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Angiotensin receptor subtypes in the uterine artery during ovine pregnancy

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Abstract

This study was undertaken to determine if changes in receptor density or affinity could account for the reduced vascular sensitivity to angiotensin II seen during pregnancy. Angiotensin receptor subtypes in the uterine arteries of non-pregnant, pregnant and postpartum ewes were investigated using saturation and competition receptor binding techniques with the specific receptor antagonists, losartan (DuP-753) and 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). Receptor density and affinity of total angiotensin receptors, as well as the angiotensin AT_1 and AT_2 receptor subtypes in uterine arteries were compared with those in the mesenteric artery and aorta. The uterine artery contains both AT_1 and AT_2 receptor subtypes, whereas the mesenteric artery and aorta contain primarily the AT_1 receptor subtype. In uterine arteries from pregnant sheep, angiotensin receptor density was increased because AT_2 receptors were increased. AT_1 receptor density was not altered. This change was not seen in the aorta. In the uterine artery, receptor affinity for [Sar^1,Ile^8]angiotensin II decreased in mid-gestation (IC_{50} $7.7 \pm 1.2 \times 10^{-9}$ M) compared with non-pregnant ewes (IC_{50} $3.0 \pm 0.6 \times 10^{-9}$ M, $P = 0.006$), and there was decreased affinity of angiotensin AT_1 receptors for losartan during pregnancy (IC_{50} $2.8 \pm 1.0 \times 10^{-4}$ M) compared with non-pregnant ewes (IC_{50} $2.2 \pm 1.3 \times 10^{-6}$ M, $P = 0.025$). Our results show changes in the density and affinity of the angiotensin receptor subtypes in the uterine artery which could explain its reduced responsiveness to circulating angiotensin II during pregnancy.

Keywords: Angiotensin AT_1 receptor; Angiotensin AT_2 receptor; Losartan; PD-123319; Uterine artery; Pregnancy; (Sheep)

1. Introduction

During normal pregnancy there is activation of the renin-angiotensin system with increased levels of renin and angiotensin II, but reduced vascular sensitivity to infused angiotensin II which is associated with a reduction in its pressor response (Chesley et al., 1965; Lumbers, 1970; Rosenfeld and Gant, 1981). Although there was early conflicting evidence regarding the effect of angiotensin II on the uteroplacental circulation (reviewed in Naden and Rosenfeld, 1989), it now appears that during pregnancy the uterine vasculature is even less responsive to angiotensin II than other systemic vascular beds (Naden and Rosenfeld, 1989). It is important for normal fetal growth that uteroplacental blood flow is maintained in spite of the high levels of circulating angiotensin II, so that the developing fetus receives adequate nutrition and oxygen.

As the pressor response to angiotensin II is mediated via specific vascular receptors, it has been proposed that alterations in the vascular angiotensin receptors may occur during pregnancy, which could explain the decreased responsiveness to circulating angiotensin II. Following the development of specific angiotensin receptor antagonists, angiotensin receptors can be classified into a number of subtypes: the two major subtypes (AT_1 and AT_2) have been cloned and sequenced (Murphy et al., 1991; Sasaki et al., 1991; Mukoyama et al., 1993; Kambayashi et al., 1993). Using the selective angiotensin AT_1 antagonist losartan, it has been shown that AT_1 receptors mediate the vasoconstrictor and sodium and water retaining actions of angiotensin II (Wong et al., 1990). Angiotensin AT_2 receptors are abundant in fetal tissues (Grady et al., 1991; Tsutsumi et al., 1991) but in the adult they are restricted to the adrenals and uterus (Whitebread et al., 1989), heart

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(Rogg et al., 1990) and brain (Tsutsumi and Saavedra, 1991). The function of AT_2 receptors is not understood. They may be involved in growth and differentiation (Grady et al., 1991), but are not directly involved in myometrial smooth muscle contraction (Cox et al., 1993).

A number of possible mechanisms responsible for uteroplacental refractoriness have been suggested. Initially, it was proposed that down-regulation of angiotensin receptors could be responsible for the reduced vascular response to angiotensin II (Siddiqi et al., 1983). However, more recently, in the rat mesenteric artery (Parent et al., 1991), the sheep aorta, mesenteric and uterine arteries (Mackanjee et al., 1991; Rosenfeld et al., 1993; Cox et al., 1996a) and uterine arteries from pregnant women (Cox et al., 1996b), it has been shown that the total number of angiotensin vascular smooth muscle receptors is not altered during pregnancy.

It has been suggested that the proportions of the angiotensin AT_1 and AT_2 receptor subtypes may change during pregnancy, without altering the total angiotensin receptor density. In their two recent studies on pregnant ewes and women, Cox et al. (1996a,b) found no change in the expression of AT_1 and AT_2 receptor subtypes during pregnancy. Both these studies used competition binding techniques only to measure the affinity and density of total angiotensin receptors, as well as the AT_1 and AT_2 receptor subtypes. Receptor density of the two receptor subtypes was expressed as a percentage after extrapolation from the percentage of inhibition observed when specific displacement curves were compared.

We undertook a study in pregnant and non-pregnant ewes to see if any changes in the density of angiotensin receptor subtypes in the uterine artery could be demonstrated using saturation, as well as competition binding techniques, which could explain the reduced responsiveness of the pregnant uterine vasculature to circulating angiotensin II. Our findings contradict those of Cox et al. (1996a) who carried out only competition binding assays. In addition, we report the effects of surgery and anaesthesia on the AT_2 receptor subtype in the uterine artery during pregnancy.

2. Materials and methods

2.1. Animals

These experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales.

19 non-pregnant ewes were studied. Uterine arteries from 15 non-pregnant ewes were collected within 10 min of death at a slaughterhouse. Ovaries and uteri were inspected to confirm that the ewes were not pregnant. 10 paired uterine arteries were used for saturation assays, and 5 for competition assays. As well saturation assays were carried out on uterine arteries and abdominal aortae collected from 4 non-pregnant ewes that had delivered their lambs 16 days earlier.

35 pregnant ewes (gestation age ranging from 65–147 days, where term is 150 days) were studied. Uterine arteries ($n = 35$; 27 saturation assays; 8 competition assays), abdominal aortae ($n = 16$; 12 saturation assays; 4 competition assays) and mesenteric arteries ($n = 11$; 6 saturation assays; 5 competition assays) were collected.

The 4 postpartum and 26 pregnant ewes were housed in our laboratory and given water and chaff ad libitum until they were killed by rapid intravenous injection of pentobarbitone sodium (Lethabarb: Virbac Australia, Peakhurst, Australia; 100 mg/kg). As these ewes had no previous treatment, they are referred to as untreated ewes.

The remaining 9 pregnant ewes were anaesthetised with 1 g sodium thiopentone (Pentothal: Abbott Australasia, Sydney, Australia) and maintained with 1–2% halothane in oxygen (Fluothane: ICI, Macclesfield, Cheshire, UK), while catheters were placed in a maternal femoral artery and vein, in the fetal bladder, and in a femoral artery and tarsal vein, using methods already described (Lumbers and Stevens, 1983). This group of pregnant ewes had been prepared for another study, and were either used as control animals, or could not be studied further because of technical problems (e.g., blocked catheters, failure to thrive following surgery). They were killed by rapid intravenous injection of pentobarbitone sodium 2–10 days after surgery. This group of ewes is referred to as treated ewes.

Paired uterine arteries (through 3–5 orders of branching) were dissected from the internal iliac artery until they entered the myometrium. The aorta (from the renal arteries to the bifurcation), and mesenteric artery (from the aorta to the wall of the small intestine, through approximately 5 orders of branching) were dissected.

As the arteries were removed, they were wrapped in foil and immediately frozen in liquid nitrogen, and stored for up to 6 months at -70°C until assayed. The endothelium was not removed. To check whether binding was affected by storage, repeated competition assays were performed using fetal glomeruli prepared from 1 cm^3 blocks of fetal kidney stored at -70°C. There was no decreased binding affinity after 6 months storage.

2.2. Preparation of vascular plasma membranes

Tissues were thawed to 4°C, and the connective tissue surrounding the arteries removed. The arteries (wet weight 2–3 g) were finely chopped and homogenised (Ultra-Turrax T125, IKA Labortechnik, Staufen) in cold 20×10^{-3} M sodium bicarbonate. The homogenate was incubated for 1 h at 4°C and stirred each 30 min. After centrifugation at $160 \times g$ to remove large tissue particles, the supernatant was centrifuged again at $30\,000 \times g$ for 45 min at 4°C. The pellet was resuspended in Tris incubation buffer (50×10^{-3} M Tris, 5×10^{-3} M $MgCl_2$, 10^{-3} M EGTA, pH

7.4) and incubated for 30 min at 4°C (Yang et al., 1994). The final membrane protein concentration for each assay was measured by the method of Lowry et al. (1951). The mean concentration ($\pm$S.E.) of all membrane preparations assayed was 151.9 ± 5.2 μg protein/ml membrane suspension ($n = 80$).

2.3. Preparation of radioligand

[Sar^1,Ile^8]Angiotensin II (Auspep, Australia) was labelled with ^{125}Iodine, using lactoperoxidase (Sigma Chemicals, St. Louis, MO, USA) and purified by reverse phase high performance liquid chromatography (HPLC) using a C18 selectosil column (Phenomenex, Torrance, CA, USA). The specific activity of the ligand (1181 ± 122 Ci/mM) was calculated from self displacement in a radioimmunoassay. Aliquots of [^{125}I][Sar^1,Ile^8]angiotensin II were stored in 0.5% bovine serum albumin (BSA, Sigma Chemicals, St. Louis, MO, USA) at −20°C and used within 7 weeks of preparation.

2.4. Saturation binding assays

Saturation binding assays were performed on membranes from uterine arteries (prepared from 10 non-pregnant, 27 pregnant and 4 postpartum ewes), aortae (12 pregnant and 4 postpartum ewes), and mesenteric arteries (6 pregnant ewes). Paired uterine arteries from the same pregnant and postpartum animal were used in each assay. However, in the case of the 10 non-pregnant ewes, uterine arteries were pooled from 2 ewes (because of insufficient tissue from one individual) for the 5 assays conducted. 18 of the pregnant ewes were untreated, and 9 had previous surgery under general anaesthesia 2–10 days before. The 18 untreated pregnant ewes were divided into 2 groups based on gestation age; mid-gestation (mean 82 ± 4 days, range 65–93 days, $n = 7$) and late gestation (mean 129 ± 4 days, range 111–147 days, $n = 11$).

Saturation binding studies were performed to measure the density of all angiotensin receptors, as well as the density of the AT_1 and AT_2 subtypes. Freshly prepared membranes in Tris buffer were divided into 4 aliquots.

(1) No antagonist added – Both angiotensin AT_1 and AT_2 receptors were available for binding.

(2) AT_2 antagonist (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; 10^{-6} M) added – Only AT_1 receptors were available for binding.

(3) AT_1 antagonist (losartan, DuP 753, DuPont Merck Pharmaceutical Company, Wilmington, DE, USA; 10^{-4} M) added – Only AT_2 receptors were available for binding.

(4) 10^{-6} M PD-123319 and 10^{-4} M losartan were added, to block both AT_1 and AT_2 receptors. In all cases, there was no specific binding when both antagonists were present. Therefore, all angiotensin receptors were blocked.

Membranes were incubated with or without antagonist for 30 min.

100 μl membrane suspensions of each of these four aliquots were then incubated for 60 min at 22°C with 25 μl of varying concentrations of [^{125}I][Sar^1,Ile^8] angiotensin II ($0.1–12 \times 10^{-9}$ M in 0.3% BSA) and 25 μl of either Tris buffer, or 10^{-5} M of unlabelled [Sar^1,Ile^8]angiotensin II to measure non-specific binding.

Competition and saturation binding assays were terminated by addition of ice-cold 0.9% sodium chloride (Ajax Chemicals, Auburn, NSW, Australia), and bound and free fractions were separated through GF/C Whatman filter paper (presoaked in 1% BSA) using a Brandel cell harvester, followed by 5 rinses of the filter with 1 litre 0.9% sodium chloride. Radioactivity was measured using an automatic gamma counter (1470 Wizard, Wallac, Turku, Finland).

2.5. Competition binding assays

Competition binding assays were performed using unlabelled [Sar^1,Ile^8]angiotensin II, losartan and PD-123319. Plasma membranes from uterine arteries of 5 non-pregnant ewes and 8 untreated pregnant ewes (65–134 days gestation), aortae from 4 untreated pregnant ewes (65–127 days gestation) and mesenteric arteries from 5 untreated pregnant ewes (119–136 days gestation) were assayed. Paired uterine arteries from the same animal were used in each assay. Binding affinities of the total angiotensin receptors, and the angiotensin receptor subtypes AT_1 and AT_2 were characterised.

Parallel competition binding studies used 100 μl of membrane suspension, 25 μl of [^{125}I][Sar^1,Ile^8]angiotensin II (0.8×10^{-9} M in 0.3% BSA) and 25 μl of varying final concentrations of either unlabelled [Sar^1,Ile^8]angiotensin II (10^{-10} to 10^{-6} M), losartan (10^{-12} to 10^{-4} M) or PD-123319 (10^{-12} to 10^{-4} M), and incubated for 60 min at 22°C. Non-specific binding was measured by incubation with 10^{-5} M unlabelled [Sar^1,Ile^8]angiotensin II.

In addition, plasma membranes from 3 non-pregnant and 5 pregnant uterine arteries, and 4 pregnant mesenteric arteries were preincubated for 30 min in Tris buffer with either losartan (10^{-6} and 10^{-4} M), or PD-123319 (10^{-6} M) before competition binding assays were performed.

2.6. Calculations and statistical analysis

Specific binding was determined by subtracting the non-specific binding from the total binding.

Saturation binding assays were analysed using Prism (GraphPad Software, San Diego, CA, USA). Non-linear regression was performed using the one-site binding equation, $Y = (B_{max} \times X)/(K_d + X)$, where X is the concentration of radioligand (pM) and Y is the specific binding

(cpm). The dissociation constant (K_d), the maximum number of binding sites (B_{max}), and the goodness of fit (R^2) were calculated. The binding site density is expressed in fmol/mg protein. To investigate any cooperativity, Hill coefficients (n_H) were analysed with linear regression plotting the Log Free radioligand against Log(Bound/B_{max} − Bound).

Competition binding assays were analysed using Prism, plotting the logarithm of the concentration of inhibitor against specific binding. The one-site competition equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{X - \text{LogIC}_{50}})$, where Bottom is the Y value at the bottom plateau, Top is the Y value at the top plateau, and IC_{50} is the X value when the response is halfway between Top and Bottom was used. The following two-site competition equation was also used: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})\ [(\text{Fraction}\ \ 1/1 + 10^{X - \text{LogIC}_{50}\,1}) + (1 - \text{Fraction}\ \ 1/1 + 10^{X - \text{LogIC}_{50}\,2})]$, where Fraction 1 is the fraction of receptors that have affinity described by $\text{LogIC}_{50}\,1$, and the remainder of receptors have an affinity described by $\text{LogIC}_{50}\,2$. The concentration of the radioligand (pM) and the K_d (determined from the saturation experiments) were entered as constants. Data were fitted to a one- or two-site curve, and the affinity of the receptor for the competing drug (IC_{50}), the Hill coefficient and the goodness of fit (R^2) were calculated. Differences between mean binding site densities and affinities were determined using Student's *t*-test for independent samples (Zar, 1984). Values are expressed as means ± S.E.

3. Results

Preliminary saturation assays were carried out following preincubation of pregnant uterine artery membranes in concentrations of 10^{-6} M losartan to measure the density of the AT_2 receptor subtype. Subsequently it was found from competition assays that the IC_{50} for losartan in pregnant uterine artery preparations was 2.8×10^{-4} M losartan. Therefore a higher concentration (10^{-4} M) of losartan was used.

3.1. Total angiotensin receptors

3.1.1. Receptor density

Total angiotensin receptor density (fmol/mg protein) in uterine arteries prepared from untreated pregnant ewes increased during pregnancy reaching a maximum in late gestation ($P < 0.0001$ compared with non-pregnant ewes, Fig. 1a). By 16 days post partum total receptor density had decreased to non-pregnant levels. In the treated group of late gestation pregnant ewes that had previous surgery and anaesthesia, there was a decrease in receptor density ($P = 0.012$) compared with untreated pregnant ewes of the same gestation age (Fig. 1a).

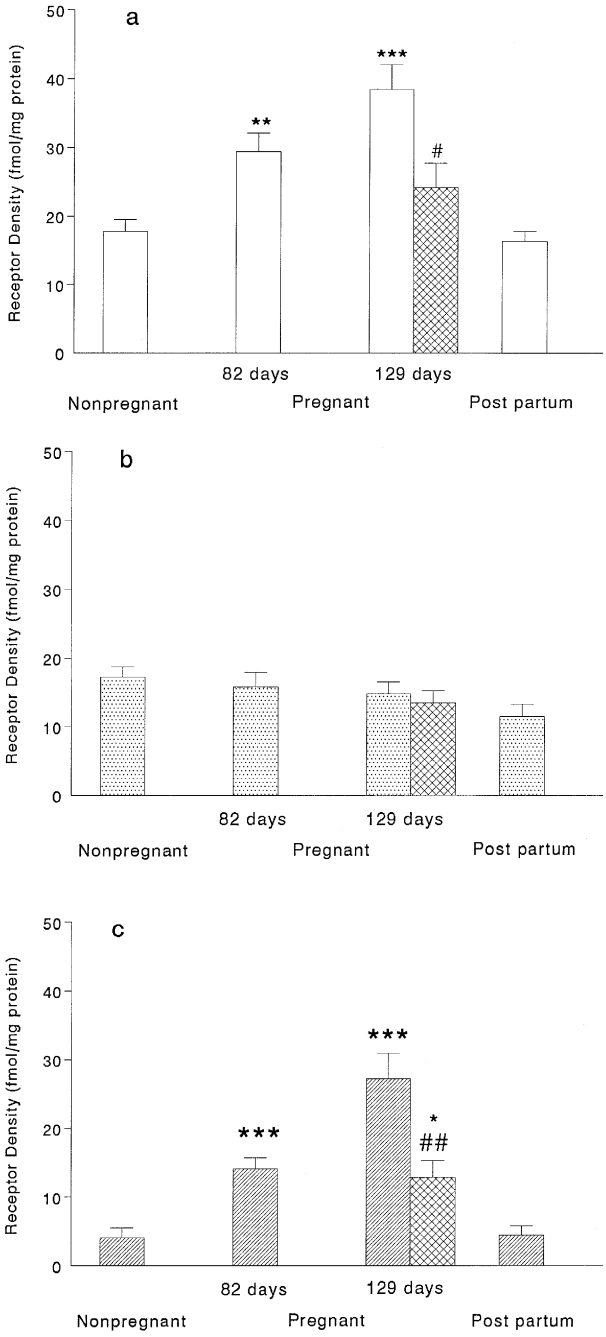


Fig. 1. Receptor density (fmol/mg protein) of (a) total angiotensin receptors (open columns: $R^2 = 0.993 \pm 0.001$), (b) AT_1 (stippled columns: $R^2 = 0.991 \pm 0.002$) and (c) AT_2 (hatched columns: $R^2 = 0.995 \pm 0.001$) subtypes in uterine artery membranes obtained from untreated non-pregnant ($n = 5$), mid gestation (82 ± 4 days gestation, $n = 7$), late gestation (129 ± 4 days gestation, $n = 11$) and postpartum ewes ($n = 4$) that had delivered their lambs 16 days earlier. Treated pregnant ewes in late gestation (cross-hatched columns: 129 ± 2 days gestation, $n = 9$, $R^2 = 0.994 \pm 0.000$) had an anaesthetic and major surgery 2–10 days before death. Values represent mean ± S.E. $^{*}\ P < 0.05$, $^{**}\ P < 0.01$, $^{***}\ P < 0.001$ different from non-pregnant ewes. $^{\#}\ P < 0.05$, $^{\#\#}\ P < 0.01$ different from untreated ewes of 127 days gestation.

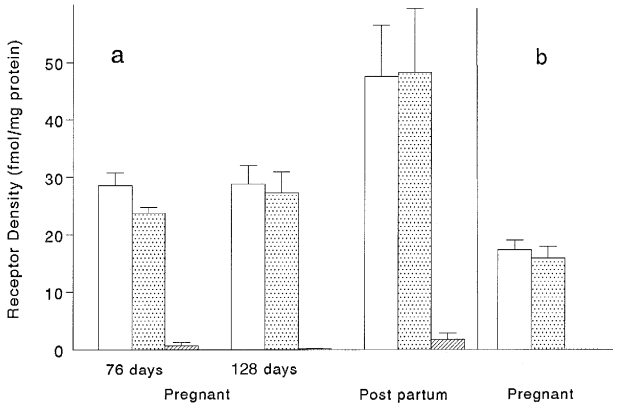


Fig. 2. Receptor density (fmol/mg protein) of (a) total angiotensin (open columns: $R^2 = 0.994 \pm 0.002$), AT_1 (stippled columns: $R^2 = 0.852 \pm 0.13$) and AT_2 (hatched columns: $R^2 = 0.995 \pm 0.001$) receptors in the aorta of pregnant ewes in mid gestation (76 ± 4 days gestation, $n = 4$), late gestation (128 ± 2 days gestation, $n = 8$), and postpartum ewes ($n = 4$) that had delivered their lambs 16 days earlier; (b) total angiotensin (open columns: $R^2 = 0.994 \pm 0.001$) and AT_1 (stippled columns: $R^2 = 0.997 \pm 0.13$) receptors in the mesenteric artery of pregnant ewes (121 ± 9 days gestation, $n = 6$). Values represent mean ± S.E.

In the aorta there was no difference in total angiotensin receptor density between pregnant (28.7 ± 2.3 fmol/mg protein) and postpartum ewes (47.6 ± 8.9 fmol/mg protein), nor did it change during gestation (Fig. 2). The density of angiotensin receptors in mesenteric arteries of pregnant sheep (17.3 ± 1.7 fmol/mg protein) was less than in the aorta ($P = 0.004$).

3.1.2. Receptor affinity

In uterine arteries from non-pregnant, pregnant (both treated and untreated) and postpartum ewes, the dissociation constants (K_d) for total angiotensin receptors were similar (Table 1), so that there was no change in the affinity of the receptors for the radioligand. During pregnancy, the affinity of angiotensin receptors for the radioligand was higher in the uterine artery than in the aorta ($P = 0.008$) and mesenteric artery ($P < 0.001$, Table 1). In the postpartum ewes, the affinity of the total angiotensin receptors in the uterine artery was no different from the aorta.

In competition assays, displacement of [^{125}I][Sar^1,Ile^8]angiotensin II by unlabelled [Sar^1,Ile^8]angiotensin II revealed a single population of binding sites (Fig. 3). The IC_{50} for uterine artery receptors in mid gestation was higher ($P = 0.006$) than in the non-pregnant ewes (Table 2), but although the IC_{50} in late gestation was lower than in mid gestation, there was no statistical difference ($P = 0.08$). Thus, the affinity of total angiotensin receptors in the uterine artery decreased in mid gestation compared to values in the non-pregnant ewes. The IC_{50} for aortic receptors was higher than for mesenteric artery receptors ($P = 0.022$), and for non-pregnant ($P = 0.001$) and pregnant uterine artery ($P = 0.002$), indicating that the affinity of angiotensin receptors in the aorta was lower than in the other vessels examined.

The Hill coefficient in non-pregnant ($n_H = 0.97 \pm 0.01$), pregnant uterine arteries ($n_H = 0.97 \pm 0.01$), aorta ($n_H = 0.95 \pm 0.01$) and mesenteric artery ($n_H = 0.97 \pm 0.02$) indicated that no cooperativity was involved.

3.2. Angiotensin AT_1 receptor

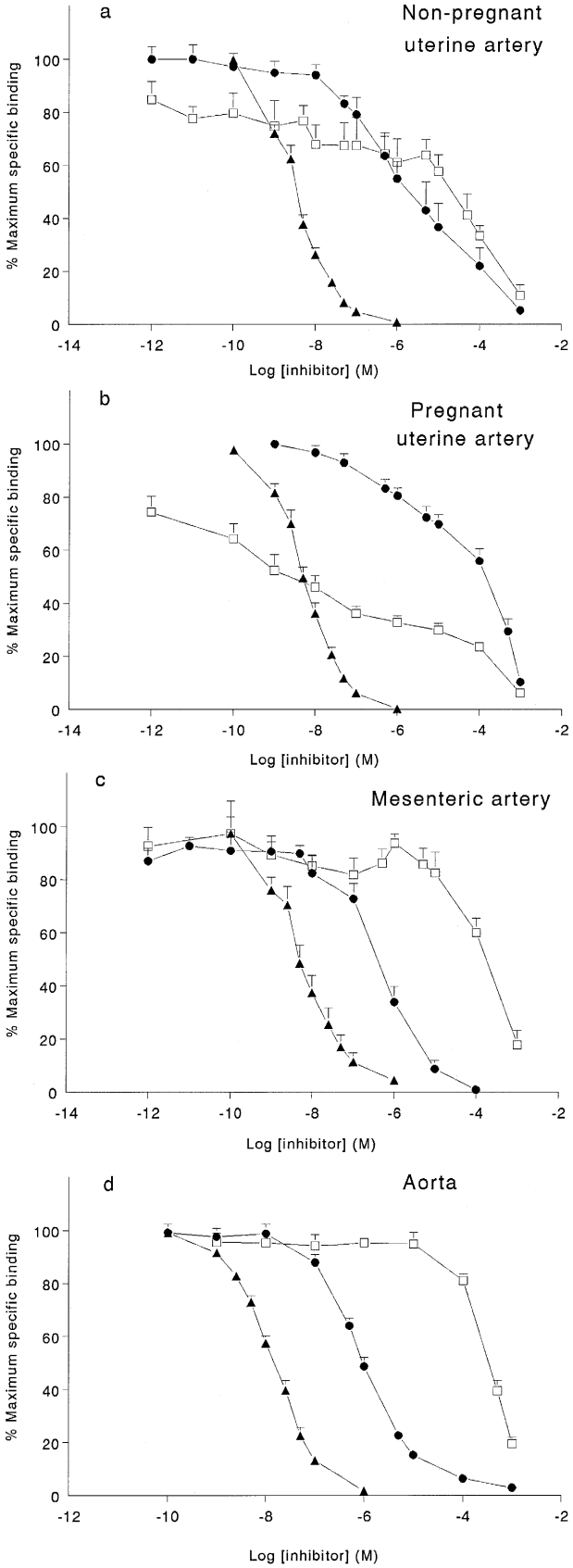
3.2.1. Receptor density

In the uterine artery the density of the angiotensin AT_1 receptor subtype did not change with pregnancy nor was it altered by anaesthesia or surgery (Fig. 1b). During preg-

Table 1
Equilibrium dissociation constants (K_d, $\times 10^{-10}$ M)

Artery	n	Total angiotensin receptors	AT_1 receptors	AT_2 receptors
Pregnant uterine artery	18	5.3 ± 1.0	15.7 ± 1.8	17.9 ± 2.8
Mid gestation	7	4.6 ± 0.7	16.3 ± 2.7	12.0 ± 1.9
Late gestation	11	5.7 ± 1.7	15.3 ± 2.5	21.7 ± 4.1
Non-pregnant uterine artery	9	6.8 ± 1.0	12.5 ± 1.7	12.7 ± 2.2
Postpartum	5	8.4 ± 1.9	15.7 ± 3.1	10.1 ± 3.2
Anoestrus	4	5.4 ± 0.4	9.9 ± 0.7	15.3 ± 2.9
Pregnant aorta	12	9.5 ± 1.1 [a]	9.4 ± 0.5 [a]	2.7 ± 1.8 ($n^* = 2$)
Mid gestation	4	12.5 ± 1.5	10.2 ± 0.8	0.9 ($n^* = 1$)
Late gestation	8	7.9 ± 1.1	9.0 ± 0.7	4.4 ($n^* = 1$)
Nonpregnant aorta				
Postpartum	4	11.5 ± 1.5	12.8 ± 2.6	52.7 ± 7.8
Pregnant mesenteric artery	6	13.4 ± 1.3 [b]	13.9 ± 1.1 [c]	

Equilibrium dissociation constants (K_d, $\times 10^{-10}$ M) derived from saturation binding data using [I^{125}][Sar^1,Ile^8]angiotensin II and membranes prepared from the uterine arteries, aorta and mesenteric artery from non-pregnant, pregnant or postpartum ewes, and preincubated in: Tris buffer (to measure the total angiotensin receptors), Tris buffer plus PD-123319 (to measure AT_1 receptors), and Tris buffer plus losartan (to measure AT_2 receptors). The results for pregnant uterine artery and pregnant aorta were subdivided on the basis of stage of gestation: mid gestation (82 ± 4 days) and late gestation (129 ± 4 days). The results for the non-pregnant uterine artery were derived from anoestrus ewes and postpartum ewes (that had delivered their lambs 16 days before). Values are expressed as means ± S.E. n refers to the number of assays performed. n^* refers to the number of assays where AT_2 receptor binding occurred. [a] $P < 0.01$, [b] $P < 0.001$ compared with pregnant uterine artery, [c] $P < 0.05$ compared with aorta.



nancy, the AT_1 receptor density in the aorta (26.1 ± 2.5 fmol/mg protein, $n = 12$) was greater than in the mesenteric (15.9 ± 2.0 fmol/mg protein, $n = 6$, $P = 0.017$) and uterine arteries (15.2 ± 1.3 fmol/mg protein, $n = 18$, $P = 0.000$, Figs. 1 and 2).

3.2.2. Receptor affinity

In the uterine artery, the K_d of the angiotensin AT_1 receptor subtype was not changed by or during pregnancy (Table 1). During pregnancy, the K_d of AT_1 receptor subtypes in aortic membranes was lower than in the uterine ($P = 0.008$) and mesenteric arteries ($P = 0.001$), thus the affinity for the radioligand was higher in the aorta than in the uterine and mesenteric arteries.

When losartan was used to displace the radioligand in competition binding assays, a single binding site was demonstrated (Fig. 3). In the uterine artery during pregnancy, the IC_{50} was higher than in the non-pregnant uterine artery ($P = 0.025$), mesenteric artery ($P = 0.025$) and the aorta ($P = 0.025$, Table 2), i.e. the affinity of the AT_1 receptors in the pregnant uterine artery was lower than in the other blood vessels studied. The affinity of the AT_1 receptors in the aorta was lower than in the mesenteric artery ($P < 0.001$). The Hill coefficient in non-pregnant ($n_H = 0.95 \pm 0.01$), pregnant uterine arteries ($n_H = 0.957 \pm 0.016$), aorta ($n_H = 0.966 \pm 0.007$) and mesenteric artery ($n_H = 0.951 \pm 0.01$) indicated that no cooperativity was involved.

3.3. Angiotensin AT_2 receptors

3.3.1. Receptor density

Only the uterine artery preparations had significant levels of AT_2 receptors (Fig. 1c, Fig. 2 and Fig. 3). The density of the AT_2 receptor subtype increased significantly during pregnancy reaching a maximum in late gestation (Fig. 1c). In uterine arteries from non-pregnant ewes, the AT_2 receptors were $21.5 \pm 6.6\%$ of total angiotensin receptors. By mid gestation they were $48.5 \pm 4.3\%$ ($P = 0.005$ compared with non-pregnant values), and by late gestation they were $71.1 \pm 6.5\%$ ($P < 0.001$). After delivery AT_2 receptor density decreased to non-pregnant levels (Fig. 1c), i.e. they fell to $26.3 \pm 7.3\%$ of total angiotensin receptors, which was not different from values obtained in

Fig. 3. Inhibition of the specific binding of [^{125}I][Sar^1,Ile^8]angiotensin II by unlabelled [Sar^1,Ile^8]angiotensin II (▲), losartan (●) and PD-123319 (□) in membranes prepared from: (a) non-pregnant uterine artery ($n = 5$) – the R^2 values with [Sar^1,Ile^8]angiotensin II, losartan and PD-123319 are 0.987 ± 0.004; 0.9 ± 0.03; and 0.84 ± 0.05, respectively; (b) pregnant uterine artery (gestation range 65–137 days, $n = 8$) – the R^2 values are 0.992 ± 0.001; 0.885 ± 0.018 and 0.993 ± 0.02, respectively; (c) pregnant mesenteric artery (gestation range 119–136 days, $n = 5$) – the R^2 values are 0.979 ± 0.004; 0.952 ± 0.018 and 0.799 ± 0.07, respectively; (d) pregnant aorta (gestation range 65–134 days, $n = 4$) – the R^2 values are 0.997 ± 0.001; 0.994 ± 0.003 and 0.986 ± 0.005, respectively. Values represent mean ± S.E.

Table 2
Values for half-maximum displacement (IC_{50}, M) derived from competition binding assays using the angiotensin receptor antagonist [Sar[1],Ile[8]]angiotensin II, and the subtype specific antagonists, losartan and PD-123319

Artery	*n*	[Sar[1],Ile[8]]Angiotensin II	Losartan	PD-123319
Pregnant uterine artery	8	$6.0 \pm 1.0 \times 10^{-9}$	$2.8 \pm 1.0 \times 10^{-4}$	$2.3 \pm 1.6 \times 10^{-9}$ $1.6 \pm 0.9 \times 10^{-4}$
Mid gestation	4	$7.7 \pm 1.2 \times 10^{-9}$ [c]	$1.6 \pm 0.5 \times 10^{-4}$	$1.5 \pm 1.5 \times 10^{-9}$ $2.3 \pm 1.7 \times 10^{-4}$
Late gestation	4	$4.2 \pm 1.2 \times 10^{-9}$	$4.0 \pm 1.8 \times 10^{-4}$	$3.0 \pm 3.0 \times 10^{-9}$ $0.9 \pm 0.9 \times 10^{-4}$
Non-pregnant uterine artery	5	$3.0 \pm 0.6 \times 10^{-9}$	$2.2 \pm 1.3 \times 10^{-6}$ [a]	$5.6 \pm 1.5 \times 10^{-5}$ [f]
Pregnant aorta	4	$15.5 \pm 2.7 \times 10^{-9}$ [b,d]	$865 \pm 52 \times 10^{-9}$ [a]	$8.2 \pm 1.2 \times 10^{-4}$
Pregnant mesenteric artery	5	$5.8 \pm 2.0 \times 10^{-9}$ [e]	$294 \pm 49 \times 10^{-9}$ [a,g]	$2.1 \pm 0.8 \times 10^{-4}$ [f]

Values for IC_{50} derived from competition binding data by inhibition of [I[125]][Sar[1],Ile[8]]angiotensin II using unlabelled [Sar[1],Ile[8]]angiotensin II, losartan or PD-123319, in membranes prepared from uterine arteries, aorta and mesenteric arteries from pregnant ewes and uterine arteries from non-pregnant ewes. Results from uterine artery preparations from the 8 pregnant ewes were subdivided based on the stage of gestation: mid gestation (72 ± 7 days, $n = 4$), and late gestation (132 ± 2 days, $n = 4$). [a] $P < 0.05$, [b] $P < 0.01$ compared with pregnant uterine artery. [c] $P < 0.01$, [d] $P < 0.001$ compared with non-pregnant uterine artery. [e] $P < 0.05$, [f] $P < 0.01$, [g] $P < 0.001$ compared with aorta. Values are expressed as means $\pm$ S.E. *n* refers to the number of assays performed.

non-pregnant ewes. In the treated group of pregnant ewes, i.e. animals that had surgery and anaesthesia up to 10 days before, the density of the AT_2 receptor subtype was significantly lower ($P = 0.007$) than untreated ewes at the same stage of gestation (Fig. 1c). This reduction in AT_2 receptor was greatest in the days immediately following surgery and had not returned to control values by 10 days. There was a linear relationship between AT_2 receptor density and days after surgery ($r = 0.64$, $P = 0.05$, Fig. 4).

3.3.2. Receptor affinity

In the uterine artery, the K_d of the AT_2 receptor subtype following preincubation in 10^{-4} M losartan was not changed by, or during pregnancy (Table 1), nor was there any change in the K_d of the AT_2 receptor subtype in the treated group of pregnant ewes following surgery and anaesthesia.

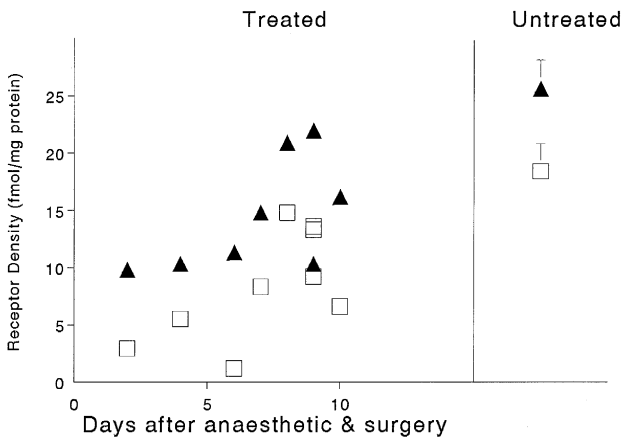


Fig. 4. Density (fmol/mg protein) of total angiotensin (▲) and AT_2 receptors (□) in uterine artery membranes from treated ($n = 9$) and untreated ($n = 11$) pregnant ewes of similar gestation age (129 days). Treated pregnant ewes had an anaesthetic and major surgery 2–10 days before death. The individual values of receptor density in the 9 treated ewes are represented on the left of the graph. On the right, the value for the 11 untreated ewes is expressed as mean $\pm$ S.E.

In competition assays, displacement by PD-123319 demonstrated a single low affinity binding site in uterine artery membranes from non-pregnant ewes (Table 2, Fig. 3a). However, in pregnant ewes, displacement by PD-

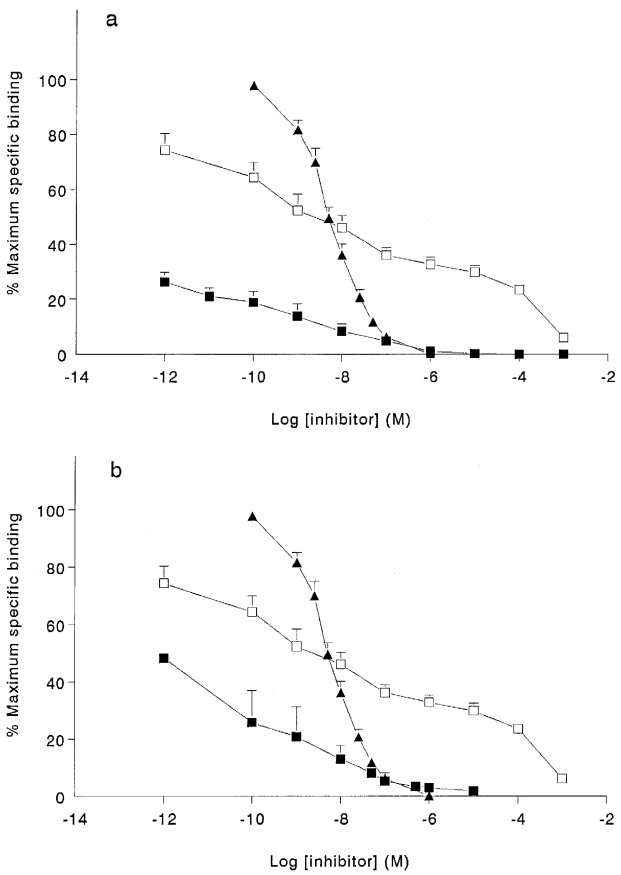


Fig. 5. Pregnant uterine artery membranes. Inhibition of the specific binding of [[125]I][Sar[1],Ile[8]]angiotensin II by unlabelled [Sar[1],Ile[8]]angiotensin II (▲, $n = 8$) and PD-123319 (□, $n = 8$) and: (a) PD-123319 after membranes were preincubated in losartan (10^{-4} M, ■, $n = 3$); (b) PD-123319 after membranes were preincubated in losartan (10^{-6} M, ■, $n = 2$). Values represent mean $\pm$ S.E.

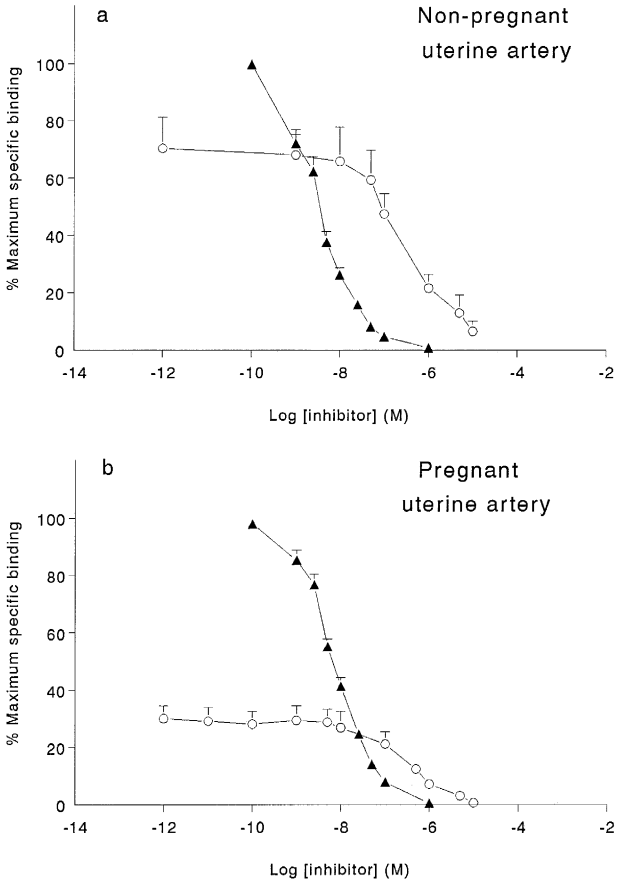


Fig. 6. (a) Non-pregnant uterine artery membranes. Inhibition of specific binding of [^{125}I][Sar^1,Ile^8]angiotensin II by unlabelled [Sar^1,Ile^8]angiotensin II (▲, $n = 5$) and losartan after membranes were preincubated in PD-123319 (10^{-6} M, ○, $n = 3$). (b) Pregnant uterine artery membranes. Inhibition of specific binding of [^{125}I][Sar^1,Ile^8]angiotensin II by unlabelled [Sar^1,Ile^8]angiotensin II (▲ $n = 8$) and losartan after membranes were preincubated in PD-123319 (10^{-6} M, ○, $n = 2$). Values represent mean ± S.E.

123319 could be resolved into 2 sites ($R^2 = 0.955 \pm 0.015$), one of high affinity, and one of very low affinity (Table 2, Fig. 3b).

To further investigate these 2 binding sites in the pregnant uterine artery and to determine if either of these sites was 'AT_1-like', we preincubated membrane preparations with losartan ($n = 3$, 10^{-4} M and 10^{-6} M, Fig. 5a and b). The low affinity site was abolished suggesting that this site could be an AT_1 receptor binding site, possibly AT_{1B}. In addition, plasma membranes of non-pregnant ($n = 3$) and pregnant (133 ± 4 days gestation, $n = 2$) uterine arteries were preincubated in 10^{-6} M PD-123319 to block the AT_2 receptor subtype (Fig. 6a and b). 30% of maximal binding was inhibited in the non-pregnant ewe, i.e. 30% of all receptors were AT_2. In the pregnant ewe 70% of maximal binding was inhibited and so 70% were AT_2 receptors. These values agree with results we obtained using saturation binding assays.

In the mesenteric artery and aorta displacement with PD-123319 also revealed a single binding site of very low affinity (Table 2). The IC_{50} of AT_2 binding sites in the aorta, mesenteric artery and non-pregnant uterine artery were similar to the IC_{50} of the low affinity binding site in the pregnant uterine artery. The affinity of AT_2 receptors in the aorta was lower than in the non-pregnant uterine artery ($P = 0.007$) and the mesenteric artery ($P = 0.003$). The Hill coefficient in non-pregnant ($n_H = 1.05 \pm 0.02$), pregnant uterine arteries ($n_H = 1.002 \pm 0.02$), aorta ($n_H = 0.83 \pm 0.25$) indicated there was no cooperativity.

Displacement of [^{125}I][Sar^1,Ile^8]angiotensin II by PD-123319 after preincubation with either 10^{-4} or 10^{-6} M losartan to block the AT_1 receptor subtype in the mesenteric arteries of 4 untreated pregnant ewes (data not shown) revealed less than 10% of maximum specific binding, demonstrating again that the mesenteric artery contained mostly the AT_1 receptor subtype.

4. Discussion

The classification of angiotensin receptors into two subtypes, AT_1 and AT_2, is made on the basis of affinity for specific antagonists. Losartan (DuP 753) binds to AT_1 receptors with high affinity, and has negligible affinity for AT_2 receptors, while the antagonist PD-123319 binds with high affinity to AT_2 receptors and with low affinity to AT_1 receptors (Timmermans et al., 1993). As well, the AT_1 receptor subtype is coupled to G-proteins, while AT_2 receptors do not interact with G-proteins (Bottari et al., 1991). Ernsberger et al. (1992) reported PD-123319-sensitive G-protein coupled receptors in rat renal mesangial cell membranes, and suggested a further classification of the AT_1 receptor subtype into AT_{1A} and AT_{1B}. AT_{1A} and AT_{1B} receptors have been cloned and characterised (Sasamura et al., 1992) but they are not easily distinguished with currently available antagonists, and they have not been distinguished on a functional basis.

A subdivision of AT_1 receptors into AT_{1A} and AT_{1B} has not been reported previously in ovine tissues. Our results with competition binding assays show that displacement with PD-123319 can be resolved into 2 sites, and the lower affinity site can be abolished with preincubation with losartan. Thus, this low affinity site behaves like an AT_1 site, and based on the findings of Ernsberger et al. (1992), is possibly an AT_{1B} site.

Our study reports an increase in the density of the total angiotensin receptors in the uterine artery of pregnant ewes in mid gestation, with a further increase in late gestation. By 16 days post partum, the total angiotensin receptor density returned to that of the non-pregnant. This increased density during pregnancy is due to an increase in the AT_2, and not the AT_1 receptor subtype. The mesenteric artery and abdominal aorta contain mainly AT_1 receptors, which are not altered during pregnancy. In the uterine artery we

found a decrease in AT_1 receptor affinity during pregnancy, and a second high affinity AT_2 receptor binding site which was not present in the non-pregnant.

Our conclusions do not agree with those from Rosenfeld's laboratory where angiotensin receptors in the uterine arteries of non-pregnant and pregnant ewes have been examined using receptor binding techniques (Mackanjee et al., 1991; Rosenfeld et al., 1993; Cox et al., 1996a). They found no differences in density or affinity of total angiotensin receptors, or the AT_1 and AT_2 receptor subtypes (Cox et al., 1996a) between non-pregnant and pregnant ewes, and concluded that the attenuated vasoconstrictor responses in the uterine artery during pregnancy are unrelated to changes in angiotensin receptor density or affinity.

There are a number of possible explanations for the contradictory results. These workers used only competition binding assays to conclude that no changes occurred in receptor density between non-pregnant and pregnant ewes. They used relative values of percentage inhibition when specific displacement curves were compared, and calculated absolute values from total binding density reported in animals from a previous study (Mackanjee et al., 1991). In addition, they preincubated membrane preparations in either 10^{-6} M losartan or PD-123319 to block the relevant receptor subtype, and competition assays were then performed. We found from competition binding assays that the IC_{50} for losartan in the pregnant uterine artery was 2.8×10^{-4} M. Therefore we chose a concentration of 10^{-4} M for preincubation of membranes to inhibit the AT_1 receptor, rather than the lower concentration of 10^{-6} M. When we repeated the experiment reported by Cox et al. (1996a) where pregnant uterine artery membranes were preincubated in 10^{-6} M PD-123319 before displacement with losartan (Fig. 6a), we found that approximately 30% of receptors bind with losartan after the AT_2 receptors have been previously bound with the preincubation in PD-123319. Cox et al. (1996a) show $> 75\%$ AT_2 receptors in all uterine arteries. We only found this in late pregnant uterine arteries. It appears that there are differences between our techniques other than the concentration of antagonists.

As well, the previous studies conducted in Rosenfeld's laboratory were carried out using a different radiolabelled ligand (tyrosyl[^{125}I][5-L-isoleucine]angiotensin II) to the radioligand used in our study. The different ligand could have a different affinity for the receptor subtypes and thus measure different subtype proportions. In the publication by Cox et al. (1996a), the angiotensin II radioligand used for the competition binding assays was not specified.

We made no attempt to remove the endothelium from the blood vessels studied, whereas in the studies reported from Rosenfeld's laboratory the endothelium had been removed. Cox et al. (1996a) used autoradiographic techniques to show that the AT_1 receptor appears to predominate in the luminal portion of the uterine artery, while the AT_2 receptor subtype is mainly in the smooth muscle. It is possible that Rosenfeld's group measured high percentages of AT_2 receptors (75–95% of total binding) in both non-pregnant and pregnant ewes, if the main location of AT_1 receptors (the endothelium) had been removed. However, it does not explain the differences we observed during pregnancy.

Our values for receptor affinity using unlabelled [Sar^1,Ile^8]angiotensin II are similar to reported values (Cox et al., 1996a). The low affinity for losartan in pregnant uterine artery membranes is also in agreement with previous studies (Cox et al., 1996a). However, the affinity of losartan for the AT_1 receptor in our preparations of non-pregnant uterine artery is higher, and in the aorta is slightly lower, than values reported by Cox et al. (1996a). We are unable to explain the different affinity for losartan in these 2 tissue preparations, but suggest that the variations in methodology between different laboratories, as described above, could account for such differences in absolute values.

There are no previous studies which have examined the effect of anaesthesia or surgery on the density or affinity of angiotensin receptors or subtypes, particularly during pregnancy. Most published data suggest that the renin angiotensin system is activated during anaesthesia and surgery, but the extent to which it is activated depends on the type of anaesthetic agent and degree of hydration prior to and during surgery (Burchardi and Kaczmarczyk, 1994).

General anaesthesia or major abdominal surgery causes raised levels of ACTH and glucocorticoids. The positive correlation between days after surgery and receptor density of both total angiotensin receptors and the AT_2 receptor subtype (Fig. 5) suggests that the up-regulation of the AT_2 receptors in the pregnant uterine artery might have been prevented by increased levels of cortisol, or angiotensin II. Dexamethasone has been shown to down-regulate renal glomerular angiotensin II receptors in rats (Douglas, 1987) and both AT_1 and AT_2 receptors in pancreatic acinar cells (Chappell et al., 1992). Cortisol infusion into fetal sheep results in a decrease in renal AT_1 receptor mRNA (Robillard et al., 1994), but AT_2 receptor expression was not examined. It is possible that cortisol may be a factor responsible for the changes in AT_2 receptor density in the treated group of ewes. In another study from our laboratory, ACTH infusion into pregnant ewes caused significant increases in plasma cortisol, and even further down-regulation of uterine artery AT_2 receptors (Burrell, Lumbers and Bernasconi, submitted manuscript).

To ascertain whether the changes in angiotensin receptors were peculiar to the uterine artery, we studied aortic (because of size and accessibility), and mesenteric artery preparations (an example of a muscular artery) for comparison. In both these vessels, there was a predominance of AT_1 receptors. No changes in receptor density were observed in the aorta during pregnancy, suggesting that AT_2 receptor subtype has a unique role in the uterine artery during pregnancy.

While AT_1 receptors have been shown to mediate the vasoconstrictor and sodium and water retaining actions of angiotensin II (Wong et al., 1990), AT_2 receptor function is not clear. Recent evidence has suggested a novel role for the AT_2 subtype in the central actions of angiotensin II (Hohle et al., 1995; Hein et al., 1995; Ichiki et al., 1995). These authors have proposed that the central AT_1 receptor-mediated actions of angiotensin II are under inhibitory control by the AT_2 receptor. At present, there is no evidence that this AT_2 receptor inhibitory control over AT_1 receptors occurs outside the central nervous system. We have shown that during pregnancy there is an up-regulation of the AT_2 receptor subtype. If the AT_1 receptors in the uterine artery are under inhibitory control of AT_2 receptors, it could account for the reduced vascular sensitivity to angiotensin II of the uterine artery during pregnancy. Another possible role for the AT_2 receptors is that they act as clearance receptors removing angiotensin II from the circulation, so lowering the local concentration of angiotensin II, resulting in a reduced contractile response. If the AT_2 receptor subtype functions in this way, an increase of this receptor subtype in the uterine artery during pregnancy could explain the reduction in sensitivity of this vessel to angiotensin II.

In summary, angiotensin receptors in the uterine artery are up-regulated during pregnancy, and this is due to the increased density of AT_2, and not the AT_1 receptor subtype. This change is not seen in the mesenteric artery or aorta, which contain primarily the AT_1 receptor subtype. Our findings support the hypothesis that AT_1 mediated responses could be under an inhibitory control by the AT_2 receptors, and help explain the reduced responsiveness of the uterine artery to angiotensin II in pregnancy. In pregnant ewes that had an anaesthetic and major abdominal surgery, the density of the AT_2 receptors was significantly reduced for at least 10 days, compared with non-treated ewes of the same stage of gestation. It is not known whether the down-regulation of AT_2 receptors after general anaesthetic and major surgery is accompanied by vasoconstriction in the pregnant uterine artery with a reduction in uteroplacental blood flow. However, these findings have important implications for angiotensin II receptor studies, suggesting that the AT_2 receptor may be particularly labile.

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