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# Comparative effects of angiotensin II and its degradation products angiotensin III and angiotensin IV in rat aorta

[1]Q. Li, J. Zhang, M. Pfaffendorf & P.A. van Zwieten

Department of Pharmacotherapy, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

**1** In the present study, the contractile effects of angiotensin III (AIII) and angiotensin IV (AIV) compared with those of angiotensin II (AII) were determined in rat aortic ring preparations.

**2** All three peptides caused concentration-dependent contractions with similar maximal responses. AIII proved approximately 4 times less potent than AII, whereas AIV was about 1000 times less active than AII.

**3** The selective $AT_1$-receptor antagonist, losartan (10-300 nM) caused parallel rightward shifts of the concentration-response curves (CRC) for all three peptides. The Schild plot slopes for the effect of losartan on AIII curves were significantly lower than unity ($P<0.05$). The selective $AT_2$-receptor antagonist, PD123177 did not influence the CRCs for AII and AIV. However, the AIII curves were moderately shifted leftward in the presence of PD123177 (0.1 and 1 $\mu$M).

**4** Destruction of the endothelium or incubation with the NO-synthesis inhibitor $N^G$-monomethyl-L-arginine acetate (L-NMMA) (0.1 mM) significantly enhanced the contractile responses to all three peptides.

**5** Tachyphylaxis was investigated by constructing a second CRC for all three peptides, after an interval of 1 h. The presence of endothelium significantly enhanced the development of tachyphylaxis to all three peptides. However, in endothelium-denuded preparations, the $E_{max}$ value of the second curve elicted by AII was about 50%, compared with the first one, whereas for AIII and AIV $E_{max}$ values were as high as 90% and 100%, respectively.

**6** Our results indicate that both AIII and AIV are less potent but similarly efficacious vasoconstrictor agents compared with AII. Their contractile effects are also mediated by $AT_1$-receptors and probably modulated by endothelium. Tachyphylaxis induced by AIII and AIV proved weaker than that for AII. Tachyphylaxis appears to be enhanced by the presence of an intact endothelium.

**Keywords:** Angiotensin II; angiotensin III; angiotensin IV; rat aorta; losartan; PD123177; L-NMMA; tachyphylaxis

## Introduction

The renin-angiotensin system plays a major role in the regulation of blood pressure, blood volume, as well as sodium and water balance. Most of these modulating effects are mediated via angiotensin II (AII), acting at the $AT_1$ subtype of the AII receptor (Timmermans *et al.*, 1993). The potent vasoconstrictor effect of AII both *in vivo* and *in vitro* has been well documented (Peach, 1977). The octapeptide (AII) is known to be primarily degraded at its N-terminal position by aminopeptidase A in both plasma and vasculature (Ahmad & Ward, 1990), resulting in the formation of the heptapeptide, angiotensin III (AIII). The subsequent degradation of circulating AIII at the N-terminal by aminopeptidase B or M which are located at the cell-surface of vascular endothelium and smooth muscle (Ahmad & Ward, 1990; Abhold & Harding, 1988), leads to the hexapeptide, angiotensin IV (AIV).

AIII is considered to be a contractile agent in rabbit aorta, femoral artery and rat uterus preparations, but estimates of its potency compared to AII are subject to wide variations (Moore *et al.*, 1976; Pendleton *et al.*, 1991; Murphy *et al.*, 1993). AIV is thought to be biologically inactive because of its inability to stimulate the aforementioned classic angiotensin-dependent processes (Fitzsimons, 1971; Tonnaer *et al.*, 1982). However, recent investigations have shown the presence of a special binding site for AIV in a variety of tissues from many species (Hanesworth *et al.*, 1993; Harding *et al.*, 1994), and it has also been suggested that AIV may be implicated in the regulation of blood flow (Haberl *et al.*, 1991; Cheng *et al.*, 1994; Coleman *et al.*, 1995).

The inhibitory effect of the endothelium on the vasoconstrictor responses to AII has been demonstrated to occur in various vessels and animal species (Gruetter *et al.*, 1988; van Heiningen & Van Zwieten, 1993; Zhang *et al.*, 1994). However, the influence of the endothelium on the vascular responses to AIII and AIV so far remains unknown.

In the present study, we compared the effects of AIII and AIV with those of AII in rat aortic ring preparations, in the absence and presence of functional endothelium. We also studied the effects of the $AT_1$ subtype selective antagonist losartan (Timmermans *et al.*, 1991) and the $AT_2$ subtype selective antagonist PD123177 (Chiu *et al.*, 1989) on the responses induced by AII, AIII and AIV, in order to characterize the receptor subtypes that are involved.

It is well known that AII can induce pronounced tachyphylaxis in rat aorta preparations. Recent studies have shown that *in vitro* tachyphylaxis to AII is associated with changes in the affinity of AII for the $AT_1$-receptor (Kuttan & Sim, 1993). However, *in vivo*, tachyphylaxis does not occur (Sim & Radhakrishnan, 1994). For these reasons, we also compared the tachyphylaxis induced by AII, AIII and AIV in rat aortic ring preparations.

## Methods

### *Preparation of the rat aorta*

Male Wistar rats (IFFA CREDO, Les Oncins, France) weighing 250-350 g were killed by stunning and exsanguination. The thoracic aorta was dissected carefully and immediately placed in a Krebs solution (at room temperature) of

[1] Author for correspondence.

the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.2 and glucose 8.3, gassed with 95% $O_2$ + 5% $CO_2$. Adhesive fat and connective tissue were removed carefully. Since it has been shown that endothelium can inhibit the vasoconstrictor effect of AII and enhance tachyphylaxis to AII and AIII in rat aorta (van Heiningen & Van Zwieten, 1993; Gruetter *et al.*, 1988), experiments were performed in preparations with intact endothelium, as well as in aortae where the endothelium had been removed by gentle rubbing of the intimal surface with a wooden rod to avoid the inhibitory role of functional endothelium to angiotensin peptides and antagonists. Each aorta was cut into 4 mm wide transverse rings. The rings were suspended between two L-shaped stainless steel hooks in a 10 ml organ bath with Krebs solution at 37°C (pH = 7.4). Each preparation was connected by a silk thread to a force transducer (UFL Co. California, U.S.A.) and the isometric force was recorded by a MacLab/8-computer system (A.D. Instruments Ltd, London, U.K.). A resting tension of 1 g was maintained, this setting allowed for the optimum observation of maximal contractile response to angiotensin peptides.

### *Experimental protocol*

After an equilibration period of 60 min, tissues were exposed to a depolarizing potassium solution to test the viability. In most cases, a maximal contractile response ranging from 0.8–1.1 g could be induced. The preparations with a response below 0.6 g were considered insufficiently viable and discarded. The potassium solution had the same composition as the Krebs buffer used, except that NaCl had been completely replaced by an equimolar amount of KCl. Once the contraction had reached a plateau, the preparations were washed with Krebs solution four times and left for a further 40 min equilibration period at a re-adjusted tension of 1 g.

To avoid tachyphylaxis, only a single cumulative concentration-response curve (CRC) for an angiotensin peptide was obtained in each ring preparation. Appropriate controls were run at the same time in different rings obtained from the same aorta.

At the end of each experiment, after the drugs had been washed out with Krebs solution four times, methacholine (1 μM) was added when a maximal response to noradrenaline (0.3 μM) had been obtained, in order to assess the presence or absence of functional endothelium. A rapid and marked reduction of the noradrenaline-induced tone was taken as evidence that a significant amount of functional endothelium was present. The absence of a relaxant response was taken as indicative of the disappearance of functional endothelium.

### *Effects of AII and its degradation products (AIII and AIV) in endothelium-denuded aortic ring preparations*

After equlibration, cumulative CRCs for AII (1–100 nM), AIII (3 nM–1 μM) or AIV (1 μM–0.1 mM) were constructed in endothelium-denuded aortic ring preparations. The peptides were then washed out with Krebs solution four times. Subsequently, noradrenaline (1 μM)-induced contractions were imposed and methacholine (0.1 μM) was added to test whether functional endothelium was present. The effects caused by AII, AIII and AIV were expressed as absolute values (g) of contractile force.

### *Effects of angiotensin receptor antagonists on the responses to AII, AIII and AIV in endothelium-denuded aortic ring preparations*

After equilibration, the preparations were exposed to different concentrations of losartan (10, 100 or 300 nM) or PD123177 (0.01, 0.1 or 1 μM). Thirty minutes later, cumulative CRCs of AII, AIII or AIV were obtained in the presence of either of these two antagonists. The angiotensin peptide-induced contractions were expressed as absolute values (g) of contractile force.

### *Influence of the endothelium on the effects of AII, AIII and AIV*

After equilibration, cumulative CRCs for AII, AIII or AIV were constructed in both endothelium-intact and endothelium-denuded aortic ring preparations of the same aorta. The contractions of the angiotensin peptides were expressed as absolute values (g).

### *Influence of the nitric oxide (NO) synthesis inhibitor (L-NMMA) on the contractile effects of AII, AIII and AIV in endothelium-intact aortic ring preparations*

In separate experiments, cumulative CRCs for AII, AIII or AIV were constructed in endothelium-intact aortic rings after 30 min of incubation with $N^G$-monomethyl-L-arginine (L-NMMA, 0.1 mM). Preliminary experiments have shown that 1 μM methacholine failed to cause significant relaxation on tone induced by 0.3 μM noradrenaline after 30 min of incubation with 0.1 mM L-NMMA, which confirmed that the concentration of L-NMMA was sufficiently high to inhibit the synthesis of NO. The responses of the angiotensin peptides were expressed as the absolute values (g) of contractile force.

### *Tachyphylaxis induced by AII, AIII and AIV*

After cumulative CRCs for AII, AIII or AIV had been obtained in both endothelium-intact and endothelium-denuded aortic ring preparations, the rings were rinsed four times and re-equilibrated for 1 h. The second series of cumulative CRCs for AII, AIII or AIV were then constructed. Angiotensin peptide-induced contractions were expressed as a percentage of the maximal responses obtained in the first CRCs.

### *Drugs used*

AII, AIII and AIV (Bachem, Hannover, F.R.G.); losartan, PD123177 (1-[(4-amino-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazol [4,5-C] pyridine-6-carboxylic acid) (Thomae GmbH; Biberach/Riss, F.R.G.); methacholine bromide (Janssen Chimica, Beerse, Belgium); $N^G$-monomethyl-L-arginine acetate L-NMMA), noradrenaline (Sigma, St. Louis, U.S.A.). All drugs were dissolved in saline except for PD123177 which was taken up in a small volume of 1 M NaOH and subsequently diluted with saline.

### *Statistical analysis*

The data are presented as means ± s.e.mean for $n$ observations. The CRCs for the agonists were analysed by means of a computer program (GraphPad, Institute for Scientific Information, U.S.A.), and the $pD_2$ value [−log effective concentration which produces 50 % of the maximal effect ($EC_{50}$)] and the maximal effect ($E_{max}$) were thus obtained. According to the basic criteria of Schild analysis (Arunlakshana & Schild, 1959), when angiotensin curves obtained in the presence of the antagonists were parallel with the control curves and no significant depression of the maximal effect occurred, angiotensin concentration-ratios ($r$) were calculated. Accordingly, the concentration of the agonist in the presence of antagonist, which produced the same response as that caused by the $EC_{50}$ in the absence of antagonist, was divided by the $EC_{50}$. The results were visualized by means of a Schild plot, as log(r − 1) versus log antagonist concentration (log[B]), and a regression of log(r − 1) on log[B] was calculated. Only when the regression was linear and the slope was not significantly different from unity, the $pK_B$ value of the antagonist was calculated. The statistical significance of differences was analysed by means of one way analysis of variance (ANOVA) or Student's $t$ test, and $P$ values less than 0.05 were considered significant.

## Results

### *AII, AIII and AIV induced contractions in endothelium-denuded aortic ring preparations*

In endothelium-denuded aortic ring preparations, the cumulative addition of AII (1–100 nM), AIII (3 nM–1 μM) and AIV (1–100 μM) caused concentration-dependent contractions with comparable maximal responses (Figure 1 and Table 1). AIII was 3–4 times less active than AII, whilst AIV was approximately 1000 times less potent than AII. The slope of the CRC of AIII was significantly less steep ($P<0.01$) than those of the AII curves (Figure 1 and Table 1). Similar slopes were observed for the CRCs of AII and AIV, respectively. Functional endothelium was absent in this preparation which was confirmed by the lack of vasodilator effect of 1 μM methacholine after precontraction with 0.3 μM noradrenaline.

### *Effects of AII receptor antagonists on the contractile effects of AII, AIII and AIV in endothelium-denuded aortic ring preparations*

Losartan (10, 100 or 300 nM) concentration-dependently shifted the CRCs of AII, AIII to the right, without depressing the maximal response (Figure 2). The $pK_B$ value of losartan for AII curves amounted to 8.34±0.06. The Schild plot slope for the effect of losartan on AIII curves amounted to 0.63±0.08, which was significantly different from unity ($P<0.05$), so that the $pK_B$ value could not be calculated. Losartan (10 nM) also caused a parallel rightward shift of the CRCs for AIV, whereas lower concentrations (1 and 3 nM) of losartan caused no significant change of the CRCs for AIV (data not shown). Since excessively high concentrations of AIV (≥10 mM) would be required to produce maximal contraction in the presence of ≥10 nM losartan (Figure 2), the $pK_B$ value of losartan on AIV could not be assessed.

PD123177 (0.01, 0.1 or 1 μM) caused no significant change of the CRCs for AII and AIV (data not shown). However, over the same concentration-range, PD123177 caused concentra-

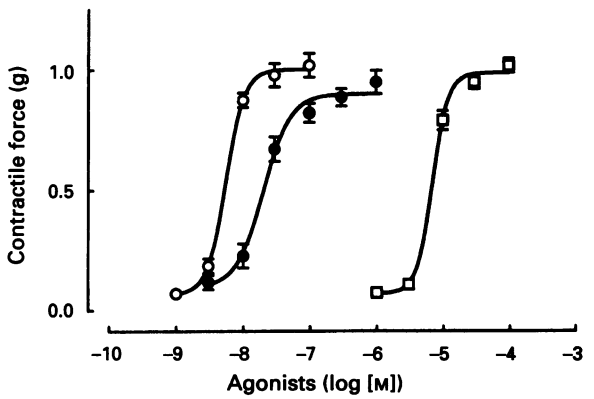


**Figure 1** Concentration-response curves for the contractile effects of angiotensin II (○), angiotensin III (●) and angiotensin IV (□) in rat aortic ring preparations. Data are shown as means±s.e.mean, expressed as the absolute values (g), s.e.mean values lower than 0.03 (g) are not shown.

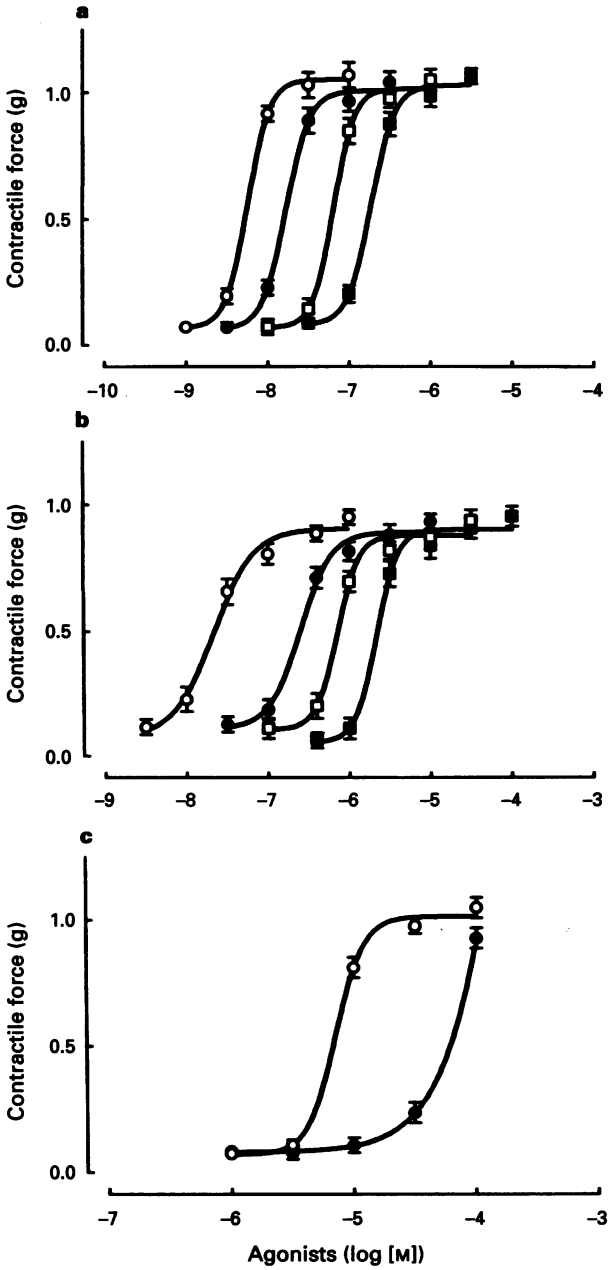


**Figure 2** Concentration-response curves for the contractile effects of (a) angiotensin II, (b) angiotensin III and (c) angiotensin IV in rat aortic ring preparations, in the absence (○) and presence of losartan (●, 10 nM; □, 100 nM and ■, 300 nM). Results are expressed as the absolute values (g); means±s.e.mean ($n=8$), s.e.mean values lower than 0.03 (g) are not shown.

**Table 1** Maximal contractile force ($E_{max}$), $pD_2$ and slope values of the CRCs for the contractile effects of angiotensin II (AII), AIII and AIV in rat aortic ring preparations

| *Agonist* | n | $E_{max}$ (g) | *$pD_2$* | *Slope* |
|---|---|---|---|---|
| AII | 20 | 1.00±0.05 | 8.25±0.03 | 3.09±0.22 |
| AIII | 20 | 0.92±0.04 | 7.69±0.08* | 1.90±0.29* |
| AIV | 12 | 0.99±0.03 | 5.19±0.07* | 3.57±0.28 |

Data are shown as means±s.e.mean.
*Significant difference from AII group ($P<0.01$).

tion-independent leftward shifts of AIII CRCs with increases in the maximal response. No significant changes occurred in the CRCs for AIII in the presence of the lower concentration (0.01 μM) of PD123177 (data not shown), whereas at the concentration of 0.1 μM, PD123177 significantly ($P<0.01$) shifted the AIII CRCs to the left and increased the maximal responses by 14.7±1.9%, compared with AIII curves generated from the vehicle-treated preparations (Figure 3). The $pD_2$ values amounted to 8.08±0.05. Higher concentrations of PD123177 (≥1 μM) did not cause further leftward shifts and increases of the maximal response of the AIII curves. The $pD_2$ values of the CRCs for AIII in the presence of PD123177 (1 μM) amounted to 7.96±0.04 ($P<0.05$) and the maximal response increased by 9.1±2.1% compared with the vehicle (Figure 3).

### *Influence of the endothelium on the contractile effects of AII, AIII and AIV*

In endothelium-intact aortic ring preparations, AII (1–100 nM), AIII (3 nM–1 μM) and AIV (1–100 μM) caused concentration-dependent increases in contractile force (Figure 4). In endothelium-denuded aortic ring preparations, the CRCs for AII, AIII and AIV were significantly shifted to the left and the maximal responses were substantially enhanced (Figure 4; $P<0.01$). Table 2 shows the calculated $pD_2$ and $E_{max}$ values of the various curves.

### *Effects of an NO synthesis inhibitor (L-NMMA) on AII, AIII and AIV induced contractions in endothelium-intact aortic preparations*

L-NMMA (0.1 mM) significantly enhanced the contractile effects caused by AII, AIII and AIV in endothelium-intact aortic rings (Figure 4; $P<0.01$). The $pD_2$ and $E_{max}$ values shown in Table 2 illustrate the significant leftward shifts of the CRCs and the increased maximal responses. The basal tone of the preparations remained unchanged by L-NMMA.

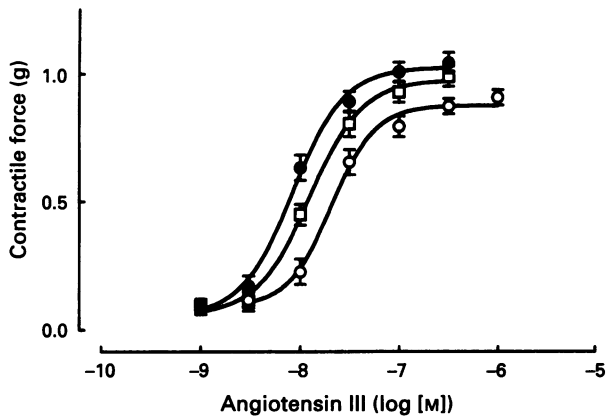


**Figure 3** Concentration-response curves for the contractile effects of angiotensin III in rat aortic ring preparations in the absence (○) and presence of (●) 0.1 μM and (□) 1 μM PD123177. Results are expressed as the absolute values (g); means±s.e.mean ($n=8$), s.e.mean values lower than 0.03 (g) are not shown.

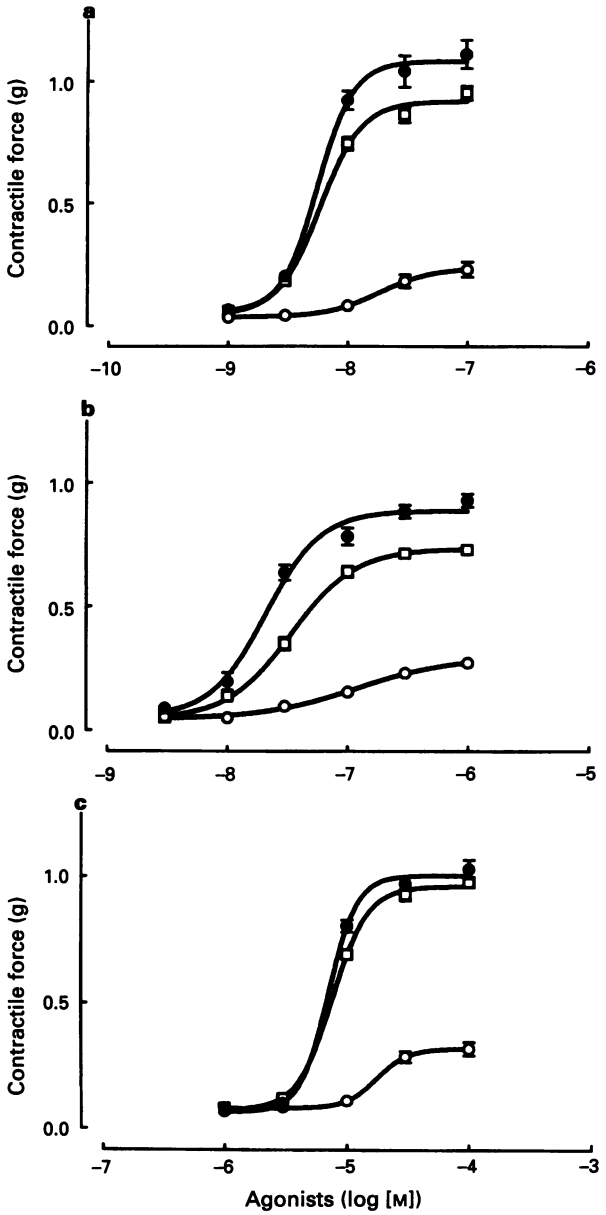


**Figure 4** Concentration-response curves for the contractile effects of (a) angiotensin II, (b) angiotensin III and (c) angiotensin IV in rat aortic ring preparations in the presence (○), absence (●) of endothelium, and in the presence (□) of endothelium and $N^G$-monomethyl-L-arginine (L-NMMA) (0.1 mM). Points represent the absolute values (g); means±s.e.mean ($n=6$), s.e.mean values lower than 0.03 (g) are not shown.

**Table 2** Maximal contractile force ($E_{max}$) and $pD_2$ values of the CRCs for the contractile effects of angiotensin II AII, AIII and AIV in endothelium-intact and -denuded aortic ring preparations and in preparations pretreated with L-NMMA (0.1 mM)

| | *AII* | | *AIII* | | *AIV* | |
|---|---|---|---|---|---|---|
| | $pD_2$ | $E_{max}$ (g) | $pD_2$ | $E_{max}$ (g) | $pD_2$ | $E_{max}$ (g) |
| +endothelium | 7.74±0.02 | 0.23±0.03 | 6.93±0.10 | 0.27±0.02 | 4.76±0.03 | 0.32±0.03 |
| −endothelium | 8.25±0.05* | 1.11±0.06* | 7.68±0.09* | 0.93±0.03* | 5.15±0.05* | 1.03±0.04* |
| +endothelium +L-NMMA (0.1 mM) | 8.23±0.08* | 0.95±0.03* | 7.47±0.02* | 0.73±0.02* | 5.12±0.03* | 0.98±0.02* |

Data are shown as means±s.e.mean ($n=6$).
*Significant differences from '+endothelium' group ($P<0.01$).

### *Tachyphylaxis induced by AII, AIII and AIV*

In endothelium-intact aortic preparations AII, AIII and AIV did not provoke significant vasoconstriction when administered cumulatively 1 h after the first CRC had been constructed (data not shown). In contrast, in endothelium-denuded aortic ring preparations AII, AIII and AIV caused a concentration-dependent increase in contractile force after the first CRC had been constructed. The $E_{max}$ values of the second CRCs for AII achieved only 50.7±2.1% of that of the first one, whereas for AIII and AIV the corresponding values were 90.9±2.5% and 98.6±2.2% of that of the first one, respectively. The calculated $pD_2$ values of the second curves for AII, AIII and AIV amounted to 7.98±0.06, 7.03±0.03 and 4.82±0.05, respectively (Figure 5).

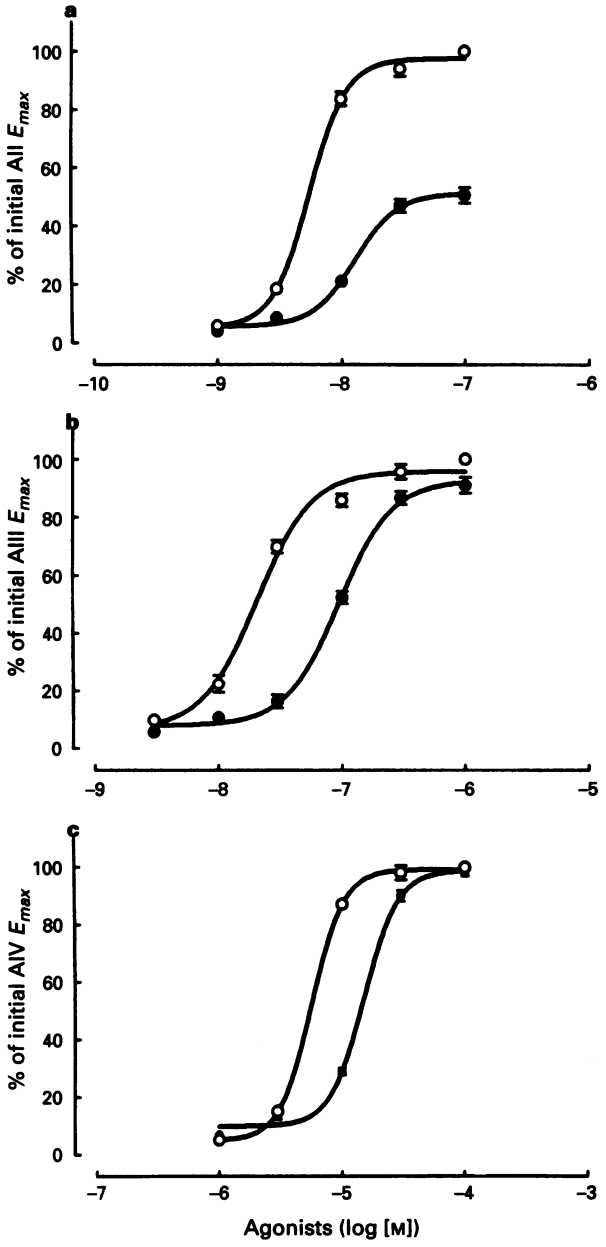


**Figure 5** First (○) and second (●) CRCs for the contractile effects of (a) angiotensin II, (b) angiotensin III and (c) angiotensin IV in endothelium-denuded aortic preparations, established with an interval of 1 h. The increase in force was expressed as a % of the initial maximal responses; means±s.e.mean ($n$=8), s.e.mean values lower than 3% are not shown.

## Discussion

Both AIII and AIV are known to be important degradation products of AII. They naturally occur in substantial concentrations in various species (Peach, 1977). Quantitative data on pharmacological properties of these two degradation products are scarce and controversial, and we therefore investigated various effects of these two peptides under carefully standardized conditions, and compared them with those of their precursor, AII.

AIII and AIV each elicited concentration-dependent contractions of rat aortic rings, with a similar maximal effect as AII, indicating a similar efficacy of these three peptides in this preparation. Similar observations have been obtained in several other species and tissues (Pendleton *et al.*, 1991; Robertson *et al.*, 1992; Cheng *et al.*, 1994; Nossaman *et al.*, 1995). AIII proved only 3–4 times less potent than AII on the basis of $pD_2$ values. Similar findings have been obtained in rat uterus (Moore *et al.*, 1976), but in rabbit aortic strips AIII proved to be approximately 40 times less potent than AII (Robertson *et al.*, 1992). We found AIV to be a thousand times less potent than AII, although studies in the pulmonary vascular bed of cat and rat (Cheng *et al.*, 1994; Nossaman *et al.*, 1995) have indicated a smaller difference in potency between the two peptides. Thus, it seems that the variations of compared potency may occur either in the same tissue of different species or in different tissues of the same species. These differences may be due to variations in receptor reserve, efficacy of coupling and possible receptor heterogeneity. Several studies have demonstrated that the vasoconstrictor responses to AII vary in different regional beds and among vascular smooth muscle preparations (Papadimitriou & Worcel, 1974; Toda & Miyazaki, 1978; Gruetter *et al.*, 1988). Such differences may result from local AII formation, noradrenaline release and differences in the properties of AII receptors (Oliver & Sciacca, 1984; Thorin & Atkinson, 1994). It has also been shown (Chen *et al.*, 1995) that the vasoconstrictor response to AII in rat aorta only involves the activation of $AT_1$-receptors located on vascular smooth muscle cells, whereas the responses in mesenteric artery and tail artery involve the stimulation of both vascular and endothelial $AT_1$-receptors. So far, the details concerning direct and indirect mechanisms involved in the vascular effects of AIII and AIV remain unclear.

Several studies have indicated the influence of endogenous protease activity on the CRCs for angiotensin peptides (Robertson *et al.*, 1992; Fujimoto *et al.*, 1992). We observed that the slope of AIII CRCs was shallower than that of the AII curves, although no significant difference between those of AIV and AII was found. This difference might for instance be explained by rapid enzymatic degradation of AIII (Robertson *et al.*, 1992). Accordingly, the potency of AIII could be slightly underestimated in the present study. Since the possible effects of endogenous protease and effective protease inhibition on CRCs for AIV so far remain unknown, it is uncertain whether the potency of AIV may be underestimated in a similar manner.

The newly developed nonpeptide angiotensin antagonists have led to the identification of at least two different AT-receptor populations: the angiotensin $AT_1$- and $AT_2$-receptor subtypes (Chiu *et al.*, 1989; Timmermans *et al.*, 1993). Antagonists selective for $AT_1$- or $AT_2$-receptors have revealed that the vast majority of the actions of AII, including vasoconstriction, appear to be mediated by the $AT_1$-receptor, whereas the functional relevance of the $AT_2$-receptor remains poorly defined (Smith *et al.*, 1992; Timmermans *et al.*, 1993). However, a few recent studies indicate that $AT_2$-receptors mediate vasodilatation in certain central and peripheral vessels (Brix & Haberl, 1992; Scheuer & Perrone, 1993), and others suggest the possible participation of $AT_2$-receptor in functions previously ascribed to the $AT_1$-receptor. For instance, the stimulation of the $AT_2$-receptor may inhibit the natriuretic responses to blood pressure increases (Lo *et al.*, 1995) or blunt the vasopressor responses to the angiotensin peptides (Nossaman *et al.*, 1995).

Our analysis with losartan, a selective $AT_1$-receptor antagonist (Timmermans *et al.*, 1991) and PD123177, a selective $AT_2$-receptor antagonist (Chiu *et al.*, 1989) indicates that the contractile effects of AII, AIII and AIV are mediated by $AT_1$-receptors. Interestingly, $AT_2$-receptors may be involved, although to a modest degree, in the effects of AIII, as indicated by the leftward shift and increased $E_{max}$ of CRCs by PD123177 (0.1 and 1 $\mu$M). This presumption is substantiated by two other observations in the present study. Firstly, the slope of the CRCs for AIII was significantly less steep than that of AII and AIV. Secondly, the slope of the Schild plot concerning the influence of losartan on AIII curves was significantly lower than unity. These results indicate that the contraction induced by AIII is not merely mediated by $AT_1$-receptors. It may therefore be hypothesized that $AT_2$-receptors are involved in the contractile process triggered by AIII, thus acting as an inhibitory factor in the $AT_1$-receptor mediated contraction. This hypothesis is supported by a recent finding showing that PD123319, a structural analogue of the $AT_2$-receptor antagonist used in this study, enhances the pressor responses to angiotensin peptides in the isolated blood perfused lung of the rat (Nossaman *et al.*, 1995).

Unlike rabbit aorta, adult rat aorta contains a relatively high density (28% ~ 36%) of $AT_2$-receptors, although the expression of $AT_2$-receptors is dramatically decreased in most organs just after birth (Viswanathan *et al.*, 1991; Chang & Lotti, 1991). This may explain why in rabbit aorta or in *in vivo* preparations of the rat (Abdelrahman & Pang, 1992), no stimulation of $AT_2$-receptors by AIII could be demonstrated. Binding experiments indicate that AII has a greater affinity for the $AT_1$-receptor than does AIII, whereas the affinities of the two peptides for the $AT_2$-receptor are roughly equivalent (Whitebread *et al.*, 1989; Bumpus *et al.*, 1991). It may thus be possible that the $AT_1$-receptor-mediated contractile response by AII more effectively masks the $AT_2$-receptor-mediated inhibition than the response by AIII. Higher concentrations ($\geqslant 1$ $\mu$M) of PD123177 neither caused a further leftward shift nor an increase in $E_{max}$ of the AIII curves. This finding may be explained by the fact that high concentrations of PD123177 may also block $AT_1$-receptors (Timmermans *et al.*, 1993). So far it cannot be decided whether signal transduction may be involved as well.

Specific binding sites for AIV have been identified in several tissues and species (Miller-Wing *et al.*, 1993; Harding *et al.*, 1994), but there are no reasons to believe that the contractile effects of AIV in rat aortae are mediated by other, so far unknown receptors rather than by $AT_1$-receptors.

AII-induced contractions are counteracted by the endothelium in various tissues and species (Gruetter *et al.*, 1988; van Heiningen & Van Zwieten, 1993; Zhang *et al.*, 1994). In the present study, we also found that destruction of the endothelium markedly enhanced AII, AIII and AIV-induced contractions, and the spasmogenic threshold concentrations of AII and its degradation products proved lower than in intact preparations, indicating an increase in tissue sensitivity and responsiveness to all three angiotensin peptides. It seems likely that the endothelium-induced inhibition of AII effects is mediated by the endogenous vasodilator endothelium derived relaxing factor (EDRF; Gruetter *et al.*, 1988; Zhang *et al.*, 1995). EDRF is assumed to be identical with NO or a closely related compound (Palmer *et al.*, 1987) and vascular endothelial cells are known to synthesize NO (Palmer *et al.*, 1988a). In the present study, by using L-NMMA, an inhibitor of endothelial NO synthesis (Palmer *et al.*, 1988b; Rees *et al.*, 1990) without antagonistic activity on muscarinic receptors (Buxton *et al.*, 1993), the contractile responses to AII, AIII and AIV in endothelium-intact preparations were significantly enhanced. This finding demonstrates that NO is likely to play an important role in the inhibition of AII-, AIII- and AIV-induced contractions in rat aorta, but it does not necessarily indicate that angiotensin peptides stimulate the release of NO from the endothelium.

After construction of the first CRC in endothelium-intact rat aortic ring preparations, subsequent CRCs for AII, AIII and IV could virtually not be obtained. However, when the endothelium had been destroyed, AII, AIII and AIV caused significant concentration-dependent increases in contractile force after the first CRC had been obtained. It has been shown previously that the presence of endothelium markedly enhances the development of tachyphylaxis to AII and AIII (Gruetter *et al.*, 1987). Our findings are the first demonstration of endothelium-enhanced tachyphylaxis to AIV in rat aorta. In endothelium-denuded rat aortic ring preparations, the $E_{max}$ value of the second CRC for AII was much less than that of AIII and AIV, indicating that the endothelium appears to be less influential on AII-induced tachyphylaxis. Although two mechanisms (endothelium-dependent and endothelium-independent) may be involved in the development of the tachyphylaxis to angiotensin (Gruetter *et al.*, 1987), the details remain unclear.

In separate experiments, after the first CRC of AII or AIII had been obtained in endothelium-denuded preparations, 10 nM losartan or 1 $\mu$M PD123177 were added and then the second CRC was constructed. It was found that the maximal responses of the second curves were decreased in the presence of either antagonist. Losartan significantly shifted the second curves to the right, whereas PD123177 had no effect on these curves, compared with the vehicles (data not shown). These data, taken together with other results in this study, suggest that the contractile response to angiotensin peptides is mediated by $AT_1$-receptors and that angiotensin-induced tachyphylaxis may involve the down-regulation of $AT_1$-receptors.

It has been suggested (Kuttan & Sim, 1993) that tachyphylaxis induced by AII is associated with changes at the receptor level, involving changes in both affinity and coupling efficiency. Modulating factors which influence these parameters can be released from the endothelium or from the smooth muscle cell. Tachyphylaxis may involve the activation of such factors and uncoupling of the receptor-signal transduction system, resulting in a reduction in receptor affinity and tissue response (Kuttan & Sim, 1991). Therefore, we presume that the tachyphylaxis induced by AII, AIII and AIV in the presence of endothelium is probably triggered by the binding of these peptides to the AT-receptor, thus initiating the activation of the modulator derived from the endothelium (endothelium-dependent mechanism).

In 1989, Oshiro *et al.* hypothesized the existence of a regulatory site (tachyphylactic site) which is different from the agonistic site on the AT-receptors, whereas only those angiotensin peptides that bind to the receptor and interact with the regulatory site may be expected to be subject to tachyphylaxis. Boulay *et al.* (1992) demonstrated the existence of an allosteric site modulating the affinity of AII for its $AT_1$-receptor, whilst Shimuta *et al.* (1993) showed that a $Na^+$-dependent regulatory site on the $AT_1$-receptor was associated with the tachyphylaxis induced by AII. The present findings suggest that when AII, AIII and AIV bind to the $AT_1$-receptor to cause contraction, AII also interacts with the tachyphylactic site of the receptor and hence induces down-regulation of $AT_1$-receptors, whereas AIII and AIV do not. This mechanism is endothelium-independent. Our results suggest that the endothelium-dependent mechanism appears to be involved more clearly in AIII- and AIV-induced tachyphylaxis, whereas an important trigger of AII-induced tachyphylaxis may occur at the level of the smooth muscle and may be endothelium-independent.

In conclusion, the present study has demonstrated that AIII and AIV cause significant vasoconstrictor effects in the rat isolated thoracic aorta with similar efficacy, although they are less potent than AII. The contractile responses to AIII and AIV are both mediated by angiotensin $AT_1$ receptors, although $AT_2$-receptors may play a moderate role in AIII-induced contractions as well. Destruction of the endothelium or inhibition of NO synthesis significantly enhanced the responses to AII, AIII and AIV in this preparation. The well known tachyphylaxis for AII is enhanced by the endothelium, and similar findings were obtained for AIII and AIV. However, after destruction of the endothelium, AII still showed substantial tachyphylaxis, whereas AIII and AIV did not.

## References


ABDELRAHMAN, A. & PANG, C.C. (1992). Competitive antagonism of pressor responses to angiotensin II and angiotensin III by the angiotensin II-1 receptor ligand losartan. *Can. J. Physiol. Pharmacol.*, **70**, 716–719.

ABHOLD, R.H. & HARDING, J.W. (1988). Metabolism of angiotensin II and III by membrane-bound peptidase from rat brain. *J. Pharmacol. Exp. Ther.*, **245**, 171–177.

AHMAD, S & WARD, P.E. (1990). Role of aminopeptidase activity in the regulation of the pressor activity of circulating angiotensins. *J. Pharmacol. Exp. Ther.*, **252**, 643–650.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BOULAY, G., SERVANT, G., LUONG, T.T., ESCHER, E. & GUILLEMETTE, G. (1992). Modulation of angiotensin II binding affinity by allosteric interaction of polyvinyl sulfate with an intracellular domain of the DuP753 sensitive angiotensin II receptor of bovine adrenal glomerulose. *Mol. Pharmacol.*, **41**, 809.

BRIX, J. & HABERL, R.J. (1992). The $AT_2$-receptor mediates endothelium-dependent dilation of rat brain arterioles (Abstract). *FASEB J.*, **6**, A1264.

BUMPUS, F.M., CATT, K.J., CHIU, A.T., DEGASPARO, M., GOODFRIEND, T., HUSAIN, A. & PEACH, M.J. (1991). Nomenclature for angiotensin receptors: a report of the nomenclature committee of the council for high blood pressure research. *Hypertension*, **17**, 720–721.

BUXTON, I.L.O., CHEEK, D.J., ECKMAN, D., WESTFALL, D.P., SANDERS, K.M. & KEEF, K.D. (1993). $N^G$-nitro-L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ. Res.*, **72**, 387–395.

CHANG, R.S.L. & LOTTI, V.J. (1991). Angiotensin receptor subtypes in rat, rabbit and monkey tissues: relative distribution and species dependency. *Life Sci.*, **49**, 1485–1490.

CHEN, L., MCNEILL, J.R., WILSON, T.W. & GOPALAKRISHNAN, V. (1995). Heterogeneity in vascular smooth muscle responsiveness to angiotensin II: role of endothelin. *Hypertension*, **26**, 83–88.

CHENG, D.Y., DEWITT, B.J., DENT, E.L., NOSSAMAN, B.D. & KADOWITZ, P.J. (1994). Analysis of responses to angiotensin IV in the pulmonary vascular bed of the cat. *Eur. J. Pharmacol.*, **261**, 223–227.

CHIU, A.T., HERBLIN, W.F., ARDECKY, R.J., MCCALL, D.E., CARINI, D.J., DUNCIA, J.V., WEXLER, R.R., WONG, P.C., JOHNSON, A.L. & TIMMERMANS, P. B.M.W.M. (1989). Identification of angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.*, **165**, 196–203.

COLEMAN, J.K.M., KREBS, L., ONG, B., HANESWORTH, J.M., LAWRENCE, K.A., SARDINIA, M. & HARDING, J.W. (1995). Changes in renal blood flow due to intra-arterial infusions of angiotensin II(3-8) [AIV] and AIV analogs (Abstract). *FASEB J.*, **7**, A437.

FITZSIMONS, J.T. (1971). The effect on drinking of peptide precursors and of shorter chain peptide fragments of angiotensin II injected into the rat's diencephalon. *J. Physiol.*, **214**, 295–303.

FUJIMOTO, M., MIHARA, S., SHIGERI, Y. & ITAZAKI, K. (1992). Possible implication of peptidase activity in different potency of angiotensins II and III for displacing [$^{125}$I]angiotensin II binding in pig aorta. *Eur. J. Pharmacol.*, **215**, 259–264.

GRUETTER, C.A., RYAN, E.T., LEMKE, S.M., BAILLY, D.A., FOX, M.K. & SCHOEPP, D.D. (1988). Endothelium-dependent modulation of angiotensin II-induced contraction in blood vessels. *Eur. J. Pharmacol.*, **146**, 85–95.

GRUETTER, C.A., RYAN, E.T. & SCHOEPP, D.D. (1987). Endothelium enhances tachyphylaxis to angiotensins II and III in rat aorta. *Eur. J. Pharmacol.*, **143**, 139–142.

HABERL, R.L., DECKER, P.J. & EINHAUPL, K.M. (1991). Angiotensin degradation products mediate endothelium-dependent dilation of rabbit brain arterioles. *Circ. Res.*, **68**, 1621–1627.

HANESWORTH, J.M., SARDINIA, M.F., KREBS, L.T., HALL, K.L. & HARDING, J.W. (1993). Elucidation of a specific binding site for angiotensin II(3-8), angiotensin IV, in mammalian heart membranes. *J. Pharmacol. Exp. Ther.*, **266**, 1036–1042.

HARDING, J.W., WRIGHT, J.W., SWANSON, G.N., HANESWORTH, J.M. & KREBS, L.T. (1994). AT(4) receptors: specificity and distribution. *Kidney Int.*, **46**, 1510–1512.

KUTTAN, S.C. & SIM, M.K. (1991). Isolation and purification of the putative EDMF from the rabbit aortic endothelium (Abstract). *Pharmacologist*, **33**, P470.

KUTTAN, S.C. & SIM, M.K. (1993). Angiotensin II-induced tachyphylaxis in aortas of normo- and hypertensive rats: changes in receptor affinity. *Eur. J. Pharmacol.*, **232**, 173–180.

LO, M., LIU, K.L., LANTELME, P. & SASSARD, J. (1995). Subtype 2 of angiotensin II receptors controls pressure-natriuresis in rats. *J. Clin. Invest.*, **95**, 1394–1397.

MILLER-WING, A.V., HANESWORTH, J.M., SARDINIA, M.F., HALL, K.L., WRIGHT, J.W., SPETH, R.C., GROVE, K.L. & HARDING, J.W. (1993). Central angiotensin IV binding sites: distribution and specificity in guinea pig brain. *J. Pharmacol. Exp. Ther.*, **266**, 1718–1726.

MOORE, A.F., HALL, M.M. & KHAIRALLAH, P.A. (1976). A comparison of the effects of angiotensin II and heptapeptide on smooth muscle (vascular and uterine). *Eur. J. Pharmacol.*, **39**, 101–107.

MURPHY, T.J., NAKAMURA, Y., TAKEUCHI, K. & ALEXANDER, R.W. (1993). A cloned angiotensin receptor isoform from the turkey adrenal gland is pharmacologically distinct from mammalian angiotensin receptors. *Mol. Pharmacol.*, **44**, 1–7.

NOSSAMAN, B.D., FENG, C.J., KAYE, A.D. & KADOWITZ, P.J. (1995). Analysis of responses to ANG IV: effects of PD-123319 and DuP-753 in the pulmonary circulation of the rat. *Am. J. Physiol.*, **268**, L302–L308.

OLIVER, J.A. & SCIACCA, R.R. (1984). Local generation of angiotensin II as a mechanism of regulation of peripheral vascular tone in the rat. *J. Clin. Invest.*, **74**, 1247–1251.

OSHIRO, M.E.M., SHIMUTA, S.I., PAIVA, T.B. & PAIVA, A.C.M. (1989). Evidence for a regulatory site in the angiotensin II receptor of smooth muscle. *Eur. J. Pharmacol.*, **166**, 411–417.

PALMER, R.M., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PALMER, R.M., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

PAPADIMITRIOU, A. & WORCEL, M. (1974). Dose-response curves for angiotensin II and synthetic analogues in three types of smooth muscle: existence of different forms of receptor sites for angiotensin II. *Br. J. Pharmacol.*, **50**, 291–297.

PEACH, M.J. (1977). Renine-angiotensin system: Biochemistry and mechanism of action. *Physiol. Rev.*, **57**, 313–369.

PENDLETON, R.G., GESSNER, G. & HORNER, E. (1991). Comparative effects of angiotensin II and angiotensin III in rabbit adrenal and aortic tissues. *J. Pharmacol. Exp. Ther.*, **256**, 614–620.

REES, D.D., PALMER, R.M., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

ROBERTSON, M.J., CUNOOSAMY, M.P. & CLARK, K.L. (1992). Effects of peptidase inhibition on angiotensin receptor agonist and antagonist potency in rabbit isolated thoracic aorta. *Br. J. Pharmacol.*, **106**, 166–172.

SCHEUER, D.A. & PERRONE, M.H. (1993). Angiotensin type 2 receptors mediate depressor phase of biphasic pressure response to angiotensin. *Am. J. Physiol.*, **264**, R917–R923.

SHIMUTA, S.I., KANASHIRO, C.A., FERREIRA, A.T., OSHIRO, M.E., PAIVA, T.B. & PAIVA, A.C. (1993). Role of $Na^+$ and protein kinase C in angiotensin desensitization and tachyphylaxis in the guinea-pig ileum. *Naunyn Schmied. Arch. Pharmacol.*, **347**, 425–431.

SIM, M.K. & RADHAKRISHNAN, R. (1994). In vivo study of angiotensin II tachyphylaxis in freely moving normo- and hypertensive rats. *Pharmacol. Toxicol.*, **74**, 223–227.

SMITH, R.D., CHIU, A.T., WONG, P.C., HERBLIN, W.F. & TIMMERMANS, P.B. (1992). Pharmacology of nonpeptide angiotensin II receptor antagonists. *Annu. Rev. Pharmacol. Toxicol.*, **32**, 135–165.

THORIN, E. & ATKINSON, T. (1994). Modulation by the endothelium of sympathetic vasoconstriction in an *in vitro* preparation of the rat tail artery. *Br. J. Pharmacol.*, **111**, 351–357.

TIMMERMANS, P.B., WONG, P.C., CHIU, A.T. & HERBLIN, W.F. (1991). Nonpeptide angiotensin II receptor antagonists. *Trends Pharmacol. Sci.*, **12**, 55–59.

TIMMERMANS, P.B.M.W.M., WONG, P.C., CHIU, A.T., HERBLIN, W.F., BENFIELD, P., CARINI, D.J. & LEE, R.J. (1993). Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol. Rev.*, **45**, 205.

TODA, N. & MIYAZAKI, M. (1978). Regional and species differences in the response of isolated arteries to angiotensin II. *Jpn. J. Pharmacol.*, **28**, 495–497.

TONNAER, J.A., WIEGANT, V.M., DE JONG, W. & DE WIED, D. (1982). Central effects of angiotensins on drinking and blood pressure: structure activity relationships. *Brain Res.*, **236**, 417–428.

VAN HEININGEN, P.N. & VAN ZWIETEN, P.A. (1993). Influence of the endothelium on $Ca^{2+}$ dependency of angiotensin II-induced contractions of rat aortic rings. *Fundam. Clin. Pharmacol.*, **7**, 227–233.

VISWANATHAN, M., TSUTSUMI, K., CORREA, F.M. & SAAVEDRA, J.M. (1991). Changes in expression of angiotensin receptor subtypes in the rat aorta during development. *Biochem. Biophys. Res. Commun.*, **179**, 1361–1367.

WHITEBREAD, S., MELE, M., KAMBER, B. & DEGASPARO, M. (1989). Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.*, **163**, 284–291.

ZHANG, J., PFAFFENDORF, M., ZHANG, J.S. & VAN ZWIETEN, P.A. (1995). Influence of the vascular endothelium on angiotensin II-induced contractions in rabbit renal artery. *Fundam. Clin. Pharmacol.*, **9**, 25–29.

ZHANG, J.S., VAN MEEL, J.C., PFAFFENDORF, M., ZHANG, J. & VAN ZWIETEN, P.A. (1994). Endothelium-dependent, nitric oxide-mediated inhibition of angiotensin II-induced contractions in rabbit aorta. *Eur. J. Pharmacol.*, **262**, 247–253.

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