Stimulation of Endothelial Cell Prostaglandin Production by Angiotensin Peptides
Characterization of Receptors

Neelam Jaiswal, Debra I. Diz, Mark C. Chappell, Mahesh C. Khosla, and Carlos M. Ferrario

Angiotensin II stimulates prostaglandin release in blood vessels via activation of angiotensin receptors present in endothelium, vascular smooth muscle cells, or both. We evaluated the response of angiotensin II, angiotensin I, and [des-Phe⁸]angiotensin II (angiotensin-(1-7)) on prostaglandin release in porcine aortic endothelial cells. Incubation of cell monolayers with angiotensin I and angiotensin-(1-7), but not angiotensin II, stimulated the release of prostaglandin E₂ and prostaglandin I₂ in a dose-dependent manner (10⁻¹⁰ to 10⁻⁶ M) with an EC₅₀ of approximately 1 nM. In addition, we characterized the angiotensin receptor subtypes mediating prostaglandin synthesis by using subtype-selective antagonists. Angiotensin I–stimulated prostaglandin synthesis was not altered by either of the nonselective classical angiotensin receptor antagonists [Sar¹,Thr⁸]angiotensin II or [Sar¹,Ile⁸]angiotensin II. In contrast, either the angiotensin subtype 1 (AT₁) antagonist DuP 753 or the subtype 2 (AT₂) antagonist CGP42112A significantly attenuated the prostaglandin release in response to angiotensin I. However, PD123177, another AT₂ antagonist, did not inhibit angiotensin I–stimulated prostaglandin release. Angiotensin-(1-7)–induced prostaglandin release was significantly attenuated by [Sar¹,Thr⁸]angiotensin II (10⁻⁶ M) and PD123177 (10⁻⁶ M) but not by [Sar¹,Ile⁸]angiotensin II, DuP 753, or CGP42112A. Higher doses (10⁻³ M) of DuP 753 and CGP42112A attenuated the angiotensin-(1-7) response. These data suggest that in porcine aortic endothelial cells, angiotensin I and angiotensin-(1-7) but not angiotensin II are potent stimuli for prostaglandin synthesis. Angiotensin-(1-7)–stimulated prostaglandin synthesis occurs through activation of a receptor subtype distinct from AT₁ and AT₂ angiotensin receptor but recognizable by [Sar¹,Thr⁸]angiotensin II or PD123177 and at high concentrations by both DuP 753 and CGP42112A. Because angiotensin II was devoid of agonistic actions for prostaglandin release and the nonselective classical peptide antagonists did not block the angiotensin I response, these findings suggest that angiotensin I does not act through an angiotensin II receptor. The explanation for the blockade of angiotensin I–stimulated prostaglandin synthesis by either AT₁- or AT₂-selective angiotensin receptor antagonists remains unknown. (Hypertension 1992;19[suppl II]:II-49–II-55)

The vascular renin-angiotensin system is one example of tissue angiotensin-generating systems¹ that may participate in the long-term local regulation of vascular tone. Abundant data suggest that vascular endothelium is involved in the metabolism of several biologically active substances, including angiotensins²–⁴. Angiotensin II (Ang II) stimulates prostaglandin (PG) synthesis in a variety of organs as well as in components of the vasculature.⁵ PGS synthesized by vascular endothelium play an important role in modulation of the hemodynamic effect of various vasoactive agents, including angiotensins. Studies with isolated vessels have shown that the intimal surface has the greatest potential for the synthesis of prostacyclin as compared with vascular smooth muscle or fibroblasts.⁶–⁹ Prostacyclin is an extremely potent vasodilator and platelet antiaggregatory agent. Our recent studies have shown that the N-terminal heptapeptide of Ang II, angiotensin-(1-7) (Ang-(1-7)), also stimulates PG synthesis in vas deferens,⁴⁶ C6 glioma,¹¹ astrocytes,¹² and porcine smooth muscle cells (unpublished observation from our labo-
In the present study, we determined the subtype or subtypes of angiotensin receptor mediating PG synthesis in response to angiotensin peptides in porcine aortic endothelial cells (PAECs) using subtype-selective angiotensin receptor antagonists.

**Materials**

PGs were purchased from Sigma Chemical Co., St. Louis, Mo. [3H]PGE2, [3H]6-keto-PGF1α, and 125I-angiotensin I (Ang I) (2,200 Ci/mmol) were purchased from DuPont–New England Nuclear, Boston, Mass. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, penicillin, and streptomycin were obtained from GIBCO, Grand Island, N.Y. Angiotensin peptides were synthesized by M.C. Khosla. DuP 753 and PD123177 were gifts from P.B.M.W.M. Timmermans at DuPont, and CGP42112A was a gift from M. de Gasparo at CIBA-GEIGY. All reagents were of the highest purity available.

**Cell Culture**

PAECs were cultured according to published procedures. Briefly, thoracic aorta segments were split longitudinally, rinsed with serum-free medium, and the exposed intimal surface digested with collagenase (2 mg/ml in serum-free medium) for 15 minutes at 37°C. The detached endothelial cell patches were gently collected and placed in primary culture. Cells were grown in DMEM/F12 (1:1) medium containing 5% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO2. At confluence, cells were subcultured by trypsin-EDTA at a 1:3 ratio. Cells were used between passages 3–11. Endothelial cells grown in monolayers with a cobblestone appearance tested for factor VIII antigen by commercially available factor VIII antibodies.

**Determination of Prostaglandins by Radioimmunoassay**

The medium was directly assayed for PGE2 and 6-keto-PGF1α (the stable metabolite of PGI2) as previously reported in detail. The cross-reactivity of each antibody was less than 0.5% for other PGs. Experimental Protocol

All experiments were performed in monolayers of cells adherent in 24-well plates. Cell monolayers were washed twice with Hanks’ balanced salt solution and then incubated with different doses of angiotensin peptides (10^{-10} to 10^{-6} M) for 15 minutes at 37°C. At the end of the incubation, the medium was removed to measure PG by radioimmunoassay. For experiments that investigated the effect of angiotensin receptor antagonists, cells were pretreated for 5 minutes with the antagonist before incubation for 15 minutes with the angiotensin peptide (10^{-8} M). PG release was measured in the media as described above. Metabolism of 125I-Ang I (0.2 nM) by the cells was studied over 60 minutes at 37°C in Hanks’ balanced salt solution according to procedures published previously by us.

**Results**

In all experiments, basal release of prostacyclin was greater than that found for PGE2 release (24±3.2 versus 1.9±0.2 ng/mg protein). The effects of angiotensin peptides on PG release were investigated over a dose range of 10^{-10} to 10^{-6} M, and the results are shown in Figure 1. Ang-(1-7) stimulated both PGE2 and PGI2 release, producing a maximal response at 10^{-8} M, with a half maximal effect (EC50) at approximately 1 nM. Although apparently less potent than Ang-(1-7), Ang I also stimulated PGE2 and PGI2 release during the 15-minute collection, with the maximal response averaging 142±29% and 104±23% above basal, respectively. The EC50 value for Ang I was also in the nanomolar range. In contrast to Ang-(1-7) and Ang I, PG release was not significantly stimulated by Ang II even at concentrations as high as 10^{-6} M. Bradykinin (10^{-6} M) stimulated PGE2 and PGI2 release by 89±25% and 162±34% above basal, respectively (p<0.05).
We also examined the time course of PG release in response to 10^{-7} M angiotensin peptides (Figure 2). Ang-(1-7) produced maximal release of both PGE_2 and PGI_2 at 15 minutes, and these effects were maintained for 60 minutes of observation. Although less potent than Ang-(1-7), Ang I stimulated release of PGE_2 over a similar time course, with peak values found at 15 minutes and remaining stable for 60 minutes. In contrast, the release of PGI_2 in response to Ang I did not reach a plateau during the 60 minutes of study. At 60 minutes, however, the level of stimulation was comparable to that produced by Ang-(1-7).

To determine whether Ang I was converted to Ang-(1-7), we studied the metabolism of 125I-Ang I in the PAECs and analyzed the products by high-performance liquid chromatography. At 15 minutes, 90±0.1% of Ang I (n=2) remained intact. Ang-(1-7) represented 3±0.6% and Ang II represented 3±0.4% at this time point. At 60 minutes, Ang I still represented the major component (80±1%) of the total radioactivity, with 6±1% identified as Ang-(1-7) and 5±0.2% as Ang II (n=2). These results indicate that the majority of the added Ang I remains intact, even at the longer incubation time, suggesting that the Ang I effects during the 60-minute incubation are not likely to be a result of total conversion to Ang-(1-7).

Effects of Angiotensin Receptor Antagonists on Angiotensin-(1-7)-Stimulated Prostaglandin Release

The classical nonselective angiotensin receptor antagonists [Sar',Thr^8]Ang II and [Sar',Ile^8]Ang II have been shown to block the biological activity of Ang II in several studies.\textsuperscript{11,12} We found that these nonselective antagonists also display differential ability to block the effects of Ang II versus Ang-(1-7) in C6 glioma and human astrocytes, suggesting their ability to discriminate among angiotensin receptor subtypes.\textsuperscript{11-12} In PAECs, [Sar',Thr^8]Ang II but not [Sar',Ile^8]Ang II reduced Ang-(1-7)-elicited PGE_2 and PGI_2 release from 130±35% to 6±16% (p<0.0005) and from 65±25% to 23±9% (p<0.05) above basal, respectively (Figure 3). [Sar',Thr^8]Ang II and [Sar',Ile^8]Ang II (10^{-6} M) alone stimulated PGI_2 by 127±9% and 124±24% above basal, respectively (p<0.05), with no effect on PGE_2 release.

The actions of the subtype-selective angiotensin receptor antagonists DuP 753, PD123177, and CGP42112A were also investigated against the PG-releasing action of Ang-(1-7) in PAECs. As shown in the top panels of Figure 4, DuP 753 (10^{-6} M), a nonpeptide AT_1 antagonist,\textsuperscript{17} did not inhibit Ang-(1-7)-induced PGE_2 or PGI_2 production. Likewise, CGP42112A (10^{-6} M), a peptidic AT_2 antagonist,\textsuperscript{18} did not alter Ang-(1-7)-stimulated PG production (Figure 4, middle panels). DuP 753 or CGP42112A completely blocked Ang-(1-7)-enhanced PG production at a 1,000-fold higher concentration (10^{-5} M) only (data not shown). In contrast, PD123177 (10^{-7} M), a nonpeptide AT_2 antagonist,\textsuperscript{17} significantly blocked Ang-(1-7)-stimulated PG production (Figure 4, bottom panels). As shown in the bottom panels of Figure 4.
**Ang-(1-7)** -7 -6

**+Log DuP 753 (M) +Log DuP 753 (M)**

**f**

**100**

**50**

**T**

**i**

**i**

**150**

**SO'**

**•fLogPD 123177 (M) •fLogPD 123177 (M)**

**i**

**T**

**Ang-(1-7)**

**-7 -8**

**+U>gPD 123177 (M)**

**4, PD123177 at 10^-7 M reduced PGE_2 and PGI_2 release by approximately 50-75% of control values. In contrast to the potent stimulatory action of DuP 753 on PGI_2 in previous studies using different cell types, the compound at 10^-6 and 10^-5 M was completely devoid of stimulatory effects on either PGE_2 or PGI_2 release in PAECs. Neither of the AT_2 angiotensin receptor antagonists stimulated PGE_2 or PGI_2 release.**

**Effect of Angiotensin Receptor Antagonists on Angiotensin I-Stimulated Prostaglandin Release**

Neither [Sar',Thr^2]Ang II nor [Sar',Ile^2]Ang II significantly reduced Ang I-stimulated PG production (Figure 5). However, DuP 753 (10^-6 M) decreased PGE_2 production by 80% (p<0.01) and reduced PGI_2 release by 50% (p<0.05) (Figure 6A). CGP42112A significantly reduced Ang I-stimulated PGE_2 release but did not significantly inhibit PGI_2 release at the same concentrations (Figure 6B). These data suggest a difference in receptor subtypes linked to PGE_2 and PGI_2 release, as we previously found in C6 glioma cells.**

Although PD123177 inhibited Ang-(1-7)-stimulated PGE_2 and PGI_2 release, it failed to decrease Ang I-stimulated PGE_2 and PGI_2 release at similar concentrations (Figure 6C). These results again demonstrate a difference in the ability of the two subtype 2 antagonists to block the stimulatory effect of angiotensin peptides on PG release.

**Discussion**

PGs formed within blood vessels modulate the action of circulating vasoactive hormones. The effect of the vasodilator PGs (PGE_2, PGI_2, and PGD_2) or vasoconstrictor PGs (thromboxane A_2 and PGF_2a) can either offset or mediate vascular responses elicited by various hormonal systems. The ultimate response may depend, however, on both the type of PGs produced and the type of cells involved. The endothelium is a source for the production of several vasoactive peptides and PGs, and because various vasoactive agents stimulate PG production both in vitro and in vivo, the location of the cells that they affect might determine the type of PG released. For example, PGI_2 is the major product of arachidonic acid metabolism in porcine aortic vascular tissue, whereas isolated PAECs synthesize predominantly PGI_2 and PGE_2.23 In porcine smooth muscle cells, the predominant prostanoid is PGE_2.23 The results of the present study indicate that in PAECs, application of Ang-(1-7) or Ang I stimulated PGI_2 as the predominant prostanoid, although PGE_2 was also detected. Interestingly, Ang II (10^-5 to 10^-6 M) was ineffective in stimulating either PGE_2 or PGI_2 release in PAECs, in accordance with previous findings. Under the same conditions, however, Ang II produced a twofold to threefold stimulation for PG release in porcine smooth muscle cells. Moreover, in human endothelial cells, bradykinin but not Ang II stimulated PG release.21-29 Similarly, in the present study, we found that Ang I, Ang-(1-7), and bradykinin stimulated PG release. In contrast, Ang II had no effect. Gimbrone and Alexander reported that Ang II released a PGE-like material from cultures of human umbilical vein endothelial cells; therefore, it is possible that different cells in culture may express different receptors. Although this variability is often related to loss of Ang II receptors at higher passages, this did not seem to be the case in the present study, as no difference in responsiveness was observed in cells over passages 3-11.

The present study further suggests that in PAECs, Ang I and Ang-(1-7) stimulated PGE_2 and PGI_2 release via activation of different subtypes of angiotensin receptors. First, Ang I-induced PGE_2 release was blocked by DuP 753 and CGP42112A but not by...
PD123177. Second, Ang I–stimulated PGI₂ release was blocked by DuP 753 only. Third, Ang-(1–7)–stimulated PGE₂ and PGI₂ release was inhibited by low doses of PD123177 only. Moreover, the majority of Ang I was not metabolized to Ang-(1–7). That CGP42112A blocked only the Ang I–stimulated release of PGE₂ but not the release of PGI₂ further suggests that the release of the two PGS is coupled to different pools of arachidonic acid. We have previously shown this differential effect of angiotensin receptor antagonists in blocking PGE₂ versus PGI₂ release in other cells such as porcine smooth muscle cells (unpublished observation from our laboratory), rat C6 glioma, and human astrocytes. Ang II–, bradykinin–, and vasopressin–stimulated PG and thromboxane production by affecting two functionally different pools of phospholipases. Ang II–, bradykinin–, and vasopressin–stimulated PG synthesis in kidney and mesangial cells is also linked to activation of distinct lipid pools.

Two key reactions involved in PG synthesis are the mobilization of arachidonic acid from membrane.
phospholipids catalyzed by phospholipase A₂, C, or D and the conversion of arachidonic acid by the cyclooxygenase/peroxidase enzyme system. At present, it is not clear whether Ang-(1-7) and Ang I stimulate the same or different phospholipases to release arachidonic acid for PG production in PAECs. Whether the stimulation of PG synthesis by Ang I and Ang-(1-7) is due to an increase in arachidonic acid production or to enhancement in cyclooxygenase enzyme activity also is not known. In human endothelial cells, histamine released more arachidonic acid than was converted to PGs, suggesting that cyclooxygenase may be a rate-limiting step in PG synthesis. It is also possible that a particular pool of arachidonic acid is released from a lipid pool not coupled to cyclooxygenase. The difference in the time course of the stimulation of PG₁ release is compatible with the idea that Ang-(1-7) acts through a membrane receptor coupled directly to the production of PG. However, the longer time course for Ang I may reflect an internalization of the peptide via a Ca²⁺-dependent mechanism, with this phenomenon blocked by DuP 753.

The observation in the present study that Ang-(1-7) is a potent stimulus for PG production is consistent with previous observations in C6 glioma, human astrocytes, and vas deferens. Ang-(1-7) also stimulated the release of cytochrome P-450 metabolite in transporting epithelial cells of rat kidney. On the basis of these and other studies, we conclude that Ang-(1-7) is not a biologically inactive peptide. Moreover, recent studies showed that Ang-(1-7) is a principal metabolite of Ang I in neuroblastoma cells in culture and in canine brain stem. Ang-(1-7) is generated both in the presence or absence of the angiotensin converting enzyme inhibitor MK422, and it is also endogenously present in the brain. Ang I can also be metabolized to Ang-(1-7) in human umbilical vein endothelial cells, although this pathway was not particularly robust in the PAECs studied here.

There is substantial evidence that all of the essential components of the renin-angiotensin system are present in blood vessels. Although the endothelium has been shown to be the primary target for the conversion of Ang I to Ang II by converting enzyme, a recent study suggests that Ang I can be converted to Ang II in subendothelial tissue by a chymostatin-sensitive Ang II-generating enzyme. In past studies, the vascular renin-angiotensin system has been suggested to play an important role in the maintenance of high blood pressure in the chronic phase of experimentally induced hypertension. Because endothelial cells are also capable of synthesizing PG in response to Ang-(1-7), these new findings reaffirm the concept that angiotensins synthesized in vascular tissue may be directly involved in the regulation of local vascular tone.

In conclusion, the results in the present study indicate that in PAECs, Ang-(1-7) and Ang I stimulate PG synthesis through activation of different receptor subtypes based on the differential potencies of several selective angiotensin receptor antagonists. Interestingly, Ang II had no significant stimulatory effect in this cell line, even with doses up to 10⁻⁶ M, consistent with previous reports. Moreover, the pattern of blockade with classical nonselective or new subtype-selective antagonists suggests that the receptors mediating the two angiotensin peptide responses do not fit the classical concepts of Ang II receptors or that the antagonists used are not truly selective. Whereas the Ang-(1-7)-elicited PG release could be blocked by low doses of [Sar₁,Thr⁴]Ang II and PD123177, the concentrations of either DuP 753 or CGP42112A required for blockade exceeded those used in previous studies. Thus, the findings appear to rule out involvement of an AT₁ receptor, because DuP 753 was without inhibitory effects at low concentrations. However, the different actions of PD123177 and CGP42112A do not favor involvement of an AT₂ receptor as defined by these compounds in past studies. In addition, the effects of Ang I could not be blocked by the classical antagonists, even though the DuP 753 and CGP42112A compounds had inhibitory effects. This would suggest that DuP 753 and CGP42112A have actions in addition to Ang II receptor blockade, especially because Ang II was essentially devoid of stimulatory actions over a wide dose range. Nonetheless, because Ang I and Ang-(1-7) significantly enhanced PG production, these two angiotensin peptides may act as endogenous modulators for counteracting the vasoconstrictor effect of Ang II on vascular smooth muscle.

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