Angiotensin Receptor Subtype 1 Mediates Angiotensin II Enhancement of Isoproterenol-Induced Cyclic AMP Production in Preglomerular Microvascular Smooth Muscle Cells

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ABSTRACT

In a previous study, we found that angiotensin (Ang) II enhances β-adrenoceptor-induced cAMP production in cultured preglomerular microvascular smooth muscle cells (PMVSMCs) obtained from spontaneously hypertensive rats. The purpose of the present investigation was to identify the Ang receptor subtypes that mediate this effect. In our first study, we compared the ability of Ang II, Ang III, Ang (3–8), and Ang (1–7) to increase cAMP production in isoproterenol (1 μM)-treated PMVSMCs. Each peptide was tested at 0.1, 1, 10, 100, and 1000 nM. Both Ang II and Ang III increased intracellular (EC50s, 1 and 11 nM, respectively) and extracellular (EC50s, 2 and 14 nM, respectively) cAMP levels in a concentration-dependent fashion. In contrast, Ang (3–8) and Ang (1–7) did not enhance either intracellular or extracellular cAMP levels at any concentration tested. In our second study, we examined the ability of L 158809 [a selective Ang receptor subtype 1 (AT1) receptor antagonist] to inhibit Ang II (100 nM) and Ang III (100 nM) enhancement of isoproterenol-induced cAMP production in PMVSMCs. L 158809 (10 nM) abolished or nearly abolished (p < .001) Ang II and Ang III enhancement of isoproterenol-induced intracellular and extracellular cAMP levels. In contrast, PD 123319 (300 nM; a selective AT2 receptor antagonist) did not significantly alter Ang II enhancement of isoproterenol-induced intracellular or extracellular cAMP levels. We conclude that AT1 receptors, but not AT2, Ang (3–8), nor Ang (1–7) receptors mediate Ang II and Ang III enhancement of β-adrenoceptor-induced cAMP production in cultured PMVSMCs.

ABBREVIATIONS: Ang, angiotensin; PMVSMCs, preglomerular microvascular smooth muscle cells; IBMX, 3-isobutyl-1-methylxanthine

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Although angiotensin (Ang) II is a potent vasoconstrictor in vivo, recent studies indicate that in vascular smooth muscle cells cultured from conduit arteries, Ang II enhances the accumulation of cAMP in response to agonists that stimulate adenylyl cyclase. Kubalak and Webb (1993) reported that Ang II enhances cAMP production in response to isoproterenol, adenosine, and prostacyclin in cultured rat aortic smooth muscle cells, a finding later confirmed by McCumbee et al. (1996), who showed that Ang II enhances isoproterenol-induced cAMP production in rat aortic smooth muscle cells. Moreover, this effect appears to occur in intact blood vessels, because Ang II enhances vasodilation of the rat aorta by agents that elevate cAMP levels (Brizzolara-Gourdie and Webb, 1997).

Although Ang II enhances agonist-stimulated cAMP formation in smooth muscle cells from large conduit arteries, whether this occurs in smooth muscle cells from resistance vessels was, until very recently, unknown. Studies from our laboratory have demonstrated marked Ang II enhancement of isoproterenol-induced cAMP production in cultured preglomerular microvascular smooth muscle cells (PMVSMCs) obtained from both normotensive Wistar Kyoto rats and spontaneously hypertensive rats (Mokkapati et al., 1998).

Which Ang receptor mediates Ang II enhancement of agonist-induced cAMP production in vascular smooth muscle cells has not been carefully evaluated. Ang II per se interacts with two receptor subtypes designated AT1 and AT2 receptors (Timmermans et al., 1993). Because AT2 receptors, similar to cAMP, may cause vasodilation (Scheuer and Perrone, 1993; Arima et al., 1997), it is possible that Ang II enhancement of agonist-induced cAMP production is mediated by AT2 receptors. It is also possible that Ang II is metabolized by vascular smooth muscle cells to Ang (3–8) and/or Ang (1–7), two peptides that cause vasodilation via their own receptors distinct from AT1 and AT2 receptors (Kramar et al., 1997; Iyer et al., 1998).

Because Ang II enhancement of agonist-induced cAMP production may represent an important mechanism to protect vascular smooth muscle cells from too much Ang II-induced vasoconstriction and/or proliferation, it is important to fully characterize the Ang receptor subtypes that mediate...
this interesting effect. Accordingly, the purpose of this investigation was to elucidate the Ang receptor subtypes that mediate Ang II enhancement of agonist-induced cAMP production in culture vascular smooth muscle cells obtained from preglomerular microvessels.

**Materials and Methods**

**Materials.** Cell culture supplies were obtained from Gibco Laboratories (Grand Island, NY), with the exception of donor-defined fetal calf serum, which was purchased from Hyclone Laboratories (Miami, FL). Reagents and drugs were obtained as follows: iron oxide and chloroacetaldehyde were obtained from Aldrich Chemical Co. (Milwaukee, WI); Ang II, Ang III, Ang (3–8), Ang (1–7), (L)-isoproterenol and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma Chemical Co. (St. Louis, MO); PD 123319 was obtained from Research Biochemicals International (Natick, MA); L 158809 was obtained from Merck and Co. (West Point, PA); and 1-propanol was obtained from J.T. Baker (Phillipsburg, NJ). Immunohistochemicals were characterized by obtaining. All other chemicals were of the highest grade available.

**Animals.** In this study, PMVSMCs were obtained from male spontaneously hypertensive rats (12–14 weeks of age) purchased from Taconic Farms (Germantown, NY). PMVSMCs from spontaneously hypertensive rats were used because in a previous study we determined that Ang II enhancement of isoproterenol-induced cAMP production was much greater in PMVSMCs from spontaneously hypertensive rats compared with normotensive Wistar Kyoto rats (Mokkapati et al., 1998). Animals were housed in the University of Pittsburgh Animal Facility with controlled temperature, relative humidity, and light cycle (22°C, 55%, and lights on 7 AM to 7 PM, respectively). Rats were fed Prolab RMH 3000 obtained from PM Feeds (St. Louis, MO) and were given tap water ad libitum. Animal care conformed with institutional guidelines, and studies had prior approval of the Institutional Animal Care and Use Committee.

**Cell Culture.** PMVSMCs were cultured, purified, and characterized as previously described in detail (Dubey et al., 1992; Mokkapati et al., 1998). Briefly, renal microvessels were isolated by injecting iron oxide particles into the kidneys, mincing and dispersing the renal cortical tissue, and using a magnet to retain the iron-laden vessels. Microvessels were digested with collagenase and passed through a hypodermic needle to shear off glomeruli. The arteriolar vessels were treated with a millimeter model 2360, 4.6 mm mesh) was suspended in supplements DMEM with 20% fetal calf serum, plated, and incubated (37°C in 5% CO2, 95% air, and 98% humidity). The medium was changed daily until cells attained confluence. The PMVSMCs were repeatedly submitted to selective plating (Aviv et al., 1983) to reduce fibroblast contamination. Experiments were conducted at confluence in the third to fifth passage. At this passage level, cells retain their phenotypic characteristics and grow exponentially. PMVSMCs were characterized by morphology (hill-and-valley pattern of growth), functional responses (contraction to Ang II and norepinephrine), and immunofluorescence staining (positive for smooth muscle-specific α- and γ-isoactin, heavy chain myosin and desmin, and negative for von Willebrand factor). Cell culture conditions were kept at ASPET Journals on November 12, 2017 jpet.aspetjournals.org Downloaded from jpet.aspetjournals.org at ASIET Journals on November 12, 2017.
cAMP production, the degree of Ang II enhancement of isoproterenol-induced cAMP production was calculated and compared (unpaired Student's t-test; Number Cruncher Statistical System, Kaysville, UT) for control PMVSMCs versus PMVSMCs treated with a receptor antagonist. Four culture wells were grouped in an experimental block, where each experimental block consisted of control PMVSMCs and PMVSMCs treated with either isoproterenol, an Ang peptide, or isoproterenol plus an Ang peptide. For a given experimental block, isoproterenol-induced cAMP production in the absence of Ang peptides was calculated by subtracting the cAMP levels in the control cells from the cAMP levels in the isoproterenol-treated cells. Similarly, the isoproterenol-induced cAMP production in the presence of an Ang peptide was calculated by subtracting the cAMP levels in the Ang peptide-treated cells from the cAMP levels in the isoproterenol- and Ang peptide-treated cells. Then, for each experimental block, the net effect of an Ang peptide on isoproterenol-induced cAMP production was calculated by subtracting the isoproterenol-induced cAMP production in the absence of Ang peptide from the isoproterenol-induced cAMP production in the presence of an Ang peptide. Therefore, the net effect of an Ang peptide on isoproterenol-induced cAMP production for each experimental block was calculated as: (cAMP levels in Ang peptide/isoproterenol-treated cells) – (cAMP levels in Ang peptide-treated cells) – (cAMP levels in isoproterenol-treated cells) – (cAMP in control cells). Values in text and figures indicate means ± S.E.M. (n = 4).

Results

Figure 1 summarizes the effects of Ang II, Ang III, Ang (3–8), and Ang (1–7) on intracellular (Fig. 1A) and extracellular (Fig. 1B) cAMP levels in PMVSMCs treated with isoproterenol (1 μM) and IBMX (1 mM). Neither Ang (3–8) nor Ang (1–7) increased intracellular or extracellular cAMP levels. In contrast, both Ang II and Ang III markedly increased cAMP levels. When these data were fitted to a sigmoidal concentration-response curve, the basal and maximal intracellular and extracellular cAMP levels were similar for Ang II compared with Ang III (basal intracellular cAMP levels, 1683 ± 90 and 1643 ± 42 pmol/mg protein for Ang II and Ang III, respectively; maximal intracellular cAMP levels, 3088 ± 68 and 2896 ± 55 pmol/mg protein for Ang II and Ang III, respectively; basal extracellular cAMP levels, 980 ± 38 and 1019 pmol/mg protein for Ang II and Ang III, respectively; maximal extracellular cAMP levels, 1956 ± 34 and 2039 ± 26 pmol/mg protein for Ang II and Ang III, respectively). Ang II was approximately 10-fold more potent compared with Ang III in increasing both intracellular and extracellular cAMP levels, and the log EC_{50} (molar concentrations) for Ang II (extracellular, −8.68 ± 0.11; intracellular, −9.04 ± 0.16) were significantly less (p < .001) than the log EC_{50} (molar concentrations) for Ang III (extracellular, −7.86 ± 0.06; intracellular, −7.96 ± 0.11).

Figure 2 illustrates the effects of L 158809 on Ang II (100 nM) and Ang III (100 nM) enhancement of isoproterenol (1 μM)-induced intracellular and extracellular cAMP levels. L 158809 blocked the enhancement of isoproterenol-induced cAMP levels by both Ang II and Ang III (p-values < .0001 for all comparisons). In contrast, PD 123319 had no detectable effect on either Ang II or Ang III enhancement of isoproterenol-induced cAMP levels (Fig. 3).

Discussion

Previous studies have documented that Ang II enhances agonist-induced cAMP production in macrovascular (rat aorta) smooth muscle cells (Kubalak and Webb, 1993; McCumbee et al., 1996), and we recently extended this concept to microvascular (rat renal microvessels) smooth muscle cells (Mokkapatti et al., 1998). The present study demonstrates that Ang III, like Ang II, enhances agonist-induced cAMP production in cultured microvascular smooth muscle cells and unequivocally establishes that the AT1 receptor is solely responsible for Ang II and Ang III enhancement of agonist-induced cAMP production in cultured microvascular smooth muscle cells. Finally, the present study shows that neither Ang (3–8) nor Ang (1–7) enhances agonist-induced cAMP production in cultured microvascular smooth muscle cells.

Ang (3–8) and Ang (1–7) are biologically active Ang peptides. Therefore, it is conceivable that Ang II enhancement of agonist-induced cAMP production is mediated by metabolism of Ang II to Ang (3–8) and/or Ang (1–7). However, the present study demonstrates that Ang (3–8) and Ang (1–7) have no effect on agonist-induced cAMP production even at very high concentrations. This finding virtually eliminates the possibility that the effects of Ang II on agonist-induced cAMP production are mediated by conversion of Ang II to Ang (3–8) and Ang (1–7) in cultured PMVSMCs. Therefore, in cultured...
PMVSMCs, neither the Ang (3–8) receptor nor the Ang (1–7) receptor mediates the effects of Ang II on agonist-induced cAMP production. However, because we do not know whether cultured PMVSMCs possess Ang (3–8) or Ang (1–7) receptors, we cannot logically exclude the possibility that Ang (3–8) and/or Ang (1–7) receptors enhance agonist-induced cAMP production in other cell types/tissues.

Our concentration-response study demonstrates that Ang II is 10-fold more potent than Ang III with in enhancing agonist-induced cAMP production. If all the effects of Ang II are mediated by conversion to Ang III, then the potency of Ang II would be less than or equal to the potency of Ang III. Because this is not the case, Ang II must enhance agonist-induced cAMP at least in part independent of conversion to Ang III. Thus, both Ang II and Ang III directly activate Ang receptors that cross-talk in some manner with adenylyl cyclase.

The observation that Ang II is 10-fold more potent than Ang III in the enhancement of agonist-induced cAMP production is consistent with the AT₁ receptor mediating the effects of Ang II and Ang III on cAMP levels in cultured PMVSMCs. In general, in cases in which the AT₁ receptor mediates a given biological effect, Ang II is usually approximately 10-fold more potent than Ang III (Pendleton et al., 1991; Fujimoto et al., 1992; Robertson et al., 1992; Li et al., 1995, 1996, 1997). However, the reduced potency of Ang III relative to Ang II is at least in part due to increased susceptibility of Ang III to degradation by aminopeptidases (Fujimoto et al., 1992; Robertson et al., 1992), and Ang II and Ang III are equipotent in stimulating aldosterone secretion by the adrenal gland (Campbell et al., 1974), inducing pressor responses when injected into the cerebral ventricles (Wright et al., 1996) and causing vasoconstriction of the cat pulmonary circulation (Cheng et al., 1994). Also, the relative potencies of Ang II and Ang III for AT₂ receptor-mediated effects are not

![Fig. 2. Effects of L 158809 on 100 nM Ang II (A) and 100 nM Ang III (B) enhancement of isoproterenol (1 μM)-induced cAMP levels in PMVSMCs in extracellular (top) and intracellular (bottom) compartments. Values indicate means ± S.E.M. (n = 4).](image1)

![Fig. 3. Effects of PD 123319 on 100 nM Ang II enhancement of isoproterenol (1 μM)-induced cAMP levels in PMVSMCs in extracellular (A) and intracellular (B) compartments. Values indicate means ± S.E.M. (n = 4).](image2)
well characterized, because few biological responses have been discovered for the AT2 receptor. Therefore, the relative potencies of Ang II and Ang III do not definitively implicate the AT1 receptor.

To test the hypothesis that AT1 receptors mediate Ang II and Ang III enhancement of agonist-induced cAMP production in PMVSMCs, we examined the effects of Ang II and Ang III on agonist-induced cAMP production in the presence and absence of L 158809, a potent and highly selective AT1 receptor antagonist with an IC50 of 0.3 nM for the AT1 receptor versus an IC50 of >10,000 nM for the AT2 receptor (Chang et al., 1992). In our study, 10 nM L 158809 markedly inhibited Ang II and Ang III enhancement of agonist-induced cAMP production. Because the concentration of L 158809 used in the present study was 15-fold greater than the IC50 of PD123319 (2274 versus 2169 pmol/mg protein in the absence and presence of PD 123319, respectively). Because the concentration of PD123319 used in the present study was 15-fold greater than the IC50 of PD 123319 for the AT2 receptor, these results strongly implicate the AT2 receptor as that AT receptor subtype mediating Ang II and Ang III enhancement of agonist-induced cAMP production in PMVSMCs.

A final experiment was conducted to directly assess the role of the AT2 receptor in Ang II enhancement of agonist-induced cAMP production. PD 123319 is a potent and highly selective AT2 receptor antagonist with an IC50, as determined by competition binding studies, of 21 nM for the AT2 receptor versus an IC50 of >10,000 nM for the AT1 receptor (Dudley et al., 1990). In our study, 300 nM PD 123319 had no effect on Ang II enhancement of agonist-induced cAMP production. It is highly unlikely that this negative result was due to partial agonist effects of PD 123319, because isoproterenol-induced cAMP production was nearly identical in the presence and absence of PD 123319 (2274 versus 2169 pmol/mg protein in the absence and presence of PD 123319, respectively). Because the concentration of PD123319 used in the present study was 15-fold greater than the IC50 of PD 123319 for the AT2 receptor, these results rule out any involvement of the AT2 receptor in Ang II enhancement of agonist-induced cAMP production. Although we did not test the effects of PD 123319 on Ang III enhancement of agonist-induced cAMP production, given the observation that L 158809 completely abolished Ang III enhancement of agonist-induced intracellular cAMP levels, it is unlikely that any receptor subtype other than the AT2 receptor mediates Ang III enhancement of agonist-induced cAMP production in PMVSMCs.

Our conclusion that the AT1 receptor mediates Ang II and Ang III enhancement of agonist-induced cAMP production in cultured PMVSMCs is entirely consistent with recent published studies using cloned and expressed AT1 receptors. Baulak et al. (1994) reported that Ang II enhanced adenocorticotropic-induced cAMP production in COS-7 cells transfected with the rat AT1 receptor. Similarly, Klingler et al. (1998) found that Ang II enhanced vasopressin-induced cAMP production in Chinese hamster ovary cells transfected with cDNA for both AT1 and V2 receptors. Thus, these important studies indicate that in transfected cells, AT1 receptors can enhance agonist-induced cAMP production. Our studies extend this concept to native AT1 receptors residing in functional microvascular smooth muscle cells.

An important issue is the mechanism by which Ang II and Ang III enhance agonist-induced cAMP levels. Because the concentration-response study was conducted in the presence of a very high concentration of a “broad spectrum” phosphodiesterase inhibitor, the enhancement in cAMP levels caused by Ang II and Ang III cannot be attributed to changes in the rate of cAMP breakdown by phosphodiesterases. Moreover, because Ang II and Ang III enhanced both intracellular and extracellular levels of cAMP, changes in cAMP levels cannot be attributed to changes in cAMP egress from the cells. Therefore, the results of the present study implicate an effect of Ang II and Ang III on the synthesis of cAMP. Recently, we discovered that in PMVSMCs Ang II enhancement of isoproterenol-induced cAMP levels is mediated by protein kinase C (Mokkapati et al., 1998). Most likely Ang II and Ang III, via protein kinase C, augment the effects of agonists on adenylyl cyclase activity.

An important unresolved issue is the physiological significance of AT1 receptor-mediated enhancement of agonist-induced cAMP production. It is possible that in vivo Ang II enhancement of agonist-induced cAMP production protects vascular smooth muscle cells from the provasconstrictive, progrowth, and promigratory effects of Ang II. If so, this would represent an extremely important regulatory mechanism that, if malfunctioning, could lead to cardiovascular diseases such as hypertension, heart failure, and atherosclerosis.

In summary, the findings of the current study clearly indicate that Ang II and Ang III enhance agonist-induced cAMP production in PMVSMCs exclusively via the AT1 receptor. Future studies are needed to elucidate the physiological significance of this interesting interaction between Ang II and agonists that stimulate adenylyl cyclase.

References


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