at least 5 days prior to study.

Basal maternal and fetal arterial pressures, heart rates and amniotic pressure were recorded for 1 h using disposable transducers (Spectramed Medical Products, Singapore) connected to a Grass Polygraph Model 79E. Data were collected using an IBM-compatible PC. Heart rate was determined from the beat-to-beat interval. Fetal arterial pressure was corrected for amniotic pressure.

After 20 min, 5-ml maternal and 7-ml fetal arterial blood samples were collected into heparinised syringes. Arterial oxygen (PO_2) and carbon dioxide (PCO_2) tensions and pH corrected to 39.5°C were immediately measured using a Ciba-Corning 288 blood-gas analyser (Model 288, Medfield, MA). The remaining blood was centrifuged at $1100 \times g$ for 10 min at 4°C and the plasma stored at −20°C.

An i.v. infusion to the fetus (0.5 ml/h) of either cortisol (3 mg/day, hydrocortisone sodium succinate) or 0.15 M saline was then begun and continued for 3–5 days. Four fetuses (three saline and one cortisol treated) received a

5-day infusion, nine fetuses received a 3-day cortisol infusion. In vitro contractility data from the carotid arteries of the four fetuses that received a 5-day infusion were not included in the analysis due to gestation age-related differences in their contractile responses.

On the final day of infusion, arterial pressures were again recorded for 1 h and arterial blood samples obtained. The ewes were killed by i.v. 15–20 ml pentobarbitone sodium (300 mg/ml). Gestation age at post-mortem was 118 ± 0.5 days. Segments of the umbilical artery from within the umbilical cord and fetal carotid arteries were carefully dissected without stretch and studied to test their in vitro reactivity to vasoactive substances (Section 2.4). Fetal heart, aorta, and remaining umbilical arteries were frozen in liquid nitrogen and stored at −80°C until used for receptor-binding assays. We have previously reported that there are no changes in binding characteristics following storage of 1-cm^3 blocks of tissue for 6 months at −80°C (Burrell and Lumbers, 1997).

Plasma cortisol levels were measured by cortisol [^{125}I]radioimmunoassay kit (Orion Diagnostica, Espoo, Finland).

2.3. Receptor-binding assays

2.3.1. Radioligand preparation

[Sar^1,Ile^8]angiotensin II was labeled with ^{125}Iodine using lactoperoxidase and purified by reverse-phase high-performance liquid chromatography using a C18 selectosil column (Phenomenex, Torrance, CA, USA) (Burrell and Lumbers, 1997). The specific activity of the ligand (2175 μCi/mmol) was assumed to be the theoretical maximum. To check the stability of the radioligand, the specific activity was calculated using a self-displacement assay twice during the first half life. These assays showed no change in specific activity, indicating balanced decay. The radioligand was stored in 0.5% bovine serum albumin at −20°C and used within 10 weeks.

2.3.2. Tissue preparation

Tissues were thawed to 4°C, and the connective tissue surrounding the blood vessels was removed. No attempt was made to remove the vascular endothelium, nor the endocardium. Plasma membranes were prepared for saturation and competition-binding assays as previously described (Burrell and Lumbers, 1997), except that crude tissue homogenates were centrifuged twice at $400 \times g$ for 1 min prior to the final spin at $30{,}000 \times g$ for 45 min. Phosphoramidon (10 μM) was added to preparations of heart membranes. Pilot studies investigating a range of protease inhibitors showed that optimal binding occurred in blood vessels with incubation buffer alone, and in heart, after the addition of 10 μM phosphoramidon. The protein concentration of each tissue homogenate was measured by the method of Lowry et al. (1951). The mean protein concentrations of the membranes were right ventricle: 108.3 ± 4.3 mg/ml, $n = 11$; aorta: 92.3 ± 4.7 mg/ml, $n = 11$; umbilical artery: 65.0 ± 4.3 mg/ml, $n = 11$.

2.3.3. Saturation-binding assays

Saturation binding assays were performed to measure the density of total angiotensin II receptors. Freshly prepared membrane suspensions (100-μl aliquots) were incubated for 60 min at 22°C with 25 μl of one of eight concentrations of ^{125}I-[Sar^1,Ile^8]angiotensin II (1.4 pM–1.5 nM angiotensin II in 0.3% bovine serum albumin) and 25 μl of phosphate-buffered saline, or 10 μM unlabelled [Sar^1,Ile^8]angiotensin II to measure non-specific binding. In preliminary experiments, it was shown that equilibrium conditions were reached within 60 min (McMullen, 2000).

2.3.4. Competition-binding assays

Competition-binding assays were performed using unlabelled [Sar^1,Ile^8]angiotensin II, losartan and PD 123319. Freshly prepared membrane suspensions (100-μl aliquots) were incubated for 60 min at 22°C with 25 μl ^{125}I-[Sar^1,Ile^8]angiotensin II (0.1 nM) and 25 μl of increasing concentrations of either unlabelled [Sar^1,Ile^8]angiotensin II (10 pM–1 μM), losartan (1 pM–1 mM) or PD 123319 (1 pM–1 mM). Maximum binding was measured in the absence of an antagonist. Non-specific binding was measured in the presence of 10 μM unlabelled [Sar^1,Ile^8]angiotensin II.

In both saturation and competition assays, the reaction was stopped by the addition of ice-cold 0.15 M NaCl. Bound and free fractions were separated by vacuum filtration (Brandel Harvester, Gaithersburg, MD, USA) through GF/C Whatman filter paper presoaked in 1% bovine serum albumin. Radioactivity was measured in an automated gamma counter (1470 Wizard, Wallac, Turku, Finland; efficiency 78.5%).

2.4. In vitro reactivity of fetal carotid and umbilical arteries to vasoactive substances

Ring segments (3–4 mm) were cut from freshly dissected fetal carotid and umbilical arteries, mounted in organ baths and connected to force transducers (Grass Instrument, Quincy, MA, USA) via wire struts. The endothelium of umbilical arterial rings was removed by the wire struts. The endothelium was not intentionally removed from fetal carotid arterial rings to ensure that the underlying vascular smooth muscle was not damaged. Davidson and Eldemerdash (1990) reported that excessive rubbing to remove all endothelial cells from neonatal arteries from guinea pigs resulted in poor contractile responses to phenylephrine. Since a primary aim of this part of the study was to determine whether fetal systemic vessels were able to contract to angiotensin II, it was critical that the vascular smooth muscle was intact. The organ baths were filled with 10 ml Krebs solution (118.0 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.18 mM

$MgSO_4$, 25 mM $NaHCO_3$, 5 mM glucose, 0.5 mM $CaCl_2$), bubbled with 95% O_2:5% CO_2 and maintained at a temperature of approximately 37°C and pH 7.4. Transducers were connected to a Neotrace channel amplifier (Neomedix Systems, Dee Why West, NSW, Australia). Data were collected and analyzed using a MacLab/8 computer system (Analogue Digital Instruments, Castle Hill, NSW, Australia).

2.4.1. Carotid artery

After 1-h equilibration, the rings were stretched to an optimum resting tension of 1 g. The optimum resting tension was determined in preliminary experiments by comparing the tension developed by 40-mM KCl under different resting conditions. After 10 min, the contractile responses of the rings to three separate exposures to 80-mM KCl were measured. The latter two responses were averaged and used as the standard to which the responses to other drugs were compared. The response to 5-μM angiotensin II was obtained. Rings were then exposed to losartan (1, 10 or 100 μM) or PD 123319 (1 μM) for 30 min and the contractile response to 5-μM angiotensin II again measured. Previous studies indicated that rings of the fetal carotid artery did not demonstrate tachyphylaxis to angiotensin II in vitro (McMullen et al., 1997). In the current study, a number of carotid and umbilical arterial rings received consecutive doses of 5 μM angiotensin II in the absence of antagonist to again test for tachyphylaxis. Carotid arterial rings demonstrated small but significant tachyphylaxis to angiotensin II in vitro. Therefore, the contractions to angiotensin II in the presence of antagonists were compared to the responses of rings that had received a second dose of angiotensin II. Rings of umbilical artery also showed tachyphylaxis. This, along with the limited number of rings available, did not permit the construction of dose–response curves.

Following exposure to angiotensin II in the presence or absence of the angiotensin receptor antagonists, carotid arterial rings were exposed to 80-mM KCl again, and then the response to 1-μM noradrenaline obtained. When the contraction to noradrenaline was stable, 1 μM bradykinin was given.

2.4.2. Umbilical artery

A similar protocol was used except that the umbilical arterial rings were stretched to a tension of 2 g (White, 1989). As stated above, umbilical arterial rings displayed a more reduced contractile response to angiotensin II on repeated exposures, therefore, most rings were exposed to angiotensin II only once. In each experiment, a single ring was exposed three times to 5-μM angiotensin II in the absence of antagonists to quantitate tachyphylaxis. The remaining rings were incubated for 30 min with either losartan (1, 10 or 100 μM) or PD 123319 (1 μM) and the response to 5-μM angiotensin II was tested. Since umbilical arterial rings often displayed a very weak contractile response to 1-μM noradrenaline, 10-μM serotonin was used in some experiments to preconstrict the vessels prior to the addition of bradykinin.

The contractile responses of fetal arterial rings to angiotensin II were very small. In this study, 5-μM angiotensin II was used as it consistently caused both carotid and umbilical arterial rings to contract. Although, a full dose–response curve could not be constructed due to the limited number of rings available and the demonstration of tachyphylaxis (as described above) preliminary experiments indicated this concentration was near maximal.

2.5. Calculations and statistical analysis

For the receptor-binding assays, specific binding was determined by subtracting non-specific binding from the total binding. Saturation and displacement (one or two site) binding curves were fitted and B_{max}, K_d and IC_{50} values calculated using the computer program Prism (GraphPad Software, San Diego, CA, USA). Calculation of F ratios have determined that losartan and PD 123319 displacement curves from fetal aorta and right ventricle best fit a two site binding model. The proportions of angiotensin AT_1 and AT_2 receptors in each tissue were calculated from the fraction of total specific binding displaced with high affinity by losartan and PD 123319, respectively. For this purpose, binding curves were manually set to plateau at zero. Receptor densities are expressed as femtomole per milligram protein. The in vitro responses of arterial rings to angiotensin II and noradrenaline are expressed as a percentage of the KCl response. All statistical analyses were performed using SPSS for Windows (Chicago, IL, USA). Data are presented as means ± S.E. Initial and final physiological measurements were compared using Student's paired t-test. All other statistical comparisons were made using Student's non-paired t-test or analysis of variance (ANOVA). If ANOVA showed significance, it was followed by a Student–Newman–Keuls test.

3. Results

3.1. Animal experiments

By the end of the infusion period, the plasma cortisol concentrations in cortisol-treated fetuses were significantly increased compared to control animals ($P < 0.01$, Table 1). Cortisol-infused fetuses also had significantly higher systolic pressures than control fetuses at this time ($P < 0.05$, Table 1). Arterial blood gases and pH were similar in the two groups, although cortisol infusion was associated with a very small fall in PCO_2 ($P < 0.05$, Table 1).

3.2. Saturation-binding assays

Infusion of cortisol did not change the density of total angiotensin II receptors in fetal right ventricle (control:

Table 1
Plasma cortisol levels, arterial blood pressures, gases and pH before and after control (saline/no infusion) or cortisol infusion

	Control		Cortisol	
	Initial	Final	Initial	Final
Plasma cortisol (nM)	9 ± 1^{a}	12 ± 3^{a}	10 ± 1	$29 \pm 4^{b,c}$
Diastolic BP (mm Hg)	30.4 ± 0.7	32.3 ± 0.8^{b}	30.8 ± 1.3	33.9 ± 1.1^{d}
Systolic BP (mm Hg)	50.9 ± 1.4	52.2 ± 1.6	51.8 ± 1.7	$57.8 \pm 1.7^{b,e}$
Mean BP (mm Hg)	38.5 ± 0.7	40.1 ± 1.0^{d}	39.0 ± 1.3	43.2 ± 1.2^{b}
Arterial PO_2 (mm Hg)	23.0 ± 0.6	23.2 ± 0.8	21.2 ± 0.5	22.4 ± 0.7
Arterial PCO_2 (mm Hg)	53.2 ± 1.2	53.3 ± 0.6	54.7 ± 0.1	53.5 ± 0.9^{d}
Arterial pH	7.354 ± 0.011	7.359 ± 0.004	7.359 ± 0.003	7.368 ± 0.004

Values are means ± S.E. Number of animals (n): control $n = 8$, cortisol $n = 9$, unless otherwise indicated.
[a] $n = 7$.
[b] $P < 0.01$ different from initial.
[c] $P < 0.01$ different from control.
[d] $P < 0.05$ different from initial.
[e] $P < 0.05$ different from control.

143 ± 14, $n = 5$; cortisol: 172 ± 50 fmol/mg protein, $n = 5$), aorta (control: 51 ± 10, $n = 5$; cortisol: 60 ± 16 fmol/mg protein, $n = 5$) and umbilical artery (control: 74 ± 11, $n = 4$; cortisol: 107 ± 27 fmol/mg protein, $n = 5$).

Cortisol treatment also had no effect on the affinity of ^{125}I-[Sar^1,Ile^8]angiotensin II for angiotensin receptors in the right ventricle (K_d control: 0.6 ± 0.04, $n = 5$; cortisol: 0.7 ± 0.1 nM, $n = 5$), aorta (K_d control: 0.8 ± 0.1, $n = 5$; cortisol: 0.7 ± 0.1 nM, $n = 5$) and umbilical artery (K_d control: 1.2 ± 0.3, $n = 4$; cortisol: 1.3 ± 0.2 nM, $n = 5$). Using the combined data of both treatment groups, the affinity of ^{125}I-[Sar^1,Ile^8]angiotensin II for angiotensin receptors in umbilical artery (1.2 ± 0.2 nM, $n = 9$) was significantly lower than that for the receptors in the aorta (0.8 ± 0.1 nM, $n = 10$, $P < 0.05$) and the right ventricle (0.6 ± 0.04 nM, $n = 10$, $P < 0.05$).

Hill coefficients (n_H) indicated no cooperativity was involved in the binding of ^{125}I-[Sar^1,Ile^8]angiotensin II to angiotensin receptors in the right ventricle: 0.99 ± 0.01, $n = 10$; aorta: 0.96 ± 0.01, $n = 10$; umbilical artery: 0.98 ± 0.01, $n = 9$.

3.3. Competition-binding assays

Competitive-binding assays demonstrated little effect of cortisol treatment on the affinity of angiotensin receptors for their ligands or on the proportion of the angiotensin II receptor subtypes in the right ventricle, aorta and umbilical artery (see below). Curves generated using the combined data from both treatment groups (Fig. 1) demonstrate the pattern of radioligand displacement by unlabelled [Sar^1,Ile^8]angiotensin II, losartan and PD 123319, in the three tissues.

The umbilical artery contained only angiotensin AT_1 receptors. This was evident from the single high-affinity binding site of losartan (Fig. 1b, Table 2), and the lack of a high-affinity binding site for PD 123319 (Fig. 1c, Table 2).

Assays of fetal aorta demonstrated high-affinity binding sites for both losartan and PD 123319, indicating the presence of angiotensin AT_1 and AT_2 receptor subtypes (Fig. 1, Table 2). Cortisol treatment did not increase the proportion of angiotensin AT_1 receptors (control: $50 \pm 10\%$, $n = 5$; cortisol $26 \pm 10\%$, $n = 4$) nor decrease the proportion of AT_2 receptors (control: $47 \pm 9\%$, $n = 5$; cortisol: $67 \pm 11\%$, $n = 3$). There was insufficient tissue to perform both contractile studies and receptor-binding assays on fetal carotid arteries. However, in a pilot experiment, it was shown that like the fetal aorta, a carotid artery from a fetal sheep also contained both AT_1 and AT_2 receptor subtypes (approximately 40% AT_1/53% AT_2).

Assays of right ventricular tissue generally showed high-affinity binding sites for losartan and PD 123319, again indicating the presence of both angiotensin receptors (Fig. 1, Table 2). There was, however, a clear predominance of angiotensin AT_2 receptors (control: $67 \pm 8\%$, $n = 5$; cortisol: $78 \pm 5\%$, $n = 4$). The small proportion of angiotensin AT_1 receptors led to some difficulty in their estimation in this tissue, and in four out of 11 cases, the high-affinity losartan binding site was not apparent. Cortisol treatment was found to cause a small reduction in the proportion of angiotensin AT_1 receptors of the right ventricle (control: 15 ± 3, $n = 6$; cortisol: $4 \pm 3\%$, $n = 5$, $P < 0.05$), but given the low proportions measured, the biological significance of this result is unclear.

Unlabelled [Sar^1,Ile^8]angiotensin II displaced radioligand binding at a single high-affinity site in all three tissues (Fig. 1a, Table 2). Similar to the dissociation constants (K_d) in the saturation-binding assays, combined data showed that the affinity of [Sar^1,Ile^8]angiotensin II for angiotensin receptors in the umbilical artery (IC_{50} 1.4 ± 0.2 nM, $n = 11$) was similar to that for aortic receptors (IC_{50} 1.0 ± 0.1 nM, $n = 11$, NS) but lower than that for receptors from the right ventricle (IC_{50} 0.8 ± 0.1 nM, $n = 11$, $P < 0.05$). Perhaps this represents slight differences in the affinity of [Sar^1,Ile^8]angiotensin II for the angiotensin AT_1

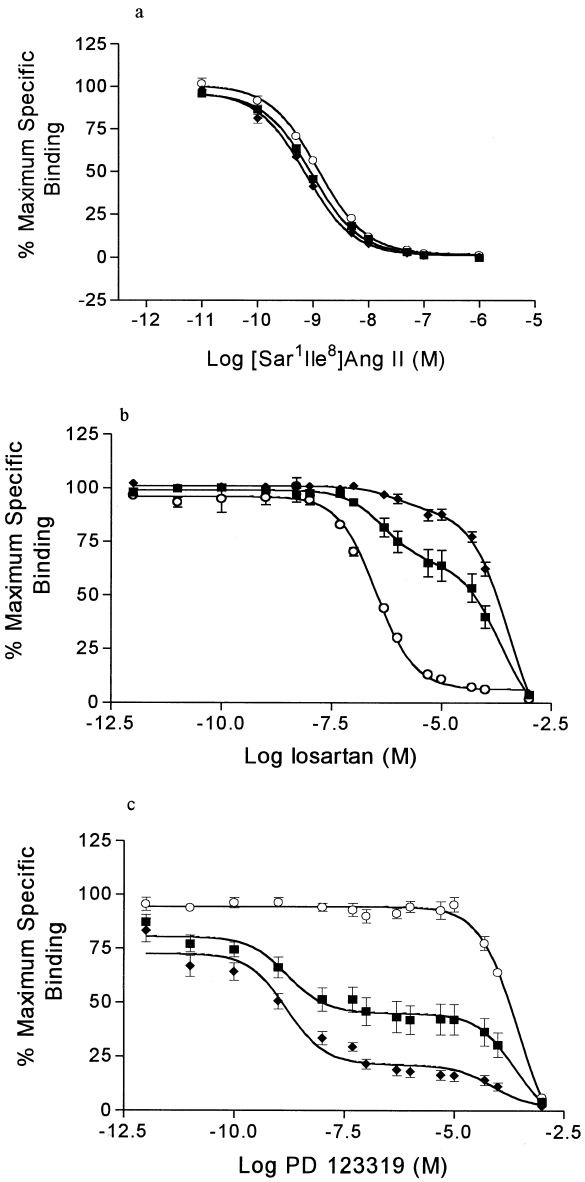

Fig. 1. Displacement of ^{125}I-[Sar^1,Ile^8]angiotensin II binding by (a) [Sar^1,Ile^8]angiotensin II, (b) losartan and (c) PD 123319, in membranes prepared from right ventricle (♦), aorta (■) and umbilical artery (○). Values represent means ± S.E. from at least 10 assays with each inhibitor.

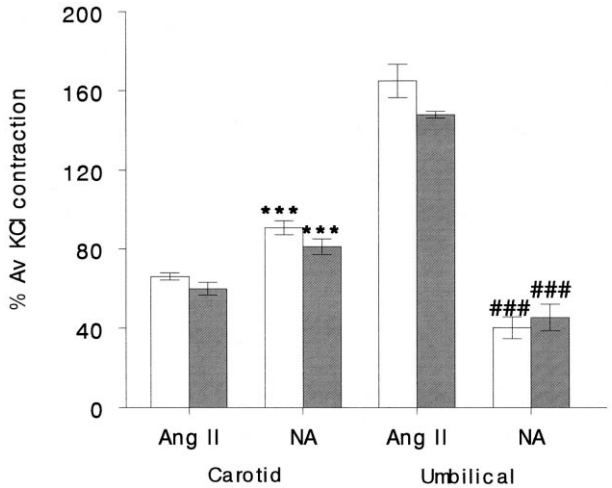

Fig. 2. Contraction of rings of carotid and umbilical artery from control (white bars) and cortisol treated fetuses (shaded bars) to angiotensin II (Ang II, 5 μM) and noradrenaline (NA, 1 μM). Values are means ± S.E. There are at least 11 rings in each group. $^{***}P < 0.001$ compared to carotid Ang II. $###P < 0.001$ compared to umbilical Ang II.

receptor, the only receptor present in the umbilical artery, and the angiotensin AT_2 receptor, the predominant receptor in the right ventricle.

3.4. In vitro contractility of fetal carotid and umbilical arteries

Rings of both carotid and umbilical artery contracted to 5-μM angiotensin II in vitro (Fig. 2). Treatment with cortisol did not have any effect on the responsiveness of these vessels to 5-μM angiotensin II nor to 1-μM noradrenaline (Fig. 2). Therefore, data from both groups were combined.

Carotid arterial rings demonstrated a small but significant degree of tachyphylaxis. Contractions induced by a second and third exposure to angiotensin II were 87 ± 3% and 84 ± 3% ($n = 9$) of the initial angiotensin-II-induced contraction ($P < 0.001$). Rings of the umbilical artery showed more marked tachyphylaxis, i.e. the second and

Table 2
Half maximal displacement values (IC_{50}, nM) of [Sar^1,Ile^8] angiotensin II, losartan and PD 123319 in right ventricle, aorta and umbilical artery

	Control			Cortisol		
	Sar	Los	PD	Sar	Los	PD
Umbilical artery	1.6 ± 0.4	468 ± 62	–	1.2 ± 0.2	415 ± 64	–
Aorta	1.0 ± 0.1	394 ± 49	6.5 ± 2.3	1.0 ± 0.2	351 ± 116	5.5 ± 1.3[a]
Right ventricle	0.9 ± 0.2	469 ± 109	3.8 ± 1.3	0.8 ± 0.1	924 ± 361[b]	5.0 ± 0.8

Values are means ± S.E. Number of tissues assayed (n): control n = at least 5, cortisol n = at least 4, unless otherwise indicated.
IC_{50}s were determined by inhibition of ^{125}I-[Sar^1,Ile^8] angiotensin II binding to membrane preparations of umbilical artery, aorta and right ventricle from control and cortisol treated fetuses by unlabeled [Sar^1,Ile^8] angiotensin II (Sar), the angiotensin AT_1 receptor antagonist losartan (Los) or the angiotensin AT_2 receptor antagonist PD 123319 (PD).
[a] $n = 3$.
[b] $n = 2$.

third contractions to angiotensin II were 73 ± 3% and 67 ± 3% ($n = 17$) of the initial angiotensin II-induced contraction ($P < 0.001$).

Preincubation with the angiotensin AT_1 receptor antagonist, losartan, reduced the contractile response of carotid and umbilical arterial rings to angiotensin II in a dose-dependent manner (Table 3). However, there was no inhibitory effect of 1-μM losartan on the responses of carotid rings and 100-μM losartan was required for complete blockade. As well, inhibition of the response of umbilical rings to angiotensin II was minimal with 1-μM losartan, and again 100-μM losartan was required for complete blockade (Table 3). Losartan had no effect on the contractile response of carotid arterial rings to noradrenaline.

Exposure to PD 123319 (1 μM) had no effect on the contractile response of carotid arterial rings to angiotensin II. Inexplicably, it did cause a minor inhibitory effect on the umbilical arterial rings ($P < 0.05$, Table 3).

Umbilical arterial rings showed greater contractility to 5-μM angiotensin II when compared with the responses of fetal carotid arterial rings (156 ± 6.9, $n = 22$ vs. 62.9 ± 1.9% average KCl contraction, $n = 51$, $P < 0.001$, Fig. 2). Since the contractions to angiotensin II were related to the contractile response of individual rings to KCl, this difference was even more marked, because umbilical arterial rings also contracted more strongly to KCl compared to carotid arterial rings (80 mM, 3.24 ± 0.14 g, $n = 52$, vs. 2.22 ± 0.08 g, $n = 51$, $P < 0.001$).

Rings from the two vessels showed remarkably different contractile reactivity to angiotensin II and noradrenaline. Whereas carotid arterial rings were more responsive to 1-μM noradrenaline than to 5-μM angiotensin II ($P < 0.001$, Fig. 2), umbilical arterial rings showed a much-reduced response to 1-μM noradrenaline compared to their response to 5-μM angiotensin II ($P < 0.001$, Fig. 2).

The presence of endothelium was tested by measuring the degree of relaxation achieved when 1-μM bradykinin was presented to rings preconstricted with 1-μM noradrenaline or 10-μM serotonin. The endothelial lining of umbilical arterial rings was removed by the wire struts, as indicated by relaxation of only 15 ± 2% ($n = 40$). A limitation of the study was that the endothelium of fetal carotid arterial rings was neither fully intact nor completely removed, indicated by relaxation of 57 ± 3% ($n = 51$). However, the bradykinin-induced relaxation of rings of the carotid artery from control- and cortisol-treated fetus was similar (control: 52 ± 6, $n = 25$; cortisol: 61 ± 3, $n = 26$); thus, valid comparisons can be made between treatment groups.

Table 3
Contractile responses of endothelium intact carotid and denuded umbilical arterial rings to 5-μM angiotensin II (Ang II) in the presence or absence of the angiotensin AT_1 receptor antagonist losartan (Los) or the angiotensin AT_2 receptor antagonist PD 123319 (PD)

Condition	Carotid		Umbilical	
	Contraction (% KCl contraction)	n	Contraction (% KCl contraction)	n
Ang II	59.0 ± 3.6	(9)	156.3 ± 6.9	(22)
Ang II + 1 μM Los	62.3 ± 3.5	(21)	118.0 ± 7.0[a]	(9)
Ang II + 10 μM Los	32.6 ± 6.3[a]	(11)	64.3 ± 7.1[a]	(7)
Ang II + 100 μM Los	5.4 ± 1.6[a]	(13)	1.2 ± 0.2[a]	(4)
Ang II + 1 μM PD	60.8 ± 2.9	(13)	129.0 ± 11.5[a]	(8)

Values are means ± S.E. n = number of arterial rings. The contractile response of carotid arterial rings to angiotensin II in the presence losartan or PD 123319 was measured after having already been exposed to angiotensin II once before. Carotid arterial rings were found to show a small but significant degree of tachyphylaxis to angiotensin II. Therefore, these responses were compared to the contractile response of rings that received a second dose of angiotensin II alone (Ang II).

[a] $P < 0.05$ different from Ang II alone.

4. Discussion

This study has shown that angiotensin AT_1 receptors are present and functional in the ovine fetal cardiovascular system. Receptor-binding studies demonstrated angiotensin AT_1 receptors in ovine fetal right ventricle, aorta and umbilical artery. Rings of umbilical artery, and for the first time, rings of fetal carotid artery were reported to contract to angiotensin II in vitro.

The effect of cortisol on the cardiovascular angiotensin receptors was also investigated. We had hypothesised that the cortisol-induced increase in arterial pressure and in vascular reactivity to angiotensin II, previously described by Tangalakis et al. (1992), was due to an action of the steroid to upregulate angiotensin AT_1 receptor density and distribution in the cardiovascular system. We further postulated that this effect would be reflected in an increased reactivity of arterial segments from cortisol-treated fetuses to angiotensin II in vitro.

Infusion of cortisol into the fetus (gestation age 118 ± 0.5 days) produced final plasma cortisol concentrations of 29 ± 4 nM, levels well within the normal physiological range found in the last fifth of gestation (Norman et al., 1985). This increase in cortisol concentration was accompanied by a rise in fetal systolic pressure (Table 1). The effect on fetal arterial pressure was not quite as great as seen in the study by Tangalakis et al. (1992) in which the cortisol concentration was raised to 36.8 ± 5.8 nM.

Despite increasing systolic pressure, cortisol infusion did not, in fact, have any effect on angiotensin-receptor densities or affinities in the tissues examined. Previous studies found that glucocorticoid treatment increased angiotensin AT_1 receptor mRNA expression in rat heart (Della Bruna et al., 1995) and angiotensin AT_1 receptor mRNA expression and receptor number in isolated adult-rat vascular smooth muscle cells (Sato et al., 1994). However, in these studies, synthetic dexamethasone was used, which

is a much more potent glucocorticoid than cortisol. Similarly, infusion of a high dose of cortisol (3 mg/h for 48 h) that elevated circulating cortisol levels to 1130 ± 230 caused an increase in cardiac angiotensin AT_1 receptor mRNA expression in fetal sheep (Segar et al., 1995). The absence of an effect of cortisol treatment on receptor number in the current study may be due to the comparatively subtle glucocorticoid exposure. In this study, we intentionally infused a much lower dose of cortisol (3 mg/day, for 3–5 days) in order to keep the circulating levels within the physiological range. It would be of interest to see if cardiovascular angiotensin AT_1 receptor protein levels were increased if the plasma cortisol concentration was raised to the level reached during the prepartum cortisol surge that occurs in the sheep (approximately 200 nM; Norman et al., 1985), which is higher than that reached in this study but much lower than the level reached in the study by Segar et al. (1995).

In addition to the lack of an effect of cortisol infusion on receptor density, cortisol did not affect the in vitro responses of either carotid or umbilical arterial rings to 5-μM angiotensin II (Fig. 2). The lack of an effect of cortisol infusion on the reactivity of isolated vessels to angiotensin II suggests that the cortisol-induced increase in pressor response to angiotensin II seen by Tangalakis et al. (1992) that accompanied the increase in fetal arterial pressure was not mediated by an increase in angiotensin-receptor density or sensitivity within the blood vessels themselves. Instead, cortisol may actually increase fetal blood pressure and responsiveness to angiotensin II by enhancing the potent central-pressor actions of angiotensin II.

We are the first to demonstrate the presence of angiotensin AT_1 receptors, in addition to angiotensin AT_2 receptors, in the ovine fetal aorta (see also Burrell et al., in press). Both receptor subtypes were also found in the right ventricle, but in this tissue, the angiotensin AT_2 receptors were clearly predominant and the low numbers of angiotensin AT_1 receptors were difficult to measure. In contrast, only angiotensin AT_1 receptors were found in the umbilical artery. The affinity of losartan for angiotensin AT_1 receptors in these fetal tissues was similar to that found for angiotensin AT_1 receptors in the adult-sheep aorta ($IC_{50} \sim 10^{-7}$ M; Burrell and Lumbers, 1997). Our finding that the ovine umbilical artery contains only angiotensin AT_1 receptors is in accordance with recently published data (Kaiser et al., 1998; Cox and Rosenfeld, 1999). However, Cox and Rosenfeld (1999) report no angiotensin AT_1 receptors in either the aorta or carotid artery of fetal sheep. Our competition-binding studies clearly demonstrate the presence of angiotensin AT_1 as well as angiotensin AT_2 receptors in the fetal aorta (Fig. 1b and c, Table 2). Furthermore, in recent studies in which fetal aorta was preincubated in 10^{-6} M PD 123319 for 30 min before losartan competition assays were performed, the low-affinity losartan binding site (10^{-4} M) disappeared and the high-affinity (10^{-7} M) binding site remained (the proportion of angiotensin AT_1 receptors and their IC_{50} were unchanged). Therefore, this high-affinity site must represent the angiotensin AT_1 receptor subtype (Burrell et al., in press). The fact that rings of fetal carotid artery contracted to angiotensin II in vitro and that this contraction was inhibited by losartan indicate the presence of functional angiotensin AT_1 receptors in this vessel also. The discrepancy between the findings of Cox and Rosenfeld (1999) and this study may be partially explained by the fact that, in the present study, endothelium was not removed from the blood vessels used in receptor-binding studies, whereas Cox and Rosenfeld (1999) denuded their vessels. However, Cox and Rosenfeld (1999) have also shown, using emulsion autoradiographic techniques, that binding of ^{125}I-[Sar^1,Ile^8]angiotensin II to the endothelium of fetal systemic arteries was unaffected by the angiotensin AT_1 receptor antagonist, losartan. Thus, it is unlikely that the population of angiotensin AT_1 receptors found in fetal aorta in the present study was derived solely from the endothelium.

Interestingly, 100-μM losartan was required for substantial blockade of angiotensin-II-induced contraction in fetal carotid and umbilical arterial rings, compared to 1-μM losartan in adult uterine arterial rings (McMullen et al., 1999). In addition, blood vessels of adult sheep and other species demonstrate marked tachyphylaxis to angiotensin II in vitro (McMullen et al., 1999; Sim and Kutten, 1992), whereas fetal arterial rings displayed minimal tachyphylaxis in this study. Thus, it appears that there are functional differences between fetal and adult angiotensin II receptors of vascular smooth muscle.

The significance of the markedly different proportions of the receptor subtypes in the various regions of the cardiovascular system will not be clear until the function of the angiotensin AT_2 receptor has been determined. A significant body of data suggests that angiotensin II acting via the angiotensin AT_2 receptor opposes its AT_1-mediated pressor effects. Angiotensin AT_2 receptor-gene-knockout mice have been shown to have an increased pressor response to angiotensin II (Ichiki et al., 1995; Hein et al., 1995). Ichiki et al. (1995) also observed an increase in basal blood pressure. Furthermore, McMullen et al. (1999) showed that angiotensin AT_2 receptor blockade enhanced the contractile response of rings of uterine artery from pregnant sheep to angiotensin II. The fetal carotid artery, like the fetal aorta, contains both angiotensin AT_1 and AT_2 receptors. Therefore, it is possible that the weaker contractile response of the carotid artery to angiotensin II compared with the umbilical artery (Fig. 2) could have been due to antagonism of the angiotensin AT_1 receptor-mediated contractile response via angiotensin AT_2 receptors. However, in the present study, unlike McMullen's experiments using adult uterine arterial rings, preincubation of fetal carotid arterial rings with the angiotensin AT_2 receptor antagonist, PD 123319, did not increase the contractile response to angiotensin II. This may be another example of

the functional differences of angiotensin receptors in fetal and adult vessels.

Angiotensin AT_2 receptors have an abundant expression during fetal life, which rapidly decreases after birth (Grady et al., 1991; Tsutsumi and Saavedra, 1991; Viswanathan et al., 1991). This, along with the fact that various studies have shown that the angiotensin AT_2 receptor plays a regulatory role in the growth and/or apoptosis of vascular smooth muscle cells (Akishita et al., 1999; Yamada et al., 1998), coronary endothelial cells (Stoll et al., 1995) and cardiomyocytes (Booz and Baker, 1996) and is therefore important in cardiovascular modeling, has led to the assumption that this receptor subtype is important in the development of the cardiovascular system. Consistent with this hypothesis is the fact that the umbilical artery, which is functionally mature during fetal life (Arens et al., 1998), lacks the angiotensin AT_2 receptor.

In conclusion, we have demonstrated that angiotensin AT_1 receptors are present and functional in the ovine fetal cardiovascular system. Infusion of a low dose of cortisol had no effect on the receptor densities or affinities, or on the in vitro contractility of rings of umbilical and carotid arteries to angiotensin II. Therefore, the increase in vascular reactivity to angiotensin II (Tangalakis et al., 1992) and the increase in blood pressure seen with cortisol infusion by Tangalakis et al. (1992) and in this study, do not appear to be due to alterations in the vascular angiotensin receptors. Rather, cortisol must mediate its effect through other mechanisms, possibly through enhancement of the powerful central-pressor actions of angiotensin II.

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