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Angiotensin II-elicited signal transduction via AT_1 receptors in endothelial cells

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1 Angiotensin II (AII) actions are mediated by two distinct types of receptors: AT_1, which includes two subtypes, AT_{1A} and AT_{1B}, and AT_2. AII produces vasoconstriction on the vascular wall acting directly on smooth muscle cells via AT_1 receptors. AII receptors have recently been demonstrated on endothelial cells. But the pharmacological characteristics of these receptors and the intracellular signal pathways coupled to them remain unclear.

2 The aim of this work was to characterize the AII receptor subtypes in rat aortic endothelial cells (RAEC) in primary culture and to evaluate the signal pathways coupled to these receptors by measuring the activation of phospholipase C (PLC) and phospholipase A_2 (PLA_2).

3 Labelled AII bound to RAEC in a specific, saturable manner. Scatchard analysis showed a K_d of 1.87 ± 0.49 nM and a B_{max} of $50.2 \pm 10.9 \times 10^3$ sites per cell. AII was displaced by the AT_1-specific antagonist, DuP753 with a K_i of 17.37 ± 1.49 nM, but not by the AT_2 receptor analogues CGP42771B or PD123177. These data were confirmed by the finding of AT_1 mRNA in endothelial cells. Analysis of RNA expression by RT-PCR showed the presence of both subtypes, AT_{1A} and AT_{1B}, in endothelial cells, whereas smooth muscle cells express only AT_{1A}.

4 The activation of PLC and PLA_2 in response to AII was evaluated by measuring inositol phosphate production and arachidonic acid release, respectively. Both were enhanced by AII in a dose-dependent manner, and inhibited by DuP753, but not by PD123177.

5 We conclude that AT_1 receptors are expressed by endothelial cells in primary culture and that phospholipase C and phospholipase A_2 are activated via this receptor.

Keywords: Angiotensin II; endothelial cells; angiotensin II receptors; angiotensin II receptor antagonists; phospholipase C; phospholipase A_2

Introduction

The renin-angiotensin system plays an important role in the regulation of blood pressure and body fluid homeostasis (Peach *et al.*, 1977). This system includes the cleavage of angiotensinogen to angiotensin I by renin and the generation of angiotensin II (AII) by the angiotensin converting enzyme both in plasma and in the tissues in the neighbourhood of its target cells (Danser *et al.*, 1995). AII, the main effector of the system, acts in several tissues, including vascular beds, adrenals, kidney and brain.

The actions of AII are mediated by two pharmacologically and biochemically distinct classes of receptors, AT_1 and AT_2. Both of them belong to the seven-transmembrane domain receptor family. While the physiological role and the intracellular signalling pathways of the AT_2 receptor are not clearly defined (Timmermans *et al.*, 1993), AT_1 receptors have been extensively studied. They are responsible for most of the known physiological actions of AII, via interactions with a G-protein which activates phospholipase C (PLC) (Morrero *et al.*, 1994). AII also stimulates phospholipase A_2 (PLA_2), leading to the release of arachidonate and its metabolic products (Ford & Gross, 1989). Genome analyses and homology cloning demonstrated that there are two subtypes of AT_1 receptors, AT_{1A} and AT_{1B}, which are encoded by two different genes in the rat (Lewis *et al.*, 1993). They have very similar amino acid sequences, pharmacological specificities and signal transductions (Chiu *et al.*, 1993). It is currently unknown whether the two subtypes have different specific functions, but they have different patterns of expression in tissues (Gasc *et al.*, 1994; Llorens-Cortes *et al.*, 1994), suggesting that they could mediate different physiological functions.

One of the principal targets of AII is the vascular wall, where it produces vasoconstriction. This effect is mediated by a direct action of AII on smooth muscle cells via the AT_1 receptor (Whitebread *et al.*, 1989; Chiu *et al.*, 1990). But, AII could interact with another vascular cell type, the endothelium and modulate its own effect on smooth muscle cells. Gimbrone & Alexander (1975) reported twenty years ago that AII caused the release of prostaglandins by cultured endothelial cells, but the presence of AII receptors in endothelial cells has only recently been observed (Feener *et al.*, 1995; Vaughan *et al.*, 1995; Stoll *et al.*, 1995). The pharmacological characteristics of these receptors are controversial and the intracellular signal pathways coupled to the receptors in these cells have not been evaluated.

This work was therefore undertaken to characterize the AII receptor subtypes in rat aortic endothelial cells (RAEC) in primary culture, by binding assays and RNA analysis. The signal pathways coupled to these receptors were also examined by evaluating the activation of PLC and PLA_2, in terms of inositol phosphate (IP) production and arachidonic acid (AA) release, respectively.

Methods

Cell culture

Endothelial cells (RAEC) and smooth muscle cells (RSMC) were isolated from rat aorta as previously described (Battle *et al.*, 1994). Briefly, the thoracic aorta were excised and rinsed.

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Fat was removed and the collateral vessels cut off. The adventitia was removed and the remaining media plus intima were sliced into fine rings and incubated in DMEM containing collagenase (1248 iu ml^{-1}) for 40 min at 37°C. The rings were flushed and filtered to dislodge the endothelial cells. The cell suspension was centrifuged, resuspended in cultured medium, and plated for 40 min in a plastic flask coated with rat fibronectin. This short period allowed only endothelial cells to adhere. RMSC were isolated by placing the aortic rings in a second enzymatic bath containing collagenase (1248 iu ml^{-1}) and elastase (17.5 iu ml^{-1}) for 60 min at 37°C. The cell suspension was centrifuged, resuspended in culture medium, and plated out in plastic flasks coated with 0.1% collagen. Confluent RAEC were detached with trypsin/EDTA, and propagated in DMEM supplemented with 15% horse serum, 5% foetal calf serum, 75 $\mu g\ ml^{-1}$ ECGS, 20 mM HEPES, 2 mM glutamin, 50 iu ml^{-1} penicillin, and 50 $\mu g\ ml^{-1}$ streptomycin. RSMC were cultured in DMEM supplemented with 15% foetal calf serum. Cells from passages 1-5 were used in these studies.

The purity of the cell preparations was verified by immunostaining with a specific rat endothelial cell antibody (Duijuvestin *et al.*, 1992) and with a monoclonal antibody for smooth muscle α-actin.

Binding and displacement assays of AII receptors in RAEC

Sarcosine^1-AII was labelled by the chloramine-T method (Freedlender & Goodfriend, 1979). Monoiodated [^{125}I]-(Sar^1)-AII (2000 Ci $mmol^{-1}$) was purified by h.p.l.c. RAEC were grown to confluence in 24-well plates. For saturation binding assays, cells were incubated for 1 h at 22°C with various concentrations of [^{125}I]-(Sar^1)AII in 50 mM Tris-HCl, 6.5 mM $MgCl_2$, 125 mM NaCl, 1 mM EDTA, 20 mM HEPES and 1 mg ml^{-1} BSA, pH 7.4. The cells were then washed with buffer solubilized in 0.5 ml 1N NaOH and counted. For competitive binding assays, cells were incubated in the same buffer with 0.5 nM [^{125}I]-(Sar^1)AII and various concentrations of competing ligands: the AT_1-specific antagonist DuP 753 or the AT_2 receptor pseudopeptidic or non-peptidic compounds CGP42112B and PD123177. Non-specific binding was determined in the presence of 10^{-6} M AII. Each experiment was carried out in duplicate. Binding data were analysed with a non-linear least-squares curve fitting procedure (Ebda-Ligand, Elsevier-Biosoft, Cambridge, UK) (Munson & Rodbard, 1980).

RNA isolation and analysis by Northern blot and RT-PCR assays

Total RNA was isolated from confluent cultures of RAEC by the guanidine thyocyanate method (Chomczynski *et al.*, 1987). For Northern blot analysis, 20 μg of total RNA were separated in a 1% agarose gel. RNA was transferred by capillarity to a Hybond-N membrane, and cross-linked by ultraviolet irradiation. Agarose gel-purified cDNA probe for AT_{1A} (bases 37–724) were labelled with [α-^{32}P]-dCTP and purified on Nick-columns. Blots were hybridized overnight at 42°C, in 50% deionised formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1 × Denhardt's solution, 50 mM sodium phosphate (pH 6.5), 0.1% sodium dodecyl sulphate (SDS), and 250 $\mu g\ ml^{-1}$ denaturated salmon sperm DNA. The blots were then washed twice with 0.5 SSC and 0.1% SDS at room temperature for 15 min and with 0.1 SSC and 0.1% SDS at 42°C for 10 min and finally exposed to Hyperfilm-MP film at −80°C. Blots were rehybridized to a probe for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to compare the RNA loading in each lane.

PCR amplifications were performed as previously described (Llorens-Cortes *et al.*, 1994). Single-stranded cDNA was synthesized from 3 μg of total RNA in the presence of Moloney murine leukaemia virus reverse transcriptase and the reverse primers. Oligonucleotides primers were chosen from the homologous parts of the coding region of the rat AT_{1A} and AT_{1B} receptor genes; the antisense primer was 5'-GCA CAA TCG CCA TAA TTA TCC-3' and the sense primer was 5'-CAC CTA TGT AAG ATC GCT TC-3'. Digestion by *Eco* RI was used to differentiate between the AT_1 receptor subtypes: this digestion does not affect the AT_{1B} product, but hydrolyses that of AT_{1A} into two fragments. Therefore, the PCR products were digested by *Eco* RI (2000 u μl^{-1}) for 90 min at 37°C. The digestion efficiency was verified in each experiment by checking the complete digestion of an internal standard.

The sense primer for AT_2 amplification, contained the 23 bp of the AT_2 receptor sequence (5'-ATG AAG GAC AAC TTC AGT TTT GC-3'). The reverse primer contained the 22 bp of the AT_2 receptor sequence (5'-CAA GGG GAA CTA CAT AAG ATG C-3') (Shanmugam *et al.*, 1995). PCR products were separated on a 1.5% agarose gel for visual verification.

Determination of inositol phosphate production

RAEC were cultured to confluence in 24-well plates, and then labelled with 1.5 μCi/well [^{3}H]-*myo*-inositol in DMEM for 16-24 h. Cells were washed with medium and preincubated with 20 mM LiCl for 10 min, and then with different concentrations of AII (10^{-10} M – 10^{-6} M) for 30 min in the presence of 20 mM LiCl. In another set of experiments, antagonists were added to cells and incubated for 30 min in the presence of 10^{-8} M AII. After purification on a Dowex 1 × 8 anion exchange resin (Bio-Rad), the total radiolabelled IP fraction was measured.

Measurement of arachidonic acid release

Confluent RAEC cultured in 24-well plates were labelled with 0.5 μCi/well [^{3}H]-arachidonic acid in DMEM for 16–24 h. Cells were washed with medium and incubated with AII (10^{-10} M – 10^{-6} M) for 30 min. The type of receptor implicated in this activation was deduced by adding the selective antagonists for each receptor, DuP753 or PD 123177 to cells and incubating for 30 min in the presence of 10^{-8} M AII. After incubations, the buffer was collected and radioactivity determined.

Materials

[^{3}H]-*myo*-inositol (15 Ci $mmol^{-1}$), [^{3}H]-arachidonic acid (150 Ci $mmol^{-1}$), Hybond-N membrane and Hyperfilm-MP were purchased from Amersham (UK). [α-^{32}P]-deoxycytidine 5'-triphosphate (dCTP) was from DuPont (New England Nuclear). Dulbecco's modified Eagle's medium (DMEM), N - 2-hydroxyethyl - piperazine - N'-2-ethanesulphonicacid (HEPES), horse serum, and trypsin/EDTA were obtained from Boehringer Mannheim (Germany). AII, (Sar^1)AII, penicillin, streptomycin, collagen, endothelial growth cell supplement (ECGS) were from Sigma Chemical Co (St Louis, U.S.A.). Collagenase was from Eurobio (France). Rat endothelial cell antibody (RECA) was from Medac Diagnosika, Hamburg (Germany) and antibody against α-actin from Dako SA, Glostrup (Denmark). DuP753 was kindly provided by Merck-Sharp Dohme, CGP 42112B by Dr de Gasparo (Ciba-Geigy) and PD123177 by Dr P. Janiack (Servier). Primers for PCR amplification were a gift of Dr Llorens-Cortes.

Results

Binding and displacement assays of AII receptors in RAEC

These experiments were performed to detect AII receptors in RAEC and to determine their type. Binding assays showed a saturable, specific binding of [^{125}I]-(Sar^1)AII to RAEC. Analysis of saturation data (Figure 1) revealed a straight line ($r = 0.95$), which is consistent with a single class of binding site.

Scatchard analysis showed a K_d of 1.87 ± 0.49 nM and B_{max} of $50.2 \pm 10.9 \times 10^3$ sites per cell ($n=8$; Table 1). Binding was selectively inhibited by the AT_1-specific antagonist DuP753 with a K_i of 17.37 ± 1.49 nM, ($n=5$; Table 1). Both CGP42112B, and PD123177, failed to displace the [^{125}I]-(Sar^1)AII bound to RAEC, at concentrations below 10^{-6} M (Table 1). These data suggest that only AT_1 receptors are present on these cells. [^{125}I]-(Sar^1)AII bound to all preparations of RAEC, and this binding capacity persisted up to the 12th passage (data not shown). However, only first passage cultures (1–5) were used in subsequent experiments.

AT_1 and AT_2RNA in RAEC and RSMC

Total RNA was extracted from RAEC and RSMC. A representative autoradiogram derived from a Northern blot analysis is shown in Figure 2a. AT_1 mRNA was detected in both endothelial and smooth muscle cells. AT_2 mRNA was not detected by Northern blot in either RSMC or RAEC. The AT_1 receptor subtypes were identified by RT-PCR analyses with RNA from both cell types. The RT-PCR products were digested with *Eco* RI which hydrolyses AT_{1A} product into two fragments, allowing the differentiation between the AT_1 receptor subtypes. Specific mRNA transcripts for AT_1 receptor were detected in both cell types. Three fragments were obtained after *Eco* RI digestion of the amplification product of RAEC (Figure 2b): one 444 bp fragment corresponding to AT_{1B} and two 269 and 175 bp fragments, corresponding to AT_{1A}. Digestion of the amplification product of RSMC yielded only the two small fragments, showing exclusively the presence of AT_{1A} receptors in these cells (Figure 2b).

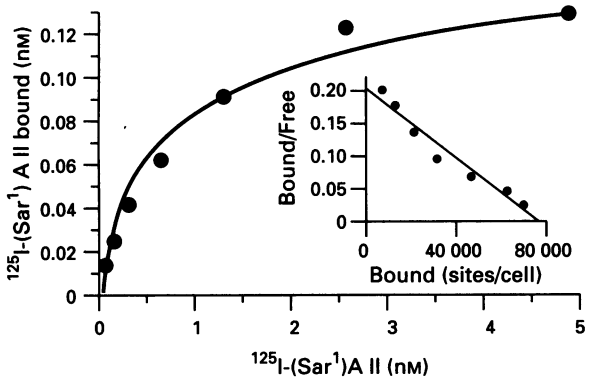

Figure 1 Specific binding of [^{125}I]-(Sar^1)AII to rat aortic endothelial cells (RAEC). Confluent cultures of RAEC in 24-well plates were incubated with different concentrations of labelled AII for 60 min. Non-specific binding was determined in the presence of 10^{-6} M unlabelled AII. Each point represents the average of two determinations in a representative experiment. Scatchard analysis of the data is shown in the inset.

Table 1 Pharmacological characteristics of AII receptors on rat aortic endothelial cells

A	*Binding parameters of [^{125}I]-AII*		
K_d (nM)		1.87 ± 0.49	(n = 7)
B_{max} (10^3 sites/cell)		50.2 ± 10.9	(n = 7)
B	*Affinities of receptors for AT-selective compounds*		
K_i (nM)	DuP753	17.37 ± 1.49	(n = 5)
	CGP42112B	8203 ± 1508	(n = 4)
	PD123177	>10000	(n = 4)

Data represented the means ± s.e.mean obtained from the indicated number of experiments (n) with each point being performed in duplicate.

Determination of inositol phosphate production

The effect of AII on IP production was dose-dependent over the range 10^{-10} M – 10^{-6} M (Figure 3), with an EC_{50} of 3.15 ± 1.46 nM. The IP production in response to 10^{-8} M AII was inhibited by the AT_1 antagonist, DuP753 in a dose-dependent manner and was completely abolished by 5×10^{-6} M DuP753. In contrast, the AT_2 antagonist, PD123177 did not reduce the angiotensin-induced IP production, suggesting that this effect is mediated only by the AT_1 receptor (Figure 4).

Measurement of arachidonic acid release

The effect of AII on PLA_2 activity was evaluated by measuring AA release in response to AII (Figure 5). This release was enhanced by AII in a dose-dependent manner, reaching 200% over basal values and having an EC_{50} of 2.71 ± 1.82 nM. The AA release in response to 10^{-8} M AII was inhibited by the AT_1 antagonist, DuP753 in a dose-dependent manner and was completely abolished at a DuP753 concentration of 5×10^{-6} M. In contrast, the AT_2 antagonist, PD123177, did not block the AII-induced release of AA, suggesting that this effect is mediated only by the AT_1 receptor (Figure 6).

Discussion

The results of this study showed that RAEC express AII receptors and that the affinity of these receptors ($K_d = 1$ nM) is similar to that reported for AT_1 receptors on vascular smooth muscle cells (McQueen *et al.*, 1984), cardiac fibroblasts (Cra-

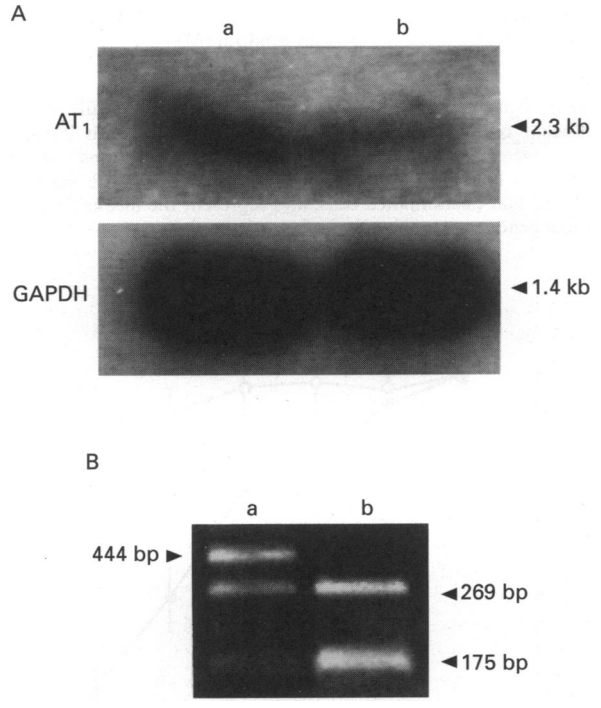

Figure 2 AT_1 receptor mRNA in rat aortic endothelial cells (RAEC) and rat smooth muscle cells (RSMC). Total RNA was extracted from cultured RAEC and RSMC. (A) Northern blot analysis by hybridisation with a specific AT_1-labelled oligonucleotide. The blots were rehybridized to a probe for the housekeeping gene GAPDH, to compare RNA loading in each lane. Similar mRNA was detected in RAEC (lane a) and in RSMC (lane b). (B) Evaluation of AT_1-receptor mRNA by RT-PCR amplification. PCR products were digested with *Eco* RI, electrophoresed on an agarose gel and visualized by adding ethidium bromide and photographing under u.v. light. RAEC (lane a) express both, AT_{1A} and AT_{1B} receptors, as there are three fragments: one of 444 bp corresponding to AT_{1B} and two of 269 and 175 bp corresponding to AT_{1A}. RSMC (lane b) express only the AT_{1A} subtype, as there are only two fragments.

bos *et al.*, 1994), liver (Gunther *et al.*, 1984) or kidney (Brown *et al.*, 1983). Binding assays did not detect AT_2 receptors in these cells.

AII receptors have been found in cultured endothelial cells (Patel *et al.*, 1989; Weimer *et al.*, 1993; Feener *et al.*, 1995; Vaughan *et al.*, 1995; Stoll *et al.*, 1995) but few studies have characterized these receptors and the results are rather heterogeneous. It has been reported that cultured endothelial cells express AT_1 receptors (Feener *et al.*, 1995), AT_2 (Wiemer *et al.*, 1993), both (Stoll *et al.*, 1995) or even a distinct form of AII receptor (Vaughan *et al.*, 1995). These different findings could be due to differences in the methods and mainly to the diversity of endothelial cell origins used in these studies. Indeed, endothelial cells were from different vascular beds (aorta, mesenteric, coronary or pulmonary arteries, brain microvessels) and/or from different species (cattle, pig or rat).

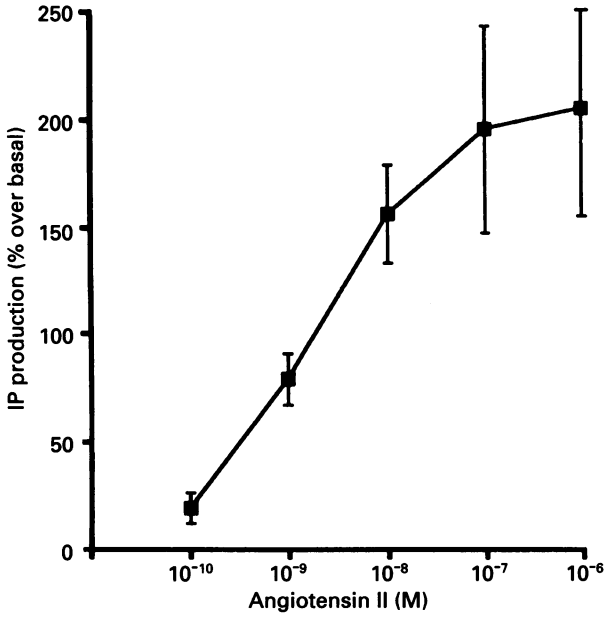

Figure 3 Stimulation of inositol phosphate production in rat aortic endothelial cells (RAEC) by AII. RAEC labelled with [^{3}H]-*myo*-inositol were incubated with different concentrations of AII. Increases in IP production are given as the ratio of the values for stimulated and unstimulated cells, and represent the means±s.e.mean of 6 independent experiments performed in triplicate.

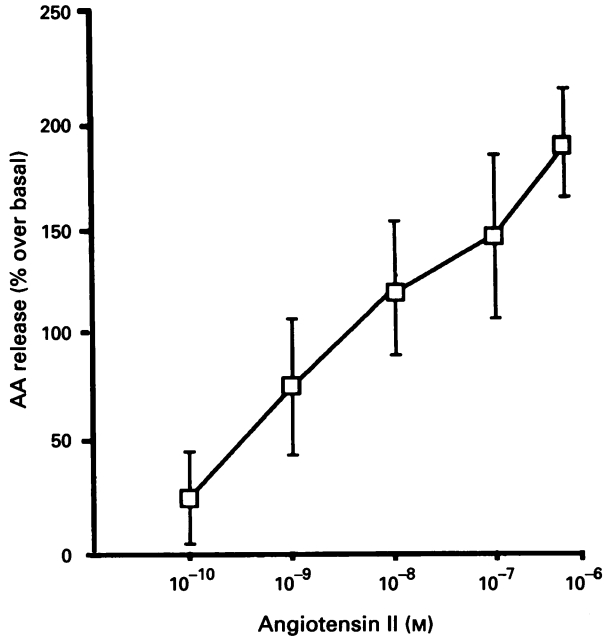

Figure 5 Stimulation of arachidonic acid (AA) release in rat aortic endothelial cells (RAEC) by AII. RAEC labelled with [^{3}H]-AA were incubated with different concentrations of AII. Increases in AA release are given as the ratio of the values for stimulated and unstimulated cells and represent the means±s.e.mean of 4 independent experiments performed in triplicate.

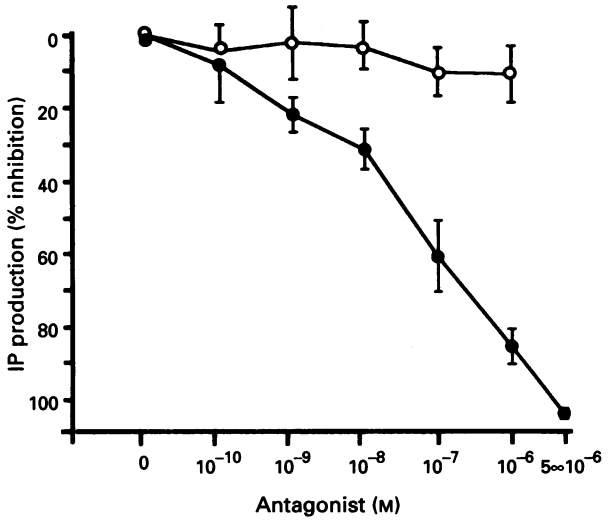

Figure 4 Effect of AII receptor antagonists on stimulation of IP production by AII. Rat aortic endothelial cells labelled with [^{3}H]-*myo*-inositol were incubated in the presence of 10^{-8}M AII with different concentrations of the AT_1-receptor antagonist, DuP753 (●) or the AT_2-receptor antagonist, PD 123177 (○). Results are given as the percentage inhibition of the values obtained with 10^{-8}M AII alone and represent the means±s.e.mean of 4 independent experiments performed in triplicate.

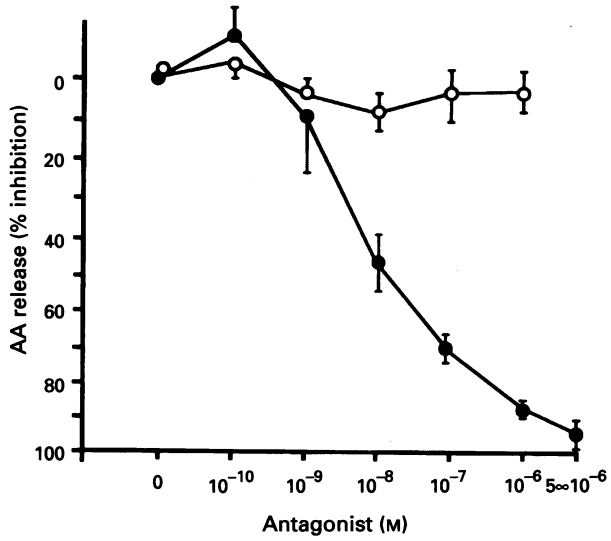

Figure 6 Effect of AII receptor antagonists on AII-induced arachidomic acid (AA) release. Rat aortic endothelial cells labelled with [^{3}H]-AA were incubated in the presence of 10^{-8}M AII with different concentrations of the AT_1 receptor antagonist, DuP753 (●) or the AT_2-receptor antagonist, PD123177 (○). Results are expressed as the percentage inhibition of the values obtained with 10^{-8}M AII alone and represent the means±s.e.mean of 3 or 4 independent experiments performed in triplicate.

Our results are consistent with those of another study on endothelial cells from rat microvessels, showing the presence of only AT_1 receptors (Feener *et al.*, 1995). However, the expression of both types of receptor has recently been reported in proliferating rat coronary endothelial cells (Stoll *et al.*, 1995). These differences in AT_2 receptor expression could be due, at least in part, to differences in culture conditions and in the growth state of the cells, quiescent or proliferating. Indeed, it has been shown that growth factors down-regulate the expression of AT_2 but not that of AT_1 (Kambayashi *et al.*, 1993), and we have detected minimal quantities of the AT_2 receptor mRNA by RT-PCR in proliferating cells cultured without growth factors (data not shown). Thus, the regulation of the AT_2 receptor expression in endothelial cells deserves further investigation.

Studies on the whole aorta, by autoradiography or RNA analysis, have demonstrated the presence of both AT_1 and AT_2 receptors, most of them belonging to the AT_1 type (Viswanathan *et al.*, 1991; Llorens-Cortes *et al.*, 1994). Both subtypes, AT_{1A} and AT_{1B}, have been found in the whole aorta (Llorens-Cortes *et al.*, 1994) but these techniques do not indicate the cell type in which receptors are found. As smooth muscle cells account for the large majority of these cells in the whole aorta, these results reflect mainly the smooth muscle cell receptor expression. The present study shows that RAEC express both AT_{1A} and AT_{1B}, whereas RSMC express only AT_{1A}. The lack of discriminatory pharmacological antagonists made it impossible to define the individual functions of the two AT_1 receptor subtypes (AT_{1A} and AT_{1B}), so that the physiological relevance of this different expression is not yet clear. Further studies will be needed to establish the specific roles of each subtype.

Stimulation of AT_1 receptors results in the activation of the PLC to generate the second messengers IP_3 and diacylglycerol. In turn, IP_3 increases the release of Ca^{2+} from intracellular stores and diacylglycerol activates protein kinase C (Dostal *et al.*, 1990; Sadoshima & Izumo, 1993). This pathway has been observed in several tissues, including the liver, adrenal cortex, kidney and vascular smooth muscle (Timmermans *et al.*, 1993). We investigated the AII-induced stimulation of PLC in endothelial cells by measuring IP production. This production was enhanced by AII in a dose-dependent manner. This effect was inhibited by the AT_1 antagonist, DuP753, but not by the AT_2 analogue, PD123177, suggesting that this effect is mediated by the AT_1 receptor.

We also evaluated the activation of PLA_2 by AII. This enzyme hydrolyses a phosphoglyceride producing lysophosphatidylcholine and arachidonic acid (AA). We demonstrated that AA release was enhanced by AII in a dose-dependent manner. The increase in AA release could be due to direct activation of a PLA_2 coupled to the AII receptor by a Pertussis toxin-sensitive G protein (Burch *et al.*, 1986); but it could also be the consequence of PLC activation, since several PLA_2 isoforms are calcium-dependent and can be activated by protein kinase C (Lambert *et al.*, 1986; Buckley *et al.*, 1991; Lin *et al.*, 1992). The PLA_2 stimulation is mediated only by the AT_1 receptor in endothelial cells, since it was completely inhibited by DuP753 and not by PD123177. In contrast, in other cell types, PLA_2 activation is mainly mediated by the AT_2 receptor (Lokuta *et al.*, 1994).

AII is known to regulate various physiological functions in target tissues. This diversity of action may result from AII receptor heterogeneity in different cell types, as well as from the multiplicity of associated intracellular signalling pathways (Bottari *et al.*, 1993). In the vascular wall, AII induces vasoconstriction by a direct action on smooth muscle cells, but this effect might be modulated by AII interaction with endothelial cells. Indeed, we show that AII activates PLA_2 and PLC in endothelial cells. PLA_2 activation could stimulate prostaglandin release, since arachidonic acid is metabolized to generate leukotrienes or prostaglandins. Furthermore, the activation of PLC leading to the increase of intracellular calcium could stimulate NO synthase. Since prostaglandins and NO are vasodilators, the AII action on endothelial cells may modulate the AII-induced construction of smooth muscle. This hypothesis is supported by studies showing that AII-induced vasoconstriction in isolated arteries is potentiated after endothelium removal and decreased by the release of endothelium-derived relaxing factors (Gruetter *et al.*, 1988; Zhang *et al.*, 1994; Caputo *et al.*, 1995).

Finally, AII, in common with other vasoactive peptides like bradykinin, endothelins or atrial natriuretic peptides acts via specific receptors on both endothelial and smooth muscle cells. AII may act preferentially on endothelial or on smooth muscle cells depending on its plasmatic or tissue origin. The interaction of AII with its endothelial receptors must be taken into account when evaluating the action of AII on the vascular wall in physiological or physiopathological situations.

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References

BATTLE, T., ARNAL, J.F., CHALLAH, M. & MICHEL, J.B. (1994). Selective isolation of rat aortic wall layers and their cell types in culture application to converting enzyme activity measurement. *Tissue Cell*, **26**, 943–955.

BOTTARI, S.P., DE GASPARO, M., STECKELINGS, V.M. & LEVENS, N.R. (1993). Angiotensin II receptor subtypes: characterization, signalling mechanisms, and possible physiological implications. *Front. Neuroendocrinol.*, **14**, 123–171.

BROWN, G.P. & DOUGLAS, J.G. (1983). Angiotensin-II binding sites in rat and primate isolated renal tubular basolateral membranes. *Endocrinology*, **112**, 2007–2014.

BUCKLEY, B.J., BARCHOWSKY, A., DOLOR, R.J. & WHORTON, A.R. (1991). Regulation of arachidonic acid release in vascular endothelium. *Biochem. J.*, **280**, 281–287.

BURCH, R.M., LUINI, A. & AXELROD, J. (1986). Phospholipase A_2 and phospholipase C are activated by district GTP-binding proteins in response to α-1-adrenergic stimulation in FRTL5 thyroid cells. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7201–7205.

CAPUTO, L., BENESSIANO, J., BOULANGER, C.M. & LEVY, B.I. (1995). Angiotensin II increases cyclic guanosine mono-phosphate content via endothelial angiotensin II AT_1 subtype receptors in the rat carotid artery. *Arterioscler. Thromb.*, **15**, 1646–1651.

CHIU, A.T., DUNSCOMB, J., KOSIEROWSKI, J., BURTON, C.R.A., SANTOMENNA, L.D., CORJAY, M.H. & BENFIELD, P. (1993). The ligand binding signatures of the rat AT_{1A} and AT_{1B} and the human AT_1 receptors are essentially identical. *Biochem. Biophys. Res. Commun.*, **197**, 440–449.

CHIU, A.T., MCCALL, D.E., PRICE, W.A., WONG, P.C., CARINI, D.J., DUNCIA, J.V., WEXLER, R.R., YOO, S.E., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1990). Nonpeptide angiotensin II receptor antagonists. VII. Cellular and biochemical pharmacology of DuP753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.*, **252**, 711–718.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162,** 156–159.

CRABOS, M., ROTH, M., HAHN, A.W.A. & ERNE, P. (1994). Characterization of angiotensin II receptors in cultured adult rat cardiac fibroblasts. *J. Clin. Invest.*, **93,** 2372–2378.

DANSER, A.H.J., CHOWDURY, S., DE LANNOY, L.M., VAN DER GIESSEN, W.J., SAXENA, P.R. & SCHALEKAMP, M.A.D.H. (1995). Conversion and degradation of (^{125}I) labelled angiotensin I in isolated perfused porcine coronary and carotid arteries. *Cardiovasc. Res.*, **29,** 789–795.

DOSTAL, D.E., MURAHASHI, T. & PEACH, M.J. (1990). Regulation of cytosolic calcium by angiotensins in vascular smooth muscle. *Hypertension*, **15,** 815–822.

DUIJVESTIJN, A.M., VAN GOOR, H., KLATTER, F., MAJOOR, G.D., VAN BUSSEL, E., VAN BREDA & VRIESMAN, J.C. (1992). Antibodies defining rat endothelial cells : RECA-1, a pan-endothelial cell-specific monoclonal antibody. *Lab. Invest.*, **66,** 459–466.

FEENER, E.P., NORTRUP, J.M., AIELLO, L.P. & KING, G.L. (1995). Angiotensin II induces plasminogen activator inhibitor-1 and -2 expression in vascular endothelial and smooth muscle cells. *J. Clin. Invest.*, **95,** 1353–1362.

FORD, D.A. & GROSS, R.W (1989). Plasmenylethanolamine is the major storage depot for arachidonic acid in rabbit vascular smooth muscle and is rapidly hydrolyzed after angiotensin II stimulation. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 3479–3483.

FREEDLENDER, A.E. & GOODFRIEND, T.S. (1979). In *Methods of Hormone Radioimmunoassay*. ed. (Jaffe, B. & Behrman, H. pp. 889–909. New York: Academic Press.

GASC, J.M., SHANMUGAM, S., SIBONY, M. & CORVOL, P. (1994). Tissue-specific expression of type 1 angiotensin II receptor subtypes. *Hypertension*, **24,** 531–537.

GIMBRONE, M.A. & ALEXANDER, R.W. (1975). Angiotensin stimulation of prostaglandin production in cultured human vascular endothelium. *Science*, **189,** 219–220.

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.

GUNTHER, S. (1984). Characterization of angiotensin II receptor subtypes in rat liver. *J. Biol. Chem.*, **262,** 7622–7629.

KAMBAYASHI, Y., BARDHAN, S. & INAGAMI, T. (1993). Peptide growth factor markedly decrease the ligand binding of angiotensin II type 2 receptor in rat cultured vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **194,** 478–482.

LAMBERT, T.L., KENT, R.S. & WHORTON, A.R. (1986). Bradykinin stimulation of inositol polyphosphate production in porcine endothelial cells. *J. Biol. Chem.*, **261,** 15288–15293.

LEWIS, J.L., SERIKAWA, T. & WARNOCK, D.G. (1993). Chromosomal localization of angiotensin II type 1 receptor isoforms in the rat. *Biochem. Biophys. Res. Commun.*, **194,** 677–682.

LIN, L.L., LIN, A.Y. & KNOPF, J.L. (1992). Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 6147–6151.

LLORENS-CORTES, C., GREENBERG, B., HUANG, H. & CORVOL, P. (1994). Tissular expression and regulation of type 1 angiotensin II receptor subtypes by quantitative reverse transcriptase-polymerase chain reaction analysis. *Hypertension*, **24,** 538–548.

LOKUTA, A.J., COOPER, C., GAA, S.T., WANG, H.E. & ROGERS, T.B. (1994). Angiotensin II stimulates the release of phospholipid-derived second messengers through multiple receptor subtypes in heart cells. *J. Biol. Chem.*, **269,** 4832–4838.

MCQUEEN, J., MURRAY, G.D. & SEMPLE, P.F. (1994). Identification of the angiotensin II receptor in the rat mesenteric artery. *Biochem. J.*, **223,** 659–671.

MORRERO, M.B., PAXTON, W.G., DUFF, J.L., BERK, B.C. & BERNSTEIN, K.E. (1994). Angiotensin II stimulates tyrosine phosphorylation of phospholipase C-γ1 in vascular smooth muscle cells. *J. Biol. Chem.*, **269,** 10395–10939.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107,** 220–239.

PATEL, J.M., YARID, F.R., BLOCK, E.R. & RAIZADA, M.K. (1989). Angiotensin receptors in pulmonary arterial and aortic endothelial cells. *Am. J. Physiol.*, **256,** 987–993.

PEACH, M.J. (1977). Renin-angiotensin system: biochemistry and mechanisms of action. *Physiol. Rev.*, **57,** 313–370.

SADOSHIMA, J.I. & IZUMO, S. (1993). Signal transduction pathways of angiotensin II-induced c-*fos* gene expression in cardiac myocytes in vitro: roles of phospholipid-derived second messengers. *Circ. Res.*, **73,** 424–438.

SHANMUGAM, S., LENKEI, Z.G., GASC, J.M., CORVOL, P. & LLORENS-CORTES, C. (1995). Ontogeny of angiotensin II type 2 (AT_2) receptor mRNA in the rat. *Kidney Int.*, **47,** 1095–1100.

STOLL, M., STECKELINGS, M., PAUL, M., BOTTARI, S.P., METZGER, R. & UNGER, T. (1995). The angiotensin AT_2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J. Clin. Invest.*, **95,** 651–657.

TIMMERMANS, P.B.M., WONG, P., CHIU, A.T., HERBLIN, W.F., BENFIELD, P., CARINI, D.J., LEE, R.J., WEXLER, R.R., SAYE, J.A.M. & SMITH, R.D. (1993). Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol. Rev.*, **45,** 205–251.

VAUGHAN, D.E., LAZOS, S.A. & TONG, K. (1995). Angiotensin II regulates the expression of plasminogen activator inhibitor-1 in cultured endothelial cells. *J. Clin. Invest.*, **95,** 995–1001.

VISWANATHAN, M., TSUTSUMI, K., CORREA, F.M.A. & 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., MEME, M., KAZBMER, B. & DE GASPARO, M. (1989). Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.*, **163,** 284–291.

WIEMER, G., SCHÖLKENS, B.A., WAGNER, A., HEITSCH, H. & LING, W. (1993). The possible role of angiotensin II subtype AT_2 receptors in endothelial cells and isolated ischemic rat hearts. *J. Hypertens.*, **11,** 234–235.

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

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