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Short Communication

ANGIOTENSIN II BINDING ACTIVITY IN CULTURED PORCINE ARTERIAL ENDOTHELIAL CELLS

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Angiotensin II (A II) binding activity was detected in the particulate fraction (100,000 *g*, 60 min precipitate) of cultured porcine aortic endothelial cells. Scatchard analysis of the binding activity indicated a single class of binding sites with a dissociation constant (K_d) of 1.1 nM and a total binding capacity (B_{max}) of 125 fmol/mg protein. The binding of [^{125}I]A II was inhibited by excess unlabelled A II, A II analogues ([Sar^1, Ile^8]A II and [Sar^1, Ala^8]A II), A I (angiotensin I) and A III (angiotensin III), but not by bradykinin. Type specific A II receptor antagonists, losartan (type 1 angiotensin II receptor) and PD123319 (type 2 angiotensin II receptor), did not inhibit the binding. These results suggest that the A II specific binding protein(s) or receptor(s) is present in arterial endothelial cells, and that it is different from typical type 1 and type 2 angiotensin II receptors.

Key words: AT_1 receptor; AT_2 receptor; losartan; PD123319

A II† has many physiological roles, mainly related to contraction of vascular smooth muscle [1] and the regulation of blood pressure. It has recently been reported that A II is also involved in the migration and proliferation of arterial smooth muscle cells [2, 3]. We have previously shown that in the interaction of A II with smooth muscle cells, A II is transported from the apical to the basolateral surface through the endothelial cell monolayers, via transcytosis [4].

Two classes of receptors for A II have been demonstrated using non-peptide antagonists [losartan (Dup 753) and PD123319, CGP42112A] [5–9]. The AT_1 receptor, which is sensitive to losartan, is G-protein coupled and mediates signal transduction through phosphoinositide specific phospholipase C and adenylate cyclase [6–8]. The AT_2 receptor, which is sensitive to both PD123319 and CGP42112A, is involved in regulation of K^+ currents and mediates inhibition of guanylate cyclase through tyrosine protein phosphatase [9]. These receptor subtypes also have different tissue distribution. The AT_1 receptor is predominant in vascular smooth muscle and adrenal cortex [5, 10], whilst the AT_2 receptor is found in the uterus and adrenal medulla [5, 11]. Patel *et al.* [12] have reported that binding sites specific for A II exist on cultured endothelial cells of porcine pulmonary artery and aorta.

In the present study, we show that the particulate fraction of cultured arterial endothelial cells exhibit A II binding activity, which is different from that of the AT_1 and AT_2 receptors.

Materials and Methods

Preparation of particulate fraction. Primary culture of endothelial cells and smooth muscle cells from porcine arterial walls were prepared by the method described previously [13, 14]. These cells were cultured in Dulbecco's modified Eagle's medium (Flow Lab., VA, U.S.A.) supplemented with 10% fetal calf serum. The particulate fraction was freshly prepared from cultured cells according to the method described previously [15].

The particulate fraction from rat adrenal medulla was prepared by the methods of Chiu *et al.* [15].

Binding assay. Assay of binding of A II by the particulate fraction was measured according to the methods described previously [16] with slight modifications. Particulate fraction (20–40 μg protein) was incubated at 22° with Tris–HCl buffer (20 mM, pH 7.5) (total volume 150 μL) containing 158 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 4.5 mM Na_2EDTA, ^{125}I-A II [5-L-isoleucine] (tyrosyl-^{125}I, 81.4 TBq/mmol, 0.36 nM) (New England Nuclear, MA, U.S.A.). The reaction was stopped by the addition of 4 mL of ice-cold stop buffer (20 mM Hepes, pH 7.5 containing 158 mM NaCl). [^{125}I]A II bound to the particulate fraction was then separated from free [^{125}I]A II on 0.45-μm HA millipore filters and measured in a gamma counter (Aloka 300, Japan).

Materials. Losartan and PD123319 were supplied by Du Pont (Wilmington, DE, U.S.A.) and Warner-Lambert Co. (Ann Arbor, MI, U.S.A.), respectively. Angiotensin related peptides and bradykinin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Results and Discussion

A II binding activity in the particulate fraction of endothelial cells. [^{125}I]A II binding to the freshly prepared particulate fraction of cultured aortic endothelial cells was investigated. The dose–response curve of the specific binding of [^{125}I]A II at 22° for 60 min is shown in Fig. 1. The binding increased linearly with time up to 120 min and was not observed at 4° (data not shown). The scatchard analysis showed that the K_d and B_{max} values of [^{125}I]A II binding were 1.1 nM and 125 fmol/mg protein, respectively (Fig. 1, inset). Patel *et al.* [12] reported the existence of a single class of high-affinity binding sites specific for A II on cultured monolayer aortic endothelial cells with a K_d

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† Abbreviations: A I, angiotensin I; A II, angiotensin II; A III, angiotensin III; K_d, dissociation constant; B_{max}, total binding capacity; AT_1 receptor, type 1 angiotensin II receptor; AT_2 receptor, type 2 angiotensin II receptor

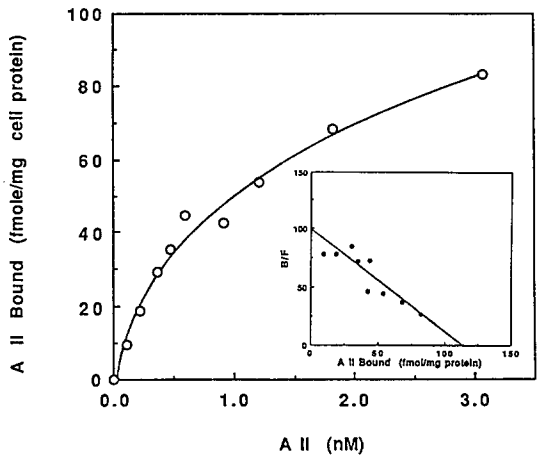

Fig. 1. Dose–response of [^{125}I]A II binding to particulate fraction of endothelial cells at 22° for 60 min. Inset: Scatchard analysis. Each point is the mean of duplicate or triplicate samples.

Table 1. Effects of A II antagonists on [^{125}I]A II binding to the particulate fractions

Addition (10^{-5} M)	[^{125}I]A II Binding (fmol/mg protein)	(%)
(a) Porcine smooth muscle cells		
None	9.72 ± 1.00	100
A II	0.68 ± 0.40	7.0
Losartan	0.54 ± 0.11	5.6
PD123319	8.59 ± 1.28	88.3
(b) Rat adrenal medulla		
None	26.1 ± 0.20	100
A II	0.30 ± 0.40	1.1
Losartan	24.9 ± 2.1	87.0
PD123319	2.00 ± 0.80	7.7
(c) Porcine endothelial cell		
None	10.2 ± 1.20	100
A II	0.48 ± 0.30	4.7
Losartan	7.54 ± 0.51	73.8
PD123319	7.88 ± 1.03	77.2

Results show means ± SEM of triplicate samples.

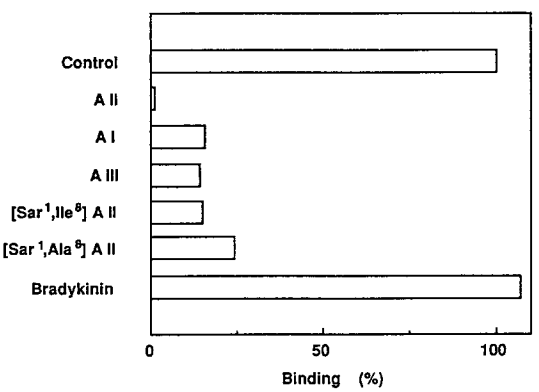

Fig. 2. Inhibitory effects of 10^{-5} M A II related peptides on [^{125}I]A II binding. Each bar represents a mean of duplicate samples. The average per cent error for these data was 4.5%.

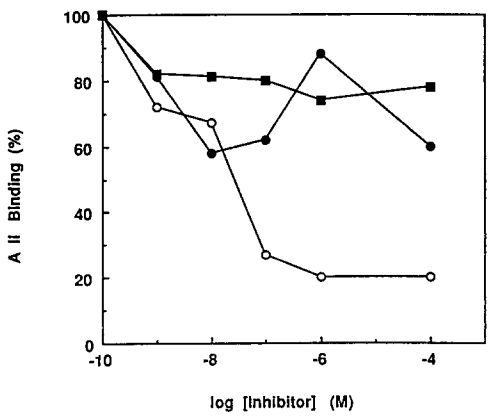

Fig. 3. Dose–response effects of A II antagonists on [^{125}I]A II binding. A II (○), losartan (●), PD123319 (■). Each point is the mean of duplicate or triplicate samples. The average per cent error for these data was 8.0%.

value of 0.81 nM and a B_{max} value of 73 fmol/mg protein. There were no significant differences in the binding affinity (K_d value) between the particulate fraction used in this study and monolayer cells of cultured endothelial cells, suggesting that the activity in the particulate fraction was derived from receptor(s) on the surface of endothelial cells.

The specificity of [^{125}I]A II binding by the particulate fraction was investigated using unlabelled A II or A II analogues (Fig. 2). An excess (10^{-5} M) unlabelled A II, [Sar^1, Ile^8]A II and [Sar^1, Ala^8]A II inhibited the binding of [^{125}I]A II. Both A I and A III inhibited the binding activity 84.2 and 86.5%, respectively. Bradykinin (10^{-5} M) had little effect on the binding.

The effect of type specific non-peptide antagonists of A II receptor. The effect of AT_1 receptor antagonist (losartan) and AT_2 receptor antagonist (PD123319) on the [^{125}I]A II binding is shown in Table 1. In porcine smooth muscle cells, losartan (10^{-5} M) inhibited the binding of [^{125}I]A II [94.4% inhibition, Table 1 (a)]. In rat adrenal medulla, PD123319 (10^{-5} M) inhibited the binding [92.3% inhibition, Table 1 (b)], while losartan (10^{-5} M) had no effect. However, in contrast to these results, neither antagonists (10^{-5} M) had any effect on binding to the endothelial cell particulate fraction [26.2 and 22.8%, respectively, Table 1(c)]. The dose–response curves of these antagonists confirmed that they had little effect on the binding of A II to the endothelial cells (Fig. 3). At a concentration of 10^{-4} M, losartan and PD123319 inhibited the binding by 38.0 and 20.0%, respectively, whilst A II inhibited the binding by 80.5%. In the presence of both losartan (10^{-5} M) and PD123319 (10^{-5} M), [^{125}I]A II binding was not affected (data not shown).

These results suggest that neither AT_1 nor AT_2 receptor is involved in the major part of A II binding activity in the endothelial cells. There are several reports on non-AT_1-non-AT_2 receptors including the receptors for angiotensin IV [A II 3–8 hexapeptide] [17, 18] and the A II binding

site in neuroblastoma cells [19]. Micro-organisms (*Acholeplasma laidlawii*) has reportedly A II binding activity [20]. The activity of endothelial cells in this work showed different characteristics from these binding proteins in respect of affinity for A II and A III.

The function of the binding proteins in the endothelial particulate fraction is, as yet, unknown. Further studies are required to elucidate whether the binding activity has any relationship with signal transduction or transcellular transport in endothelium.

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