RESEARCH ARTICLE

Differential contractile responses and receptor properties of fetal systemic arteries and uterine arteries of pregnant sheep to angiotensin II: arteries with high proportions of AT_2 receptors

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Abstract

Uterine arteries from pregnant animals are one of only a few adult blood vessels which express predominantly AT_2 receptors. Fetal systemic arteries also contain a high density of AT_2 receptors. To assess whether fetal systemic arteries, like uterine arteries from pregnant animals, were relatively insensitive to angiotensin II (Ang II), we compared the contractile responses of fetal carotid arteries, as well as the receptor binding properties of fetal aortae, with those of uterine arteries from pregnant ewes. Ang II receptor binding properties were measured in arterial membrane preparations using ^{125}I [Sar^{1}Ile^8] Ang II. Proportions of AT_1 and AT_2 receptors were determined by inhibiting ^{125}I [Sar^{1}Ile^8] Ang II with losartan (AT_1 antagonist) or PD 123319 (AT_2 antagonist). Both fetal aortae and uterine arteries contained predominantly AT_2 receptors. However, the AT_2 receptor in fetal aortae had a higher affinity than that of the AT_2 receptor in uterine arteries (P<0.05). The contractile responses of fetal carotid arterial rings and uterine arterial rings to Ang II (4 μM) with and without antagonists were examined *in vitro*. Despite having similar proportions of Ang II receptor subtypes, fetal arterial rings were more responsive to Ang II *in vitro* than uterine arterial rings (P<0.05), possibly because of their greater density of AT_1 receptors. The recognised *in vitro* phenomenon of Ang II-induced tachyphylaxis was observed in uterine arterial rings but not fetal arterial rings. In addition, Ang II induced responses in fetal carotid rings were largely unaffected by losartan (1 μM) or PD 123319 (1 μM), whereas Ang II induced contractile responses of uterine arterial rings were inhibited by losartan (1 μM) and enhanced by PD 123319 (1 μM). Thus, it would appear that Ang II receptors in systemic arteries of fetal sheep may be functionally dissimilar to those in the uterine artery of pregnant sheep.

Extra keywords : AT_1 receptor; AT_2 receptor; Ang II receptor antagonists

1. Background

Angiotensin II (Ang II), the major biologically active product of the renin-angiotensin system, acts via 2 main receptor subtypes: AT_1 and AT_2. The AT_1 receptor is expressed in a number of tissues in the adult and is responsible for the well-known actions of Ang II including vasoconstriction, aldosterone secretion, centrally mediated drinking responses, and vascular smooth muscle growth. By contrast, the AT_2 receptor provides a counter-regulatory role, in that it opposes many of the actions mediated by the AT_1 receptor.[1-5] The AT_2 receptor is expressed in few tissues in the adult.[6] Uterine arteries from pregnant women and ewes are one of only a few adult blood vessels which contain a large proportion of AT_2 receptors (>70 %).[7-9] We have found that ovine uterine arteries are relatively insensitive to the vasoconstrictor actions of Ang II *in vitro* because these AT_2 receptors inhibit AT_1 mediated contractions.[3]

In contrast to the adult, AT_2 receptors have been identified as the major receptor subtype in fetal and neonatal tissues in rodents and sheep.[10-14] Although one laboratory reported that systemic blood vessels (aortae, carotids, mesenteric and femoral arteries) from late gestation fetal sheep contain only AT_2 receptors,[12,13] we found both AT_1 and AT_2 receptors in fetal aortae and carotids, although the AT_2 receptor was predominant.[14,15] Furthermore, by Western blot analysis and immunostaining Cox *et al.* later confirmed the presence of both AT_1 and AT_2 receptors in ovine fetal carotid.[16] Although they did not make these measurements in the ovine fetal aorta, they did confirm the presence of mRNA levels for both receptors in both of these vessels.[16]

Thus, uterine arteries from pregnant ewes and systemic arteries from fetal sheep are similar in that although they contain both receptor subtypes, they have a high proportion of AT_2 receptors. In this study we compared the contractile responses of these vessels to Ang II and antagonists *in vitro.* It was postulated that fetal blood vessels, like uterine arteries would be relatively insensitive to Ang II *in vitro* because they contain a high proportion of AT_2 receptors, which in uterine arteries inhibit AT_1 mediated contractions.[3]

2. Material and methods

2.1 Animal Care

Experiments were approved by the Animal Care and Ethics Committee, University of New South Wales. Pregnant merino ewes (50.0 ± 1.5 kg) at approximately 115 days gestation (full term 145-150 days) were housed in metabolic pens in an air-conditioned room (18-23ºC) with natural day/night cycles for approximately 3-5 days. Each day they received water ad libitum, lucerne chaff (1200 g) and oats (100 g).

Ewes were killed with an overdose of pentobarbitone sodium (approx. 3.5 g, Lethabarb Euthanasia Injection, Virbac (Australia) Pty Ltd, Peakhurst, NSW, Australia). Immediately after the ewes were killed, blood vessels were taken from pregnant ewes and their fetuses. Uterine arteries were collected from 6 late gestation pregnant ewes (120-130 days), which had received 0.15 M NaCl intravenously at 0.66 ml/h for 24h.[3] This small volume infused (~16ml total) was unlikely to impact on the study as it was <0.5% of the ewe's predicted

blood volume and would have distributed throughout the entire extracellular volume. Furthermore, in preliminary studies contractile responses were similar in pregnant ewes which had not been infused. One uterine artery was placed into an ice cold Krebs solution (NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 5.0 mM; aerated with 95% oxygen : 5% carbon dioxide, pH 7.4), and the other was snap frozen in liquid nitrogen and stored at -84°C for receptor binding studies. Both carotid and iliac arteries and the descending aorta (from just distal to the aortic arch to the common iliac bifurcation) were collected from 6 fetal sheep aged 120-135 d. There was insufficient tissue to perform organ bath studies as well as receptor binding assays on each fetal blood vessel. Therefore, some fetal blood vessels were placed in Krebs solution and others were stored at -84°C for receptor binding assays.

2.2 In vitro studies

The organ bath setup has previously been described.[3] In brief, 2-3 mm ring segments of uterine artery were cut, the endothelium was removed by gently rubbing the lumen with stainless steel hooks (0.45 mm o.d.), and the rings were suspended in 10 ml water jacketed organ baths filled with Krebs solution (pH 7.4) at 37 ºC. The preparations were connected to force transducers (Grass FT 0.03, Quincy, MA) and the isometric force generated was recorded using a MacLab/8-computer system (Analog Digital Instruments Ltd, Castle Hill, NSW, Australia). Fetal arteries were suspended in organ baths as described for uterine arteries except that the endothelium was not intentionally removed from fetal arterial rings. This was to avoid unintentional damage to vascular smooth muscle cells.[17]

2.2.1 Experimental protocol

The experimental protocol for uterine arteries has been reported in detail.[3] Uterine arterial rings were allowed to equilibrate (≥ 60 min). They were then stretched to an optimal resting tension of approximately 1.5 g, and exposed to a submaximal concentration (40 mM) of KCl. Once the contractions had reached a plateau, the preparations were washed twice with Krebs solution and left for 30 min before another drug was given. After receiving at least 3 consecutive injections of KCl (40 mM), each ring received either Ang II (4 µM; Hypertensin, Val^5-hypertensin II-asp-β-amide, a gift from Ciba-Geigy Ltd, Australia) alone, Ang II after prior incubation for 30 min with the angiotensin AT_1 antagonist losartan (1µM; Du Pont Merck Pharmaceuticals, Wilmington, Delaware), or Ang II after prior incubation for 30 min with the angiotensin AT_2 antagonist PD 123319 (1 µM; (S)1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-C] pyridine-6-carboxylic acid, ditrifluoroacetate, dihydrate, Parke-Davis Pharmaceutical Research, Division of Warner Lambert Company, MI, USA). Uterine arterial rings developed Ang II induced tachyphylaxis,[3] thus the effects of Ang II with antagonists could not be measured in the same rings as Ang II alone. The KCl response was used as a standard for comparison of all other contractile responses.

A similar experimental protocol was followed for fetal vessels, except that arterial rings were stretched to an optimum resting tension of 1 g (determined by comparing the tension developed by KCl under different resting conditions). In addition, since contractile responses of fetal blood vessels to 40 mM KCl were very small (approximately 1.0g), a higher concentration was tested. 80 mM KCl elicited larger responses (approximately 3.0g) which were more reproducible, thus, this concentration was routinely used. As previously noted, there was insufficient tissue from fetal arteries to perform both contractile studies and receptor binding studies on the same vessels. Thus, fetal carotid arteries were routinely used for organ bath studies and fetal aortae were routinely used for receptor binding assays. In preliminary studies it was shown that each of the fetal arteries collected (aorta, carotid and iliac) contracted to Ang II in vitro. In each case the response to Ang II was >30% of the response to KCl.

2.3 Receptor binding assays

^{125}I labelled [Sar^{1}Ile^{8}] Ang II (Auspep, Australia) was prepared by D. Casley (Austin Hospital, University of Melbourne) using the chloramine-T method.[18] The specific activity was assumed to be the maximum theoretical specific activity of carrier free ^{125}I (2176 Ci/mmole).

2.3.1 Membrane preparation

Uterine arteries (approximately 1 g), and fetal blood vessels (aortae, carotids and iliacs, 0.5-1.0 g) were placed into 7.5 ml lysis buffer (20 mM sodium bicarbonate) and homogenised using a Polytron homogeniser (PT 2000, Kinematica AG, Littau/Luzern, Switzerland) and 10S saw tooth cutting aggregate at approximately 20 000 rpm at least 3 times for 10 s (allowing the aggregate to cool between homogenisations). The volume was made up to 50 ml with lysis buffer and inverted. The tissue suspension was incubated for 60 min on ice, and then spun at 170 g for 1 min at 4°C to remove any large tissue particles. The supernatant was centrifuged at 30 000 g for 45 min at 4°C. The pellets were resuspended in 8 ml of incubation buffer (50 mM Tris, pH 7.4, containing 5 mM $MgCl_2$ and 1 mM EGTA) for use in saturation and competition assays. 100 µl aliquots of the tissue suspensions were set aside for determination of protein concentrations using the method of Lowry *et al*..[19]

2.3.2 Saturation assays

Saturation assays were carried out to measure the total density of Ang II receptors (Bmax) and the ligand affinity (Kd). The total assay volume (150 µl) consisted of 25 µl of one of eight concentrations of the radioligand (^{125}I [Sar^{1}Ile^{8}] Ang II, approximately 50-6000 pM in 0.3 % BSA (bovine serum albumin; Sigma Chemicals, St. Louis, Mo, USA)), 25 µl phosphate buffered saline (PBS; total tubes) or unlabelled [Sar^{1}Ile^{8}] Ang II (10 µM, non-specific tubes), and 100 µl of tissue suspension.

2.3.3 Competition assays

Competition assays were carried out to measure the fractions of AT_1 and AT_2 receptors, and the affinity of the receptors for competitors (i.e. [Sar^{1}Ile^{8}] Ang II, losartan or PD 123319). The assay volume

(150 μl) contained 25 μl of ^{125}I [Sar^{1}Ile^{8}] Ang II (100 pM), 25 μl of PBS (total tubes) or [Sar^{1}Ile^{8}] Ang II (10 μM, non-specific tubes) or increasing concentrations of competitors ([Sar^{1}Ile^{8}] Ang II (10^{-12}-10^{-6} M), losartan (10^{-12}-10^{-3} M), PD 123319 (10^{-12}-10^{-3} M), and 100 μl of tissue suspension.

As described previously, for both saturation and competition assays the suspensions were left for at least 60 min at 23°C to reach equilibrium.[20] The reaction was stopped by the addition of ice-cold NaCl (0.15 M). The bound radioligand was separated from free by vacuum filtration (Brandell Cell Harvester) through glass filter fibres (GF/C filter paper, Whatman) pre-soaked in PBS (1 % BSA) for approximately 60 min. The filter paper was washed 4 times under vacuum with ice-cold NaCl (0.15 M). The filter papers were counted on an automated Gamma Counter (1470 Wizard, Wallac, Turku 10, Finland; with an efficiency of 79.5 % for ^{125}I).

2.3.4 Analysis of receptor binding data

Saturation and competition binding experiments were analysed by non-linear curve fitting using Prism (Graphpad Software Inc., San Diego, CA, USA). For saturation curves, specific binding was calculated by subtracting non-specific binding from total binding. This was plotted against the concentration of radioligand added to each tube in pM and non-linear regression was performed using the one-site binding (hyperbola) equation, Y = (Bmax * X) / (Kd + X), where X is the concentration of radioligand (pM) and Y is the specific binding (cpm). From this equation, the Kd and Bmax were derived. Hill coefficients were determined by plotting Log (B/(Bmax –B)) against Log F (where B = specific bound, F= free radioligand concentration).

For competition curves, total binding was plotted against the logarithm of the inhibitor concentration (i.e. [Sar^{1}Ile^{8}] Ang II, losartan or PD 123319). Curves were analysed using a one-site competition equation for Sar and a one or two-site competition equation for losartan and PD 123319. From these curves the fractions of Ang II receptor subtypes and the IC_{50}s were determined. Two-site competition curves fitted the losartan and PD 123319 data when both AT_1 and AT_2 receptors were present because at high concentrations ($\geq 10^{-5}$ M) these antagonists cross react at the other Ang II receptor subtype. Competition curves were set to plateau at non-specific binding prior to determination of fractions of Ang II subtypes and IC_{50}s. To make certain a two-site fit was better than a one-site fit an F test was used.[21] The 2-site model was accepted if the P-value was less than 0.05.

2.4 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics (Version 23) or GraphPad Prism (Version 7). Results are expressed as means ± standard error (S.E.M.). *N* refers to the number of animals and *n* refers to the number of arterial ring preparations. When comparing 3 groups statistical significance was determined using one-way analysis of variance (ANOVA). If the ANOVA showed significance ($P<0.05$), it was followed by Tukey's multiple comparisons test. Significance level was $P<0.05$. Statistical comparisons between 2 groups were made using Student's paired *t*-tests or unpaired *t*-tests as appropriate, with

correction for unequal variance where necessary.

3. Results

3.1 Receptor binding properties

Both uterine arteries and fetal aortae contained predominantly AT_2 receptors, although AT_1 receptors were also present (Table 1). In a preliminary study we found that fetal iliac (data not shown) and fetal carotid arteries also expressed both Ang II receptor subtypes. Representative competition curves from a uterine artery, fetal aorta and fetal carotid artery are shown in Fig. 1. The uterine arteries (from pregnant ewes) had a higher proportion of AT_2 receptors (fraction of high affinity site on PD 123319 curves) than the aortae from fetal sheep ($P<0.01$; Table 1). The proportion of AT_2 receptors in the fetal aorta and fetal carotid were similar (Fig. 1).

Table 1. Densities, proportions, and affinities of Ang II receptors in uterine arteries and fetal aortae.

Variable	N	Uterine arteries	N	Fetal aortae
Total density (fmol/mg protein)	5	34.1 ± 4.4	8	73.6 ± 6.2 ***
AT_1 (%)	5	7.5 ± 0.8	7	17.7 ± 2.8 *
AT_2 (%)	5	84.1 ± 1.0	7	73.8 ± 2.6 **
Kd (nM)	5	0.29 ± 0.03	8	0.52 ± 0.06 *
Sar IC_{50} (nM)	5	0.27 ± 0.02	8	0.39 ± 0.04 *
Los (high) IC_{50} (nM)	5	321.0 ± 91.2	7	450.7 ± 108.9
Los (low) IC_{50} (nM)	5	$2.5\times10^5 \pm 8.0\times10^3$	7	$1.7\times10^5 \pm 1.4\times10^4$ ***
PD (high) IC_{50} (nM)	5	14.5 ± 0.7	7	8.1 ± 1.5 **
PD (low) IC_{50} (nM)	5	$2.1\times10^5 \pm 3.3\times10^4$	7	$2.2\times10^5 \pm 1.6\times10^4$

N refers to the number of animals in each group. Affinity of the radioligand for total Ang II receptors (Kd), half maximal displacement (IC_{50}) for [Sar^1Ile^8]Ang II (Sar), losartan (Los), and PD 123319 (PD). High and low refer to high and low affinity sites. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to uterine arteries.

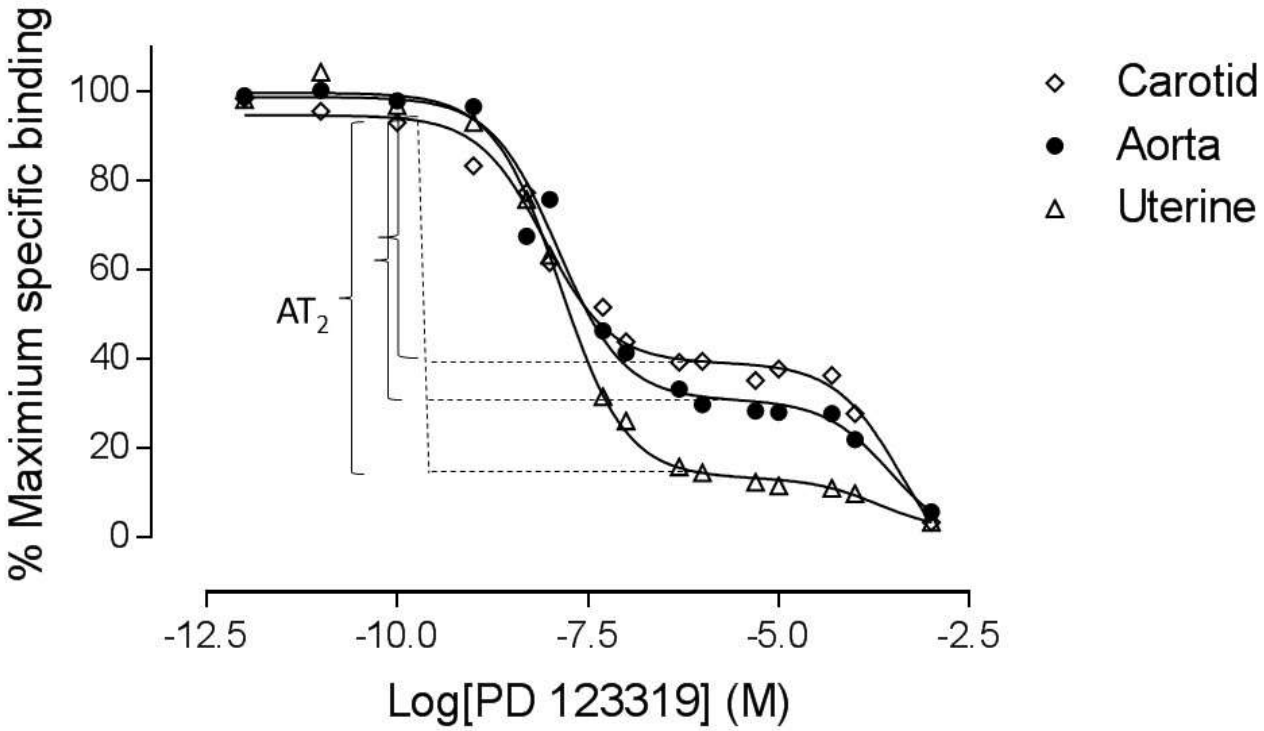

Figure 1. Representative curves from competition assays in a carotid artery from a fetal sheep (Carotid), an aorta from a fetal sheep (Aorta), and a uterine artery from a pregnant ewe (Uterine). The radioligand is displaced by PD 123319. The proportion of AT_2 receptors in each vessel is indicated.

Fetal aortae had a greater density of total Ang II receptors than uterine arteries (P<0.001; Table 1). Interestingly, the radioligand had a lower affinity (higher Kd) for Ang II receptors in fetal aortae than uterine arteries (P<0.05), and the half maximal displacement of [Sar^{1}Ile^8]Ang II (Sar IC_{50}) was higher (P<0.05). However, these differences were relatively small and may have been due to the higher proportion of AT_1 receptors in fetal aortae than uterine arteries, since [Sar^{1}Ile^8] Ang II has a 4-fold higher affinity for the AT_2 receptor than the AT_1 receptor in ovine tissues.[22] Hill coefficients were close to unity (uterine arteries n_H=0.94±0.04; fetal aortae n_H=0.96±0.02), indicating no cooperativity. The affinity of Ang II receptors for the high affinity PD 122319 site (PD (high) IC_{50}, Table 1) and the low affinity losartan site (Los (low) IC50, Table 1) was higher in fetal aortae than in uterine arteries. Both sites (PD (high) and Los (low)) correspond to binding of the radioligand to the AT_2 receptor subtype. This may suggest that the AT_2 receptor in uterine arteries and fetal aortae are different.

3.2 Contractile studies

3.2.1 Responses to KCl

The responses of fetal carotid arterial rings to KCl (80 mM) were very much lower (3.0

± 0.2 g, *n*=9, *N*=5) than the responses of uterine arterial rings from pregnant saline infused ewes to KCl (40 mM; 11.3 ± 0.5 g, *n*=52, *N*=6; P<0.0001).

3.2.2 Responses to Ang II

Fetal carotid arterial rings were more responsive to Ang II (2.4 ± 0.2 g, *n*=13, *N*=6) than uterine arterial rings (0.31 ± 0.08 g, *n*=24, *N*=6; P<0.0001). The Ang II induced responses of fetal carotid rings appeared even greater when expressed relative to KCl (fetal carotid rings (relative to 80 mM KCl): 78.3 ± 4.6 %, *n*=9, *N*=5; uterine arterial rings (relative to 40 mM KCl): 2.3 ± 0.5 %, *n*=24, *N*=6; P<0.0001).

3.2.3 Ang II induced tachyphylaxis

Uterine arterial rings displayed marked tachyphylaxis to Ang II. In uterine arterial rings exposed to a second dose of Ang II, 30 min after the first dose had been washed from the bath, a response to Ang II could not be detected (Fig 2). The contractile response of uterine arterial rings did not begin to recover until rings had been left for 60 min prior to the second dose of Ang II. By contrast, fetal carotid arterial rings did not display Ang II induced tachyphylaxis. The response of carotid arterial rings to a second dose of Ang II 30 min after the first had been washed from the bath was 95.6 ± 7.2 % (expressed as a % of the 1st exposure to Ang II, *n*=10, *N*=6; Fig 2.). In some fetal carotid arterial rings the responses of a 3rd and 4th dose of Ang II were recorded. These were 115 ± 3.5 % (*n*=5, *N*=2) and 95 ± 2.0 % (*n*=2, *N*=1) respectively (Fig. 2). Since fetal carotid rings did not show Ang II induced tachyphylaxis, individual rings could be exposed to Ang II as well as to Ang II in the presence of one of its antagonists at a later period. This was not possible with uterine arterial rings.

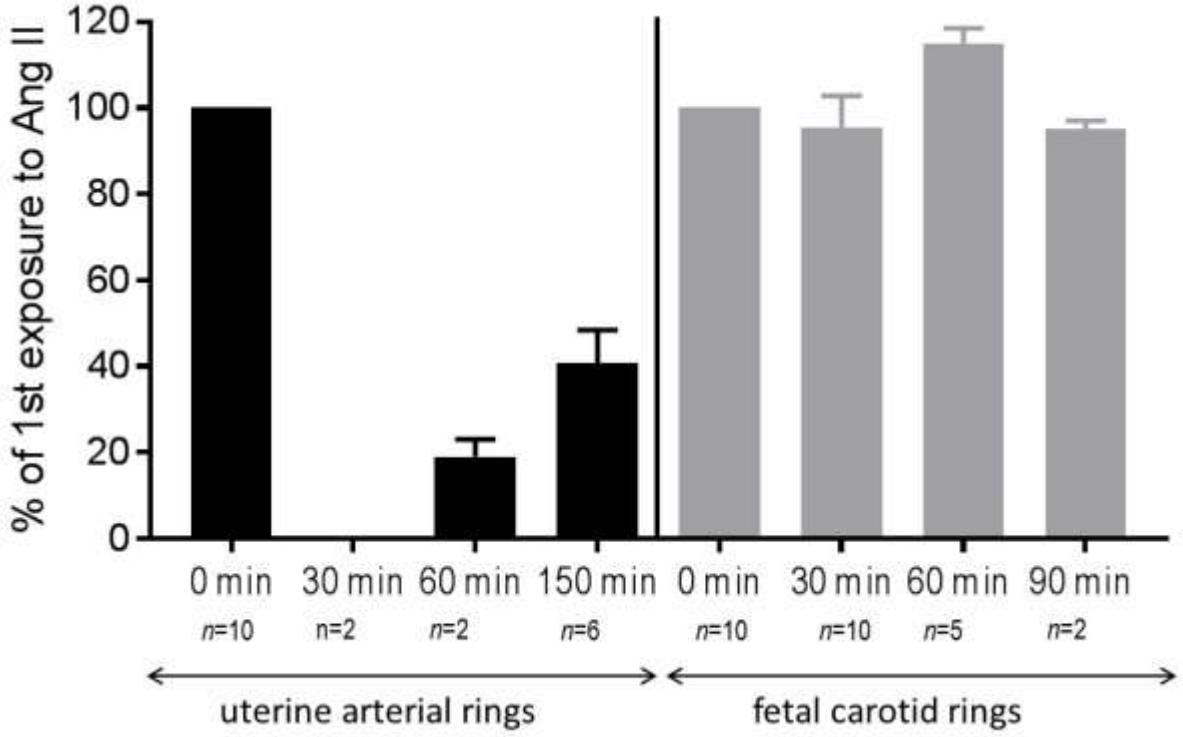

Figure 2. Responses of uterine arterial rings and fetal carotid arterial rings to an initial exposure of Ang II (0 min, expressed as 100 %) and subsequent exposure of the same rings (after the initial dose of Ang II had been washed from the organ bath) at time intervals after the 1st exposure. Subsequent responses to Ang II are expressed relative to the initial response. Fetal carotid rings were exposed to Ang II up to 4 times. Uterine arterial rings were only exposed to Ang II twice (i.e. initial exposure at 0 min followed by a second exposure at 30, 60 or 150 min).

3.2.4 Responses to Ang II antagonists

In uterine arterial rings, the AT_1 antagonist losartan inhibited (P<0.001) the contractile responses to Ang II (Fig. 3). By constrast, the AT_2 antagonist PD 123319 enhanced (P<0.001) the contractile responses induced by Ang II. Surprisingly, in carotid arterial rings from fetal sheep, the Ang II responses were not affected by losartan (1 μM), and only marginally but significantly (P<0.05) reduced by PD 123319 (1 μM; Fig. 3).

4. Discussion

We report for the first time, a comparison of the contractile responses to Ang II *in vitro* of an adult and fetal artery which both express predominantly AT_2 receptors. We also carried out Ang II receptor binding studies to see if differences in receptor density or affinity could explain the functional differences observed.

As previously reported, uterine arteries from pregnant ewes and systemic blood vessels (aorta, carotid, and iliac artery) from fetal sheep contained a high proportion of AT_2 receptors.[7,9,12-15] However, in contrast

to another group,[12,13] we were also able to find a sizeable proportion AT_1 receptors in fetal aortae, iliac and carotid arteries. This finding agrees with other studies from our laboratory which found 26 ± 10%, 35 ± 7% and 50 ± 10% AT_1 in fetal aorta and 28 ± 8% AT_1 in fetal carotid.[14,15] It is also consistent with studies using Western blot analysis which reported that AT_1 protein was present in the carotid artery in fetal sheep and increased in an age dependent manner during the last third of gestation.[16] Levels at term were approximately one third of adult levels.[16] Since contraction is mediated via the AT_1 receptor, it was not surprising that the fetal carotid arteries elicited contractile responses to Ang II *in vitro*.

In a previous study we investigated the functional significance of AT_1 and AT_2 receptors in the uterine artery by studying the contractility of this vessel to Ang II and antagonists *in vitro*.[3] Contractile responses to Ang II were small (<5% of KCl) and were inhibited by an AT_1 antagonist (losartan), which was consistent with contraction being mediated by AT_1 receptors. An AT_2 antagonist, PD 123319, significantly enhanced Ang II induced contractile responses ($P<0.05$). Thus, it was concluded that uterine arterial rings were relatively insensitive to the vasoconstrictor actions of Ang II because the uterine artery contained only a small proportion of AT_1 receptors, and because AT_2 receptors inhibited AT_1 mediated contractions in this vessel. Thus, we predicted that fetal systemic blood vessels would also be relatively refractory to Ang II for similar reasons.

Unexpectedly, fetal carotid rings were more responsive to Ang II than uterine arterial rings. The raw responses to Ang II were approximately 8-fold greater in fetal carotid arterial rings compared to those in uterine arterial rings. This difference appeared even greater when responses were expressed relative to KCl since KCl responses were 3 to 4-fold greater in uterine rings than fetal carotid rings (despite the dose of KCl being 40 mM for the uterine arteries and 80 mM for the fetal carotids). The small responses to KCl in the fetal carotid are consistent with another study in which the contractile responses of fetal blood vessels (ovine femoral artery and aorta; 130-145 days gestation) to KCl were attenuated compared with responses of the same adult blood vessels.[23] These investigators also measured contractile proteins and showed that fetal systemic vascular smooth muscle was biochemically immature.[23]

The greater response of the fetal carotid artery than the uterine artery to Ang II *in vitro* may have been due to a greater density and proportion of AT_1 receptors in the fetal carotid artery. As shown in Table 1, the total density of Ang II receptors in the fetal aorta was about twice that of the uterine artery. This, combined with the greater proportion of AT_1 receptors means that the density of AT_1 receptors in the fetal aorta was about 5-fold that of the uterine artery (approximately 13 and 2.6 fmol/mg protein respectively). Although we were not able to do these measurements in fetal carotid arteries in the current study, other studies from our laboratory have shown that the total density of Ang II receptors was higher (approximately double) in fetal carotid than aorta.[15] Since the proportion of AT_1 receptors in these two fetal vessels were similar, the density of AT_1 in the fetal

carotid tended to be greater than in the fetal aorta (approximately 24 and 12 fmol/mg protein respectively).[15] Thus, it is likely that the difference in AT_1 receptor density between the fetal carotid and the uterine artery was even greater than 5-fold.

Surprisingly, even though Ang II induced substantial contractile responses in the fetal carotid artery, these responses were by no means inhibited by the AT_1 antagonist, losartan, at a dose routinely used in pharmacological experiments (i.e 1 μM). This is in contrast to fetal renal and mesenteric arteries (but not the external umbilical artery) where this dose of losartan caused significant inhibition.[24] However, in that study,[24] the dose of Ang II that was used was lower (0.1 μM rather than 4 μM) and the fetuses were older (134-140 d rather than 120-135 d), so this difference is not necessarily due to the particular systemic vessel under study. Furthermore, we have found that to almost abolish the Ang II induced contraction in both the fetal carotid and the external umbilical artery, 100 μM losartan was required.[14] Similarly, we have noted *in vivo* that high doses of losartan (10 mg/kg) do not completely abolish the pressor response to Ang II in fetuses at 125-132 days.[25]

As well as the difference in the effect of losartan, another difference observed between the fetal carotid and the uterine arterial rings was in the response to the AT_2 antagonist, PD123319. Whereas in uterine arterial rings the contractile response to Ang II was enhanced in the presence of PD123319 (1 μM), in fetal carotid rings the Ang II induced responses were marginally inhibited ($P<0.05$). As well, PD 123319 did not enhance Ang II induced contractions in the renal and mesenteric arteries of older fetuses in the study mentioned above.[24] This failure of PD123319 to enhance the contractile response to Ang II suggests that AT_2 receptors might not have the same inhibitory effect on Ang II mediated contractions in the fetus as they do in adult uterine artery. If so, this could provide an additional explanation for why Ang II induced contractions in the fetal carotid artery were so much greater than in the uterine artery. The difference in the responses of the two vessels to PD123319 is particularly interesting in view of the higher affinity of the AT_2 receptor in fetal aortae compared to uterine arteries. However, receptors with a higher affinity do not necessarily have a greater efficacy. For example, mutations in the AT_1 receptor can increase affinity without increasing function as measured by inositol phosphate production.[26]

Another interesting finding was that fetal carotid arterial rings did not develop Ang II induced tachyphylaxis. Ang II induced tachyphylaxis can be defined as a diminution of responsiveness to repeated doses of Ang II. It is a recognised *in vitro* phenomenon which is commonly observed in adult blood vessels,[3, 27-29] and was strongly apparent in uterine arterial rings in the present study. Even 2.5 h after the first dose of Ang II had been washed from the organ bath, responses of uterine arterial rings to Ang II were still well below the initial responses. The reason for the lack of tachyphylaxis in the fetal carotid arterial rings is not clear. Furthermore, even when some degree of reduction in the response to repeated doses of Ang II has been demonstrated in fetal vessels,[14,24] the

response has been mild (eg reduction to ~ 80%) compared to the complete abolition of response seen in the uterine artery (Fig 2). Since tachyphylaxis is an *in vitro* phenomenon, the *in vivo* significance of the difference in tachyphylaxis between the fetal carotid and uterine artery is unclear. However, others have suggested that the presence of tachyphylaxis *in vitro* provides some insight into receptor function in normal and diseased states.[28]

The mechanisms responsible for tachyphylaxis to Ang II potentially involve multiple factors including AT_1 receptor modification (phosphorylation), trafficking (internalisation and recycling), down regulation, and the interaction of regulatory proteins with the cytoplasmic regions of the receptor protein,[30] with more recent literature distinguishing between the processes of tachyphylaxis and desensitization.[31,32] Whereas desensitization correlates with phosphorylation of Ser and Thr residues in the cytoplasmic domain of the AT_1 receptor, for tachyphylaxis, interaction between the AT_1 receptor and the N-terminal residues of Ang II appears to be critical.[31] Modifications in this region of the ligand can either enhance or abolish tachyphylaxis.[31] As well, for tachyphylaxis, AT_1 receptor internalization via caveolae seems to be important. Methyl-β-cyclodextrin, a drug which depletes plasma membrane cholesterol and disassembles calveolae in the plasma membrane of vascular smooth muscle cells, prevents the development of tachyphylaxis to Ang II in rat aortic rings.[33] By contrast, AT_2 receptors do not appear to be internalised and recycled after stimulation with Ang II.[5] Exposure of cells expressing AT_2 receptors, to Ang II for 2h resulted in no detectable redistribution of the cell surface receptors.[34]

One important difference that should be noted between the vessels studied is that uterine arteries were intentionally denuded of endothelium whereas fetal arterial rings were not. The endothelium was intentionally removed from uterine arterial rings because the responses of this vessel were low (Fig 3) and the presence of functional endothelium would have reduced the responses to Ang II in this vessel even further. This is because the endothelium of ovine uterine arteries contains predominantly AT_1 receptors, which when stimulated with Ang II result in synthesis of a potent vasodilator, prostacyclin.[7] In contrast, the endothelium was not intentionally removed from fetal arterial rings because we did not want to risk damaging the underlying smooth muscle. Fetal arterial rings were relatively "fragile" compared to uterine arterial rings, and rubbing inside the lumen to remove all endothelial cells in neonatal arteries from guinea pigs was reported to result in poor contractile responses.[17] We have shown that the hooks in the organ bath apparatus partially remove the endothelial lining of fetal carotid vessels, because 1 μM bradykinin only caused approximately 55% relaxation in rings preconstricted with noradrenaline.[14]

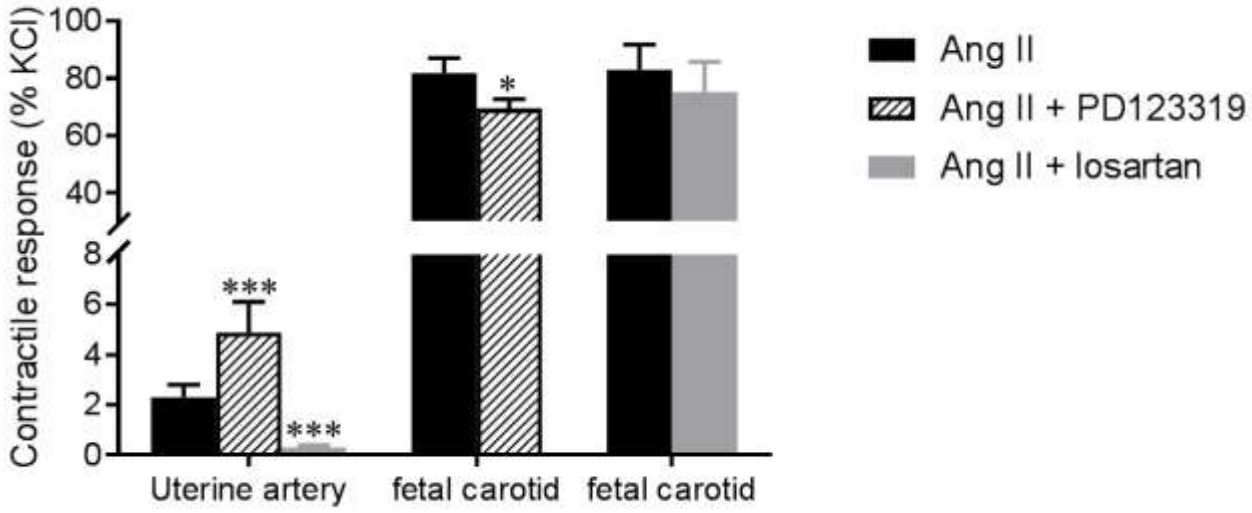

Figure 3. Contractile responses of uterine arterial rings and fetal carotid rings to Ang II (4 μM) alone and in the presence of 1 μM PD 123319 or 1 μM losartan. Statistical comparisons between responses in uterine arterial rings were made using one-way ANOVA (followed by Tukey's multiple comparison test). Responses in fetal carotid rings were compared using Student's paired t-test. * $P<0.05$, *** $P<0.001$ compared to Ang II alone. Uterine arterial rings: Ang II alone, $n=24$; Ang II + PD 123319, $n=18$; Ang II + losartan, $n=10$. Fetal carotid rings: Ang II followed by Ang II + PD 123319, $n=5$; Ang II followed by Ang II + losartan, $n=4$.

While we cannot rule out the possibility that the complete denudation of the endothelium of uterine arteries and the partial denudation of fetal arterial rings explains the different contractile responses of these vessels, we consider it unlikely for a number of reasons. Firstly, as noted above, if the endothelial lining was left intact in uterine arteries, responses to Ang II would likely have been smaller rather than greater. Secondly, neither AT_1 or AT_2 receptors were detected by immunohistochemistry in the endothelium of the carotid artery during development in fetal sheep.[16] Thirdly, the presence of endothelium enhances development of tachyphylaxis in rat aorta,[29,35] so if anything, the partial presence of endothelium in the fetal carotids but not the uterine arteries should have enhanced tachyphylaxis in the fetal carotids. Finally, it is unlikely that a partially functional endothelium would have such dramatic effects.

The unique responses of fetal carotid arterial rings to Ang II and antagonists compared to uterine arterial rings (i.e. the lack of Ang II-induced tachyphylaxis and the relative insensitivity to Ang II antagonists) may suggest that fetal vascular Ang II receptor subtypes are atypical. Similarly, even in fetal external umbilical arteries which express only AT_1 receptors, the response to Ang II was poorly blocked by 1 μM losartan, was attenuated rather than enhanced by 1 μM PD123319, and tachyphylaxis was less marked than in adult

vessels.[14] Grady *et al*. suggested that an atypical Ang II receptor subtype may exist in the rat fetus.[11] This was based on a study which showed that after AT_1 and AT_2 receptors were blocked with losartan (10^{-5} M) and PD 123317 (10^{-6} M), a small amount of residual binding to ^{125}I-[Sar^{1}Ile^8]Ang II remained. The existence of atypical AT_2 receptors has also been reported. For example, it was suggested on the basis of sensitivity to GTP analogues and pertussis toxin, that there may be more than one AT_2 receptor subtype in the brain,[36] and another investigator suggested that AT_2 receptors in the rat adrenal gland and brain were different.[37] Furthermore, an AT_4 receptor has also been characterised.[38] This receptor preferentially binds Ang IV, and although the AT_4 is particularly prominent in the brain, it is also present in the periphery and may oppose AT_1 effects.[38] To our knowledge, the ontogeny of the AT_4 receptor has not been investigated.

In summary, we have shown that the Ang II-induced contractile responses of carotid arteries from fetal sheep and uterine arteries from pregnant ewes are remarkably different, even though both vessels have a high proportion of AT_2 receptors. Fetal carotid arterial rings did not display Ang II induced tachyphylaxis and the contractile response to Ang II was relatively unaffected by the Ang II receptor antagonists losartan and PD123319. Together, this suggests that Ang II receptors in carotid arteries of late gestation fetal sheep are functionally dissimilar to those in the uterine artery of pregnant sheep.

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References

1. Ichiki T, Labosky PA, Shiota C, Okuyama S, Imagawa Y, Fogo A, et al. Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. Nature 1995; 377(6551): 748-50.

2. Höhle S, Spitznagel H, Rascher W, Culman J, Unger T. Angiotensin AT_1 receptor-mediated vasopressin release and drinking are potentiated by an AT_2 receptor antagonist. Eur J Pharmacol 1995; 275(3): 277-82.

3. McMullen JR, Gibson KJ, Lumbers ER, Burrell JH, Wu J. Interactions between AT_1 and AT_2 receptors in uterine arteries from pregnant ewes. Eur J Pharmacol 1999; 378(2): 195-202.

4. Horiuchi M, Akishita M, Dzau VJ. Recent progress in the angiotensin II type 2 receptor research in the cardiovascular system. Hypertension 1999; 33(2): 613-21.

5. McCarthy CA, Widdop RE, Denton KM, Jones ES. Update of the angiotensin AT_2 receptor. Curr Hypertens Rep. 2013; 15:25-30.

6. Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, et al. Angiotensin II receptors and angiotensin II receptor antagonists. Pharmacol Rev 1993; 45(2), 205-51.

7. Cox BE, Rosenfeld CR, Kalinyak JE, Magness RR, Shaul PW. Tissue specific expression of vascular smooth muscle angiotensin II receptor subtypes during ovine pregnancy. Am J Physiol 1996; 271(1 Pt2): H212-21.

8. Cox BE, Word RA, Rosenfeld CR. Angiotensin II receptor characteristics and subtype expression in uterine arteries and myometrium during pregnancy. J Clin Endocrinol Metab 1996; 81(1): 49-58.

9. Burrell JH, Lumbers ER. Angiotensin receptor subtypes in the uterine artery during ovine pregnancy. Eur J Pharmacol 1997;330(2-3): 257-67.

10. Tsutsumi K, Strömberg C, Viswanathan M, Saavedra JM. Angiotensin-II receptor subtypes in fetal tissues of the rat: autoradiography, guanine nucleotide sensitivity, and association with phosphoinositide hydrolysis. Endocrinology 1991; 129(2): 1075-82.

11. Grady EF, Sechi LA, Griffin CA, Schambelan M, Kalinyak JE. Expression of AT_2 receptors in the developing rat fetus. J Clin Invest 1991; 88(3): 921-33.

12. Kaiser JR, Cox BE, Roy TA, Rosenfeld CR. Differential development of umbilical and systemic arteries. I. Ang II receptor subtype expression. Am J Physiol 1998; 274(3 Pt2): R797-807.

13. Cox BE, Rosenfeld CR. Ontogeny of vascular angiotensin II receptor subtype expression in ovine development. Pediatr Res 1999; 45(3), 414-24.

14. Hegarty BD, Burrell JH, Gibson KJ, McMullen JR, Lumbers ER. Effect of cortisol on fetal ovine vascular angiotensin

II receptors and contractility. Eur J Pharmacol 2000; 406(3): 439-48.

15. Burrell JH, Hegarty BD, McMullen JR. Lumbers ER. Effects of gestation on ovine fetal and maternal angiotensin receptor subtypes in the heart and major blood vessels. Exp Physiol 2001; 86(1): 71-82.

16. Cox BE, Liu XT, Fluharty SJ, Rosenfeld. Vessel-specific regulation of angiotensin II receptor subtypes during ovine development. Pediatr Res 2005; 57:124-132.

17. Davidson D, Eldemerdash A. Endothelium-derived relaxing factor: presence in pulmonary and systemic arteries of the newborn guinea pig. Pediatr Res 1990; 27(2): 128-32.

18. Greenwood FC, Hunter WM, Glover JS. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem J 1963; 89, 114-23.

19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193(1): 265-75.

20. McMullen JR, Gibson KJ, Lumbers ER, Burrell JH. Selective down-regulation of AT_2 receptors in uterine arteries from pregnant ewes given 24-h intravenous infusions of angiotensin II. Regul Pept 2001; 99(2-3):119-29.

21. Motulsky H, Neubig R. Analyzing radioligand binding data. Curr Protoc Neurosci 2002; Chapter 7: Unit 7.5

22. McMullen JR, Gibson KJ, Lumbers ER, Burrell JH. ^{125}I[Sar^{1}Ile^8] Angiotensin II has a different affinity for AT_1 and AT_2 receptor subtypes in ovine tissues. Regul Pept 2002; 105(2):83-92.

23. Arens Y, Chapados RA, Cox BE, Kamm KE, Rosenfeld CR. Differential development of umbilical and systemic arteries. II. Contractile proteins. Am J Physiol 1998; 274(6 Pt2): R1815-23.

24. Segar JL, Barna TJ, Acarregui MJ, Lamb FS. Responses of fetal ovine systemic and umbilical arteries to angiotensin II. Pediatr Res 2001; 49:823-33.

25. Stevenson KM, Gibson KJ, Lumbers ER. Effects of losartan on the cardiovascular system, renal haemodynamics and function and lung liquid flow in fetal sheep. Clin Exp Pharmacol Physiol 1996; 23(2): 125-33.

26. Miura S, Feng Y, Husain A, Karnik SS. Role of aromaticity of agonist switches of angiotensin II in the activation of the AT_1 receptor. J Biol Chem 1999; 274(11):7103-10.

27. Sim MK, Kuttan SC. Effects of noradrenaline and prostaglandin F_{2a} on angiotensin-induced contraction and tachyphylaxis in rat aortic rings. Pharmacol Toxicol 1992; 70(1): 60-4.

28. Kuttan SC, Sim MK. Angiotensin II-induced tachyphylaxis in aortas of normo- and hypertensive rats: changes in receptor affinity. Eur J Pharmacol 1993; 232:173-180.

29. Gruetter CA, Ryan ET, Schoepp DD. Endothelium enhances tachyphylaxis to angiotensins II and III in rat aorta. Eur J Pharmacol 1987; 143:139-142.

30. Thomas WG. Regulation of angiotensin II type 1 (AT_1) receptor function. Regul Pept 1999; 79(1): 9-23.

31. Oliveira L, Costa-Neto CM, Nakaie CR, Schreier S, Shimuta SI, Paiva ACM. The angiotensin II AT_1 receptor structure-activity correlations in the light of rhodopsin structure. Physiol Rev 2007; 87: 565-92.

32. Alves FL, Oliveira VX, Miranda A. Angiotensin II analogues with N-terminal lactam bridge cyclization: an overview of AT_1 receptor activation and tachyphylaxis. Chem Biol Drug Des 2016; 88: 677-82.

33. Linder AE, Thakali KM, Thompson JM, Watts SW, Webb RC, Leite R. Methyl-β-cyclodextrin prevents angiotensin II-induced tachyphylactic contractile responses in rat aorta. J Pharmacol Exp Ther 2007; 323(1):78-84.

34. Hein L. Meinel L, Pratt RE, Dzau VJ, Kobilka BK. Intracellular trafficking of angiotensin II and its AT_1 and AT_2 receptors: Evidence for selective sorting of receptor and ligand. Mol. Endocrinol. 1997; 11(9): 1266-77.

35. Li Q, Zhang J, Pfaffendorf M, van Zwieten PA. Comparative effects of angiotensin II and its degradation products angiotensin III and angiotensin IV in rat aorta. Brit J Pharmacol 1995; 116:2963-70.

36. Tsutsumi K, Saavedra JM. Heterogeneity of angiotensin II AT_2 receptors in the rat brain. Mol Pharmacol 1992; 41:290-297.

37. Speth RC. [^{125}I]CGP 42112 binding reveals differences between rat brain and adrenal AT_2 receptor binding sites. Regul Pept 1993; 44(2): 189-97.

38. de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International Union of Pharmacology. XXIII. The angiotensin II receptors. Pharmacol Rev 2000; 52:415-72.

