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SHORT COMMUNICATION

Angiotensin Type-1 (AT1) Receptor Gene Expression in Primarily Cultured Human Arterial Umbilical Endothelial Cells

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ABSTRACT. The aim of this study was to investigate the interaction of angiotensin II (Ang II) and human umbilical arterial endothelial cells (HUAEC). Specific binding of ^{125}I-ANG II to primarily cultured HUAEC showed a K_D of 1.98 ± 0.53 × 10^{-9} M (n = 5) with a maximum binding site of 2.84 ± 1.07 × 10^{-13} mol/mg protein (n = 5). In later passages (third and fifth subculture), this binding site was no longer detectable. Gene expression analysis revealed a strong expression of the angiotensin type-1 receptor (AT1-R) in the primarily cultured HUAEC, with a decrease in additional passages. In primarily cultured HUAEC, Ang II (10^{-10} – 10^{-6} M) induced a concentration-dependent increase in intracellular free calcium ($[Ca^{2+}]_i$) that could be blocked by a preincubation with candesartan (TCV-112) but not by PD123319. These data show the expression of the AT1-R in primary cultures of HUAEC. The Ang II-induced increase in $[Ca^{2+}]_i$ seems to be mediated by this receptor. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:417–421, 1997.

KEY WORDS. angiotensin II; angiotensin type-1 receptor; endothelium; intracellular calcium; gene expression

Ang II† activates receptors in the adrenal cortex, blood vessels, kidney and brain, thus contributing to the regulation of arterial pressure, electrolyte and volume balance and tissue perfusion [1]. In addition, Ang II has been implicated in pathological states such as hypertension and left ventricular hypertrophy [2]. VSMC represent an important target tissue for circulating Ang II. The acute pressor response of vertebrates to increments of Ang II results from activation of smooth muscle cell AT1-Rs [3]. However, in 1975, Gimbrone and Alexander [4] described an Ang II-mediated release of prostaglandins by cultured vascular endothelial cells; in 1989, Patel *et al.* [5] showed the presence of Ang II receptors on porcine pulmonary arterial and aortic endothelial cells. Furthermore, in 1990, Stallone *et al.* [6] described that specific receptors for Ang II exist in the aortic endothelium of domestic fowl and may mediate an endothelium-dependent vasodilatory action. These observations raised the question as to whether AT1-R are expressed in human arterial endothelial cells.

Therefore, the present study was designed to determine the binding of ^{125}I-Ang II, the expression of AT1-R mRNA and the effect of Ang II on the $[Ca^{2+}]_i$ in HUAEC.

MATERIALS AND METHODS

Culture of HUAEC

HUAEC were isolated, cultured and characterized as described elsewhere [7].

Receptor-binding Studies

Specific binding of ^{125}I-Ang II to membrane receptors on HUAEC was measured by competitive binding studies between ^{125}I-labeled Ang II (0.2 μCi) with unlabeled Ang II at various concentrations in a total volume of 250 μL according to the method of Patel *et al.* [5]. To calculate the specific binding of ^{125}I-Ang II to HUAEC, nonspecific binding that occurred in excess of unlabeled Ang II was subtracted from total binding. The protein content of each sample was determined by the method of Bradford [8]. The B_{max}, K_D and receptor number were calculated by Scatchard analysis [9] and by the computer-assisted nonlinear curve fitting program EBDA/Ligand (Biosoft, Cambridge, United Kingdom) [10].

Measurement of $[Ca^{2+}]_i$

For the measurement of $[Ca^{2+}]_i$, cells were cultured on fresh human fibronectin-coated (5 μg/cm^2) round glass micro-

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†*Abbreviations:* AT1-R angiotensin type-1 receptor; HUAEC, human umbilical arterial endothelial cells; $[Ca^{2+}]_i$, intracellular free calcium concentration; Ang II, angiotensin II; VSMC, vascular smooth muscle cells; PCR, polymerase chain reaction; RT/PCR, reverse transcriptase/polymerase chain reaction; NO, nitric oxide; PAI, plasminogen activator inhibitor; B_{max}, maximum number of binding sites; K_D, receptor affinity.

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scope slides (diameter-13 mm). Confluent cells were incubated with 2 μM fura-2/AM at 37°C for 20 min before Ca^{2+}-fura-2 fluorescence was measured at 37°C in a fluorescence spectrofluorometer (Hitachi, Ratingen, Germany) at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 505 nm, as described elsewhere [7].

RT/PCR

Total RNA was extracted from endothelial cells by the guanidinium isothiocyanate/CsCl procedure [11]. Total RNA (4 μg) was reverse transcribed and amplified in an automated DNA thermal cycler (Perkin Elmer, model 480, Weiterstadt, Germany) according to the method of Mullis and Faloona [12] as described elsewhere [13] with the following temperature profile: denaturtion at 94°C for 1 min, primer annealing at 60°C for 1 min and primer extension at 72°C for 1.5 min. After an initial denaturation step (94°C, 5 min), the cycle was repeated 35 times when AT1-R primers (5'-AT1-R: 5'-GTCACCTGCATCATCATTTGGC-3', 3'-AT1-R: 5'-TCATAAGCCTTCTTTAGG GCCTTC-3') and 25 times when GAPDH primers [13] were used, except in the experiments in which the exponential phase of amplification was examined. The last cycle was followed by a final extension step of 20 min at 72°C; 20 μL of each sample were electrophoresed on a 1.5% regular agarose gel and stained with ethidium bromide.

Southern Blotting of PCR Products

Blotting of agarose-gel-separated and denatured cDNA onto nylonmembranes (Hybond N+), and hybridization with a γ-[^{32}P]-5' end labeled AT1-R oligonucleotide (5'-GTCCCAAAATTCAACCCTTCC-3') and washing were performed according to Sambrock *et al.* [11].

Densitometric Analysis

Densitometric analysis was performed on a two-dimensional scanning densitometer (Biometra, Göttingen, Germany) using the ScanPack software (version 14.1 A 27). The ethidiumbromide-stained agarose gels were photographed and the densitometric results of AT1-R expression were standardized to that of GAPDH expression from the same reverse-transcribed mRNA sample. Values are given as percentage of control.

RESULTS

Ang II Binding

Incubation of intact, primary cultured HUAEC with ^{125}I-Ang II resulted in a specific binding of the ligand to a high-affinity binding site, with an apparent dissociation constant (K_D) of 1.98 ± 0.53 × 10^{-9} M, with a B_{max} of 2.84 ± 1.07 × 10^{-13} mol/mg protein (mean ± SD, n = 5) (Fig. 1). Furthermore a low-affinity binding site with K_D = 1.84 ± 0.77 × 10^{-5} M and B_{max} = 1.11 ± 0.34 × 10^{-9} mol/mg protein (mean ± SD, n = 5) was found. Binding studies on HUAEC derived from a second to fifth passage revealed that only the low-affinity binding site (Fig. 1) with K_D = 1.70 ± 0.74 × 10^{-5} M and B_{max} = 1.57 ± 0.91 × 10^{-9} mol/mg protein (mean ± SD, n = 6) was detectable.

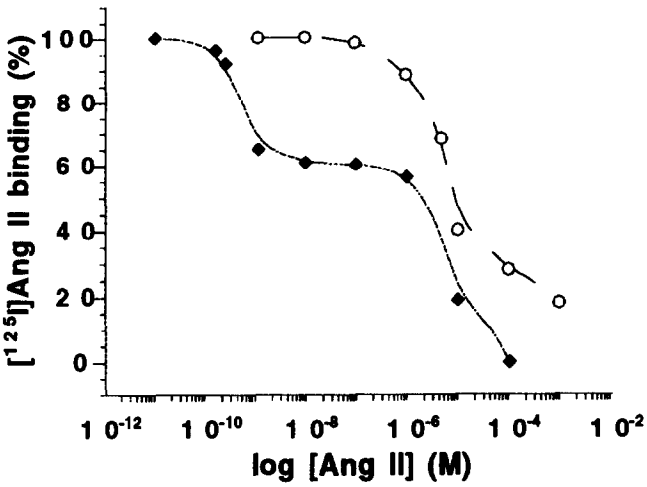

FIG. 1. Competition of binding of ^{125}I-Ang II to primarily cultured HUAEC (closed diamonds) and to HUAEC in the third and fifth cell passage (open circles). Cells were incubated with ^{125}I-Ang II. Representative experiments of five are shown.

AT1-R Gene Expression in HUAEC

To study AT1-R gene expression in HUAEC, we performed RT/PCR. Exponential phase of amplification for AT1-R mRNA was detected between 30 and 40 cycles. Therefore, further PCR experiments were performed using 35 cycles for AT1-R mRNA detection. Expression analysis for AT1-R mRNA in HUAEC showed a strong signal in the primarily cultured HUAEC, with a continuous decrease in AT1-R expression in culture passages 2–5 (Fig. 2). Figure 2A shows the ethidium-bromide-stained agarose gel and Fig. 2B the corresponding Southern blot probed with a specific AT1-R mRNA oligoprobe. Densitometric analysis of the Southern blot shows a decrease in AT1-R gene expression from 100% in primarily cultured HUAEC to 22% in the third culture passage (Fig. 2C).

Ang II-Induced Elevation of $[Ca^{2+}]_i$

We determined the $[Ca^{2+}]_i$ in HUAEC following stimulation with Ang II at different concentrations. Primary cultured HUAEC showed a concentration-dependent increase in $[Ca^{2+}]_i$ from basal level (90 ± 8 × 10^{-9} M, n = 6) to 120.0 ± 6.8 × 10^{-9} M (n = 6) at a concentration of 10^{-9} M Ang II and to 232 ± 44 × 10^{-9} M at a concentration of 10^{-6} M Ang II (Fig. 3A). This effect was blocked by the specific AT1-R blocker candesartan but not by the specific AT2-R blocker PD123319 (Fig. 3B); 10^{-8} M Ang II exerted a time-dependent increase in $[Ca^{2+}]_i$ that peaked 30 sec after stimulation and returned to a level higher than the basal

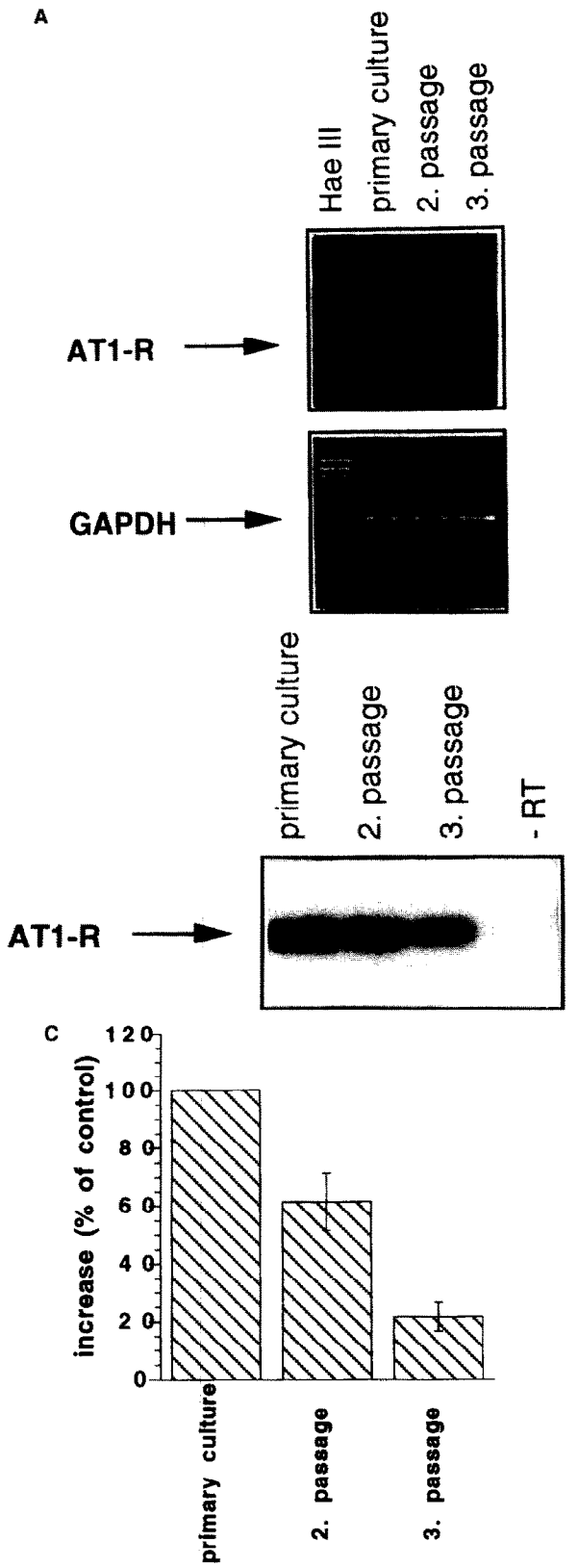

FIG. 2. Expression analysis for AT1-R in HUAEC. (A) Ethidium bromide-stained agarose gel. (B) Corresponding Southern blot probed with a specific AT1-R oligoprobe. (C) Densitometric analysis of the Southern blot (mean ± SEM, n = 3).

value 60 sec later. The following stimulation with 10^{-7} M bradykinin was performed as a positive control for the reagibility of HUAEC (Fig. 3B). As shown in Fig. 3B, pretreatment of HUAEC with candesartan (10^{-6} M) for 90 sec resulted in a complete blockade of the Ang II-induced increase of $[Ca^{2+}]_i$. In contrast, 10^{-6} M PD123319 did not inhibit the Ang II-induced elevation of $[Ca^{2+}]_i$ (Fig. 3B). In HUAEC from passages 2–5, stimulation with Ang II led to an increase in $[Ca^{2+}]_i$ from basal level ($90 \pm 8 \times 10^{-9}$ M) to $119.0 \pm 17.0 \times 10^{-9}$ M at a concentration of 10^{-6} M Ang II and to $148 \pm 3.9 \times 10^{-9}$ M (mean ± SD, n = 6) at a concentration of 10^{-4} M Ang II (Fig. 3A).

DISCUSSION

Little is known about the influence of Ang II on endothelial cells, but there have been an increasing number of reports that Ang II influences endothelial cells, e.g., induction of the expression of endothelin [14], growth-promoting effects on endothelial cells [15] or the expression of PAI [16, 17]. The question remains, by which kind of AT1-Rs are these effects mediated? In coronary endothelial cells, growth-promoting effects seem to be mediated by the AT1-R and growth inhibiting effects by the AT2-R [15]. When dealing with the expression of PAI, Feener *et al.* [16] found that in rat microvessel endothelial cells PAI-1 and PAI-2 expression are AT1-R dependent, whereas Vaughan *et al.* [17] reported that PAI-1 expression by Ang II in bovine aortic endothelial cells is neither AT1 nor AT2 receptor dependent.

Our data clearly show that there is a high-affinity binding site on primarily cultured HUAEC, with a dissociation constant (K_D) of $1.98 \pm 0.53 \times 10^{-9}$ M and a B_{max} of $2.84 \pm 1.06 \times 10^{-13}$ mol/mg protein. This binding site is similar to the binding of Ang II to other mammalian tissues. The K_D and B_{max} values for Ang II are comparable to those observed in cultured VSMC [18], in neuronal glial cells [19] and in brain membranes of rats [20]. When HUAEC were analyzed in later passages of culture, this high-affinity binding site disappeared. This result is in accordance with observations made by Stoll *et al.* in coronary endothelial cells [15]. To study AT1-R gene expression, we performed an RT/PCR analysis because of the limited cell number in the primary culture. RT/PCR analysis of AT1-R gene expression demonstrated a significant expression of this gene in primarily cultured HUAEC, with a decrease in later cell passages. This observation is consistent with the Ang II binding characteristics to the high-affinity binding site on primary cultures of HUAEC that disappears in additional cell passages. The AT1-R mediates Ang II-induced increases in $[Ca^{2+}]_i$ by activating phospholipase C and a consecutive phosphoinositide hydrolysis [21, 22]. We showed a rapid increase in $[Ca^{2+}]_i$ following stimulation of primarily cultured HUAEC, with a half-maximal effective concentration (10×10^{-9} M) close to the K_D of the high-affinity binding site for Ang II on HUAEC. Specific AT1-R an-

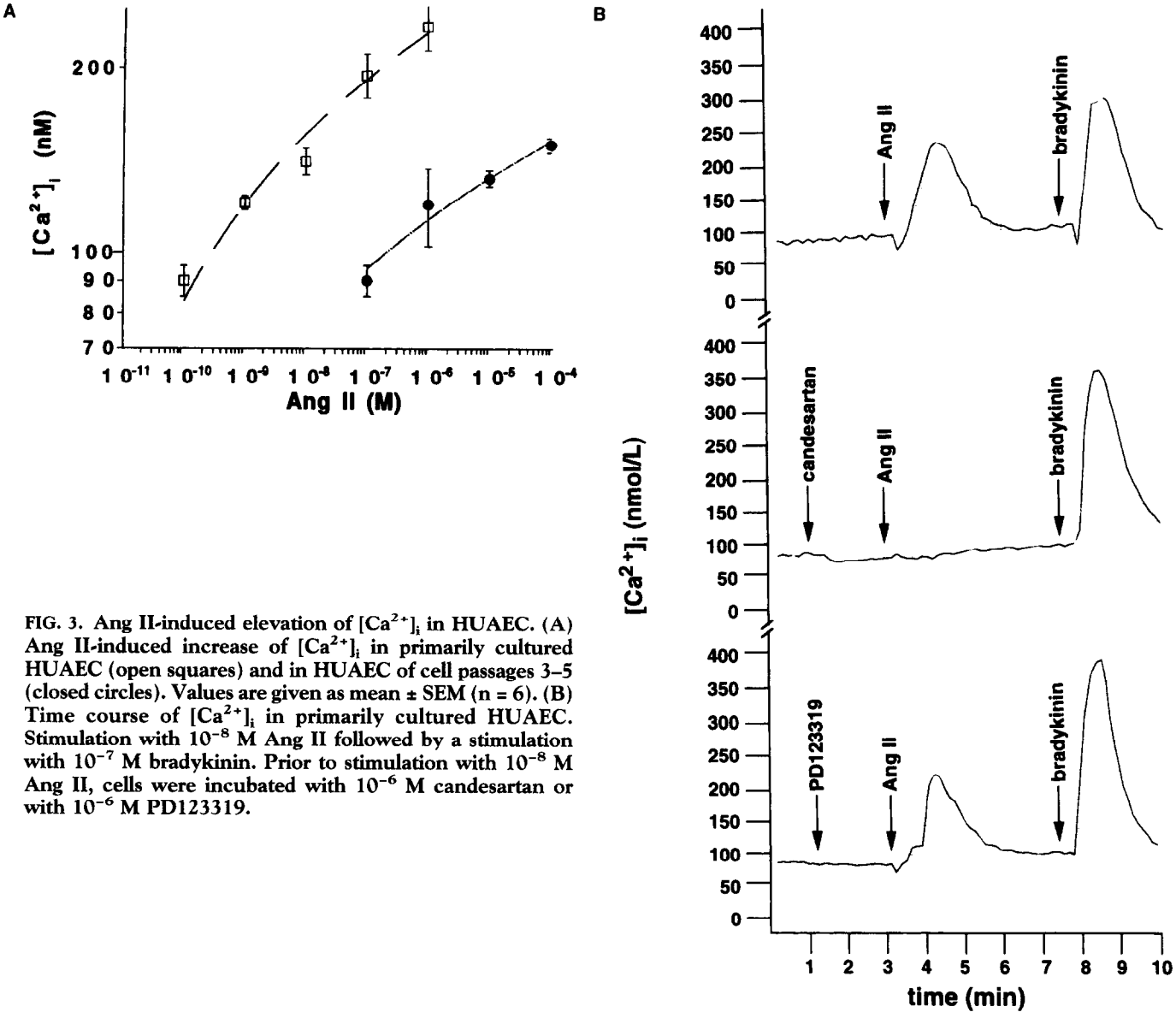

FIG. 3. Ang II-induced elevation of $[Ca^{2+}]_i$ in HUAEC. (A) Ang II-induced increase of $[Ca^{2+}]_i$ in primarily cultured HUAEC (open squares) and in HUAEC of cell passages 3–5 (closed circles). Values are given as mean ± SEM (n = 6). (B) Time course of $[Ca^{2+}]_i$ in primarily cultured HUAEC. Stimulation with 10^{-8} M Ang II followed by a stimulation with 10^{-7} M bradykinin. Prior to stimulation with 10^{-8} M Ang II, cells were incubated with 10^{-6} M candesartan or with 10^{-6} M PD123319.

tagonists such as candesartan but not the AT2-R antagonists (PD123319) prevented the Ang II-mediated increase of $[Ca^{2+}]_i$. This result is in agreement with the data provided by the ^{125}I-Ang II binding studies and analysis of AT1-R gene expression. In conclusion, our observations provide evidence for the existence of functional AT1-R on primarily cultured HUAEC.

We also showed that there is a low-affinity binding site, especially in later passages of HUAEC. Endothelial cells react to high concentrations of Ang II, with an increase in $[Ca^{2+}]_i$ that can be blocked by neither candesartan nor PD123319 (data not shown). Because it is unclear at present whether this binding site has any relevance, this question needs to be analyzed by additional experiments. In this context, one has to be aware of the various effects of Ang II on endothelial cells not mediated by the AT1-R or AT2-R [4–6, 17] but by binding sites that have not yet been classified.

In endothelial cells, a predominant effect of increasing levels of $[Ca^{2+}]_i$, e.g. by bradykinin, is the release of NO via a calmodulin-dependent pathway [23]. Endothelium-dependent vasodilatation would be the consequence. An Ang II-induced, endothelium-dependent vasodilatation has been described for dog renal and cerebral arteries [24] and for the aorta of domestic fowl [4]. Prostaglandin production, especially of PGI_2, has been associated with this vasodilatation [5] but PGI_2-independent vasodilatation has also been proposed [4]. According to our data, an increase of $[Ca^{2+}]_i$ in endothelial cells is a possible AT1-R mediated effect of Ang II associated with vasodilatation. Further investigations are necessary to analyze the possibility of an Ang II-mediated endothelial NO release.

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