Angiotensin II Relaxes Microvessels Via the AT2 Receptor and Ca$^{2+}$-Activated K$^+$ (BK$_{Ca}$) Channels

Christiana Dimitropoulou, Richard E. White, Leslie Fuchs, Hanfang Zhang, John D. Catravas, Gerald O. Carrier

Abstract—Angiotensin II (Ang II) is one of the most potent vasoconstrictor substances, yet paradoxically, Ang II may dilate certain vascular beds via an undefined mechanism. Ang II–induced vasoconstriction is mediated by the AT$_1$ receptor, whereas the relative expression and functional importance of the AT$_2$ receptor in regulating vascular resistance and blood pressure are unknown. We now report that Ang II induces relaxation of mesenteric microvessels and that this vasodilatory response was unaffected by losartan, an AT$_1$ receptor antagonist, but was inhibited by PD123,319, a selective antagonist of AT$_2$ receptors. In addition, reverse transcriptase–polymerase chain reaction studies revealed high amounts of AT$_2$ receptor mRNA in smooth muscle from these same microvessels. Ang II–induced relaxation was inhibited by either tetraethylammonium or iberiotoxin, suggesting involvement of the large-conductance, calcium- and voltage-activated potassium (BK$_{Ca}$) channel. Subsequent whole-cell and single-channel patch-clamp studies on single myocytes demonstrated that Ang II increases the activity of BK$_{Ca}$ channels. As in our tissue studies, the effect of Ang II on BK$_{Ca}$ channels was inhibited by PD123,319, but not by losartan. In light of these consistent findings from tissue physiology, molecular studies, and cellular/molecular physiology, we conclude that Ang II relaxes microvessels via stimulation of the AT$_2$ receptor with subsequent opening of BK$_{Ca}$ channels, leading to membrane repolarization and vasodilation. These findings provide evidence for a novel endothelium-independent vasodilatory effect of Ang II. (Hypertension. 2001;37:301-307.)

Key Words: angiotensin II, receptors, angiotensin, potassium channels, patch-clamp techniques

Many complications of hypertension and congestive heart failure are ameliorated by interdiction of the renin-angiotensin system, and agents that attenuate the effects of angiotensin II (Ang II) are now a first-line pharmacological antihypertensive intervention. Ang II induces constriction, hypertrophy, and proliferation of vascular smooth muscle via the AT$_1$ receptor. It is generally assumed that the therapeutic effects of AT$_1$ antagonists are due to direct receptor blockade; however, a secondary effect of these agents is increased plasma levels of Ang II,$^1$ which may then activate AT$_2$ receptors. This receptor bears only 34% sequence homology to the AT$_1$ receptor$^{2,3}$ and mediates growth inhibition$^4$ and apoptosis$^5$ of vascular smooth muscle. In contrast, very little is known about the role of AT$_2$ receptors in regulating blood pressure. Both Ang II receptors are expressed in the vasculature, but AT$_2$ receptor expression is heterogeneous with respect to vascular bed, species, and developmental stage.$^6$ Nonetheless, evidence suggests that AT$_2$ receptors modulate vascular contractility. AT$_2$ receptors mediate endothelium-dependent vasodilation of renal arterioles.$^7$ Furthermore, AT$_2$-deficient mice exhibit an increased pressor response to Ang II,$^8$ whereas overexpression of AT$_2$ receptors enhances Ang II–induced endothelium-dependent vasodilation.$^9$ These findings suggest that AT$_2$ receptors mediate Ang II–induced vascular relaxation; however, the cellular/molecular basis of Ang II–induced vasodilation is not clearly defined, nor has an effector mechanism been identified.

The purpose of the present study was to investigate the expression and physiological role of AT$_2$ receptors in the microvasculature. We now report significant expression of AT$_2$ receptors in mesenteric microvessels from adult rats and also demonstrate that Ang II relaxes these vessels via stimulation of the AT$_2$ receptor with subsequent opening of BK$_{Ca}$ channels, leading to membrane repolarization and vasodilation. These findings provide evidence for an endothelium-independent vasodilatory effect of Ang II on vascular smooth muscle cells and may help to explain the salutary effects of AT$_1$ receptor antagonists on the cardiovascular system, (eg, reduced blood pressure and infarct size$^{10}$ due to “indirect” stimulation of AT$_2$ receptors).

Methods

Isolated Microvessel Preparation

Fourth-order mesenteric arterial branches (200 to 300 μm in diameter) were isolated microscopically from male Sprague-Dawley rats.
(aged 14 to 16 weeks) in accordance with guidelines of the American Physiological Society for the humane treatment of experimental animals and prepared for measurement of intraluminal diameter by video dimension analysis as described previously.\(^1\) Vessels were allowed to equilibrate for 30 minutes and were preconstricted to \(\approx 45\%\) of their resting diameter with endothelin-1 (ET-1). After the constriction reached a steady state level, a complete concentration-response relaxation to Ang II was performed. In additional vessels a concentration-response relationship for Ang II was measured in the presence of tetraethylammonium (TEA), ibetiotrin (IBTX), losartan, or PD123,319.

Reverse Transcriptase–Polymerase Chain Reaction

Smooth muscle cells from 5 mesenteric arteries for each RNA isolation (n=15 rats, 3 RNA isolations) were harvested and pooled for total RNA measurement. As a negative control, RNA was also extracted from rat liver. Total RNA was isolated with the use of Trizol Reagent (GIBCO BRL). Phase separation was achieved by addition of chloroform, and RNA was precipitated from the aqueous phase with isopropyl alcohol. The amount of RNA was determined spectrophotometrically at \(A_{260}\). Total RNA (1 to 1.5 \(\mu\)g) was treated with DNase I (GIBCO BRL) for 15 minutes at room temperature and reverse transcribed (RT) with oligo(dT) using SuperScript (GIBCO BRL). Aliquots of the RT products were amplified with Taq DNA polymerase (GIBCO BRL) with the use of gene-specific primers. Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described previously with the use of primers derived from published cDNA sequences of the AT1B (accession X64052) and AT2 (accession D16840) receptors.\(^1\) Primer sequences for the AT1B were 5'-GGC ATT ATC CGT GAC TGT GAA A-3' (forward) and 5'-CTG CTT AGC CCA AAT GGT CCT C-3' (reverse), and the final PCR product was 446 bp in length. Primer sequences for the AT2 were 5'-GGC ATT ATC CGT GAC TGT GAA A-3' (forward) and 5'-CTG CTT AGC CCA AAT GGT CCT C-3' (reverse), and the final PCR product was 446 bp in length. PCR negative controls were (1) amplification of \(H_2 \text{O}\) and (2) non-RT (reverse), and the final PCR product was 446 bp in length. Primer sequences for the AT2 receptors were 5'-GGC ATT ATC CGT GAC TGT GAA A-3' (forward) and 5'-CTG CTT AGC CCA AAT GGT CCT C-3' (reverse), and the final PCR product was 446 bp in length. Primer sequences for the AT1B and AT2 receptors and *beta*-actin were 5'-TGCGAAACATTTCATGGACTG-3' (forward) and 5'-ACCACCATGTCGTCCTG-3' (reverse), and the final PCR product was 446 bp in length. PCRs were performed in 25 \(\mu\)l total reaction volume containing 12.5 \(\mu\)l of 2X PCR Master Mix (Promega), 1 \(\mu\)l of primer mix (0.5 \(\mu\)M forward and 0.5 \(\mu\)M reverse), 2 \(\mu\)l of RNase-DNAse free water, and 5 \(\mu\)l of cDNA template. The PCR conditions were as follows: Initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 65°C for 1 minute, and extension at 72°C for 1 minute. After PCR amplification, 10 \(\mu\)l of each PCR product was loaded into a 1.5% agarose gel and visualized with ethidium bromide stain. The sizes for the AT1B and AT2 receptors and *beta*-actin were 346, 446, and 276 bp, respectively. The gel is representative of 3 separate RNA samples from mesenteric arterial SMC.

Statistical Analysis

All data were expressed as mean\(\pm\)SE except data from tension studies, which were expressed as percentage of maximum relaxation. Statistical significance between 2 groups was evaluated with Student's \(t\) test, and a 1-way ANOVA was performed to evaluate significance between multiple groups. A \(P\) value <0.05 was considered to reflect a significant difference.

Results

Ang II Dilates Microvessels Via the AT\(_2\) Receptor

Baseline intraluminal diameter of microvessels (intraluminal pressure=40 mm Hg) was 226\(\pm\)12 \(\mu\)m (n >30). Vessels were preconstricted to \(\approx 45\%\) of the original baseline diameter with ET-1 (diameter reduced from 229\(\pm\)14 to 126\(\pm\)8 \(\mu\)m). In the absence of Ang II, ET-1–induced contraction was maintained for >60 minutes. A complete concentration-response curve for Ang II was then obtained. A typical tracing of Ang II–induced microvascular relaxation is illustrated in Figure 1A. On average, Ang II (0.1 to 300 mmol/L) induced a concentration-dependent relaxation with a maximum of 72\(\pm\)5\% (Figure 1B; n=19). Pretreating microvessels with N-nitro-l-arginine (0.1 mmol/L; 30 minutes) had no...
Ang II–induced relaxation (78±6%; n=4). A similar response to Ang II (78±3%; n=5) was obtained in microvessels pretreated for 30 minutes with 10 μmol/L losartan, a selective AT1 receptor antagonist (Figure 1B). In contrast, Ang II induced only 31±8% relaxation (n=10; Figure 1B) in microvessels pretreated for 30 minutes with 100 nmol/L PD123,319, a selective AT2 receptor antagonist. Pretreatment with these blocking agents did not affect baseline intraluminal diameter (Table). These studies indicate involvement of the AT2 receptor in Ang II–induced vasodilation, and subsequent molecular studies were designed to verify expression of this protein in rat mesenteric microvessels.

Ang II Receptor mRNA
Representative gels of AT1B and AT2 receptor mRNA from isolated mesenteric microvascular smooth muscle cells analyzed by RT-PCR are provided in Figure 1C. Each isolation was examined microscopically to ensure against contamination with other cell types; nonetheless, it is possible that some minor degree of contamination may have been present. Analysis consistently detected AT2 receptor mRNA. In contrast, amplification of liver RNA did not reveal any bands for Ang II receptor mRNA. Absence or very low copy of these Ang II receptors in liver is consistent with previous studies, whereas mRNA for β-actin was well detected in liver by RT-PCR.

Ang II–Induced Vasodilation Involves K+ Channels
We have demonstrated previously that disrupting the gradient for potassium efflux with high extracellular [K+] inhibits potassium channel–mediated vasorelaxation. To investigate whether AT2 receptor–mediated vasodilation is dependent on K+ efflux, microvessels were preconstricted with 80 mmol/L KCl. Ang II did not affect KCl-contracted arteries (data not shown). In microvessels preconstricted with ET-1, Ang II–induced relaxation was measured after a 30-minute pretreatment with a potassium channel inhibitor: 1 mmol/L TEA (n=4; Figure 2A) or 100 nmol/L IBTx (n=4; Figure 2B). In microvessels pretreated with both TEA and IBTx, Ang II–induced relaxation was abolished (data not shown).

Figure 2. Ang II–induced relaxation of microvessels is mediated by potassium channels. A, Average concentration-response relationship for Ang II relaxation of mesenteric microvessels in the presence of 1 mmol/L TEA. After addition of 1 μmol/L Ang II had no effect, subsequent addition of 10 μmol/L acetylcholine induced significant relaxation of the vessels. Each point represents the mean±SE of 4 experiments. B, Average concentration-response relationship for Ang II relaxation of mesenteric microvessels in the presence of 100 nmol/L IBTx. After addition of 1 μmol/L Ang II had no effect, subsequent addition of 10 μmol/L acetylcholine induced significant relaxation of the vessels. Each point represents the mean±SE of 4 experiments. C, Superimposed whole-cell current tracings from single mesenteric myocytes recorded with the perforated patch configuration. Cells were voltage-clamped at a holding potential of −250 mV, and currents were elicited by 100 ms depolarizing pulses to −40, −10, 20, or 50 mV before or 10 to 15 minutes after exposure to 100 nmol/L Ang II. In the right panel, the effect of subsequent addition of 1 mmol/L TEA is illustrated. Dashed line indicates baseline current. D, Complete current–voltage relationship for steady state outward current (holding potential=−50 mV) before and 10 to 15 minutes after addition of 100 nmol/L Ang II. The effect of subsequent addition of 1 mmol/L TEA on the same cell is also plotted.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Diameter, μm</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>Control</td>
</tr>
<tr>
<td>Baseline</td>
<td>229±14</td>
</tr>
<tr>
<td>Endothelin</td>
<td>126±8*</td>
</tr>
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Values are mean±SE. Neither PD123,319 nor losartan affected intraluminal diameters before or after endothelin treatment.

*Significant decrease in intraluminal diameter compared with baseline (before endothelin) conditions (P<0.05).
In the presence of these agents, even high concentrations of Ang II failed to produce significant relaxation. In contrast, subsequent treatment of microvessels with 10 μmol/L acetylcholine relaxed the microvessels 70±14% (TEA; n=4) or 73±10% (IBTx; n=4).

**Ang II Increases Potassium Currents in Single Myocytes**

Direct evidence that Ang II stimulated outward potassium currents in microvascular myocytes was obtained from whole-cell (perforated patch) patch-clamp recordings. Ang II (100 nmol/L) increased macroscopic outward current over the entire range of membrane voltages. For example, at +50 mV Ang II nearly tripled (287±35%; n=7; P<0.001) the steady state outward current. Representative tracings illustrating the effect of Ang II are provided in Figure 2C. The stimulatory effect of Ang II was reversed completely by subsequent treatment with 1 mmol/L TEA. A lower concentration of Ang II (10 nmol/L) nearly doubled (93±12% at +50 mV; n=3; P<0.05) steady state outward currents, and the effect was again abolished by 1 mmol/L TEA (decreased 27±3% below control values). The complete current-voltage relationship for these experiments is illustrated in Figure 2D. In contrast to TEA, neither 4-aminopyridine (1 mmol/L; n=3), a blocker of the delayed rectifier K⁺ channel, nor glibenclamide (10 μmol/L; n=3), a blocker of the ATP-sensitive K⁺ channel, affected Ang II–stimulated outward current (data not shown). These findings strongly suggested that the BKCa channel mediated Ang II–induced relaxation of microvessels, and subsequent single-channel studies were undertaken to prove this hypothesis.

**Ang II Stimulates BKCa Channel Activity**

Ang II stimulated the activity of single potassium channels in mesenteric myocytes. As illustrated in Figure 3A, there was minimal channel activity in cell-attached patches under control conditions (NPo<0.001 at +50 mV). However, channel activity was increased dramatically by 100 nmol/L Ang II (NPo 0.92; 15 minutes; Figure 3A), and this effect was concentration dependent (Figure 3B). Identification of this protein as the BKCa channel was demonstrated from experiments on cell-free inside-out patches in symmetrical (140 mmol/L) K⁺. Each point represents the mean±SE current of 3 to 5 patches, with the line fit by linear regression.
AT2 Receptors Mediate Ang II Stimulation of BKca Channels

Ang II receptor antagonists were used to determine which receptor mediated the stimulatory effect of Ang II on BKca channels. Channel activity stimulated by 100 nmol/L Ang II is illustrated in Figure 4 under control conditions (panel 2) or after a 30-minute pretreatment with 10 μmol/L losartan (panel 3). Blockade of AT1 receptors with losartan did not affect Ang II–induced channel activity. On average, control NPo was 0.001 ± 0.002, and after exposure to 100 nmol/L Ang II NPo increased to 0.86 ± 0.02 (n=4). Blockade of AT1 receptors, on the other hand, completely prevented the stimulatory effect of Ang II.

Figure 4. AT2 receptors mediate the stimulatory effect of Ang II on BKca channels. Channel openings (+50 mV) are upward deflections from baseline (closed state, as indicated by the dashed line). Top tracing, Recordings from the same inside-out patch with either 100 nmol/L or 100 μmol/L [Ca2+] in the solution at the cytoplasmic surface of the membrane, as indicated by the arrows. Ang II (100 nmol/L; 15-minute pretreatment) was also present in the bath solution. Second tracing, Recordings from the same cell-attached patch before and 10 minutes after addition of 100 nmol/L Ang II to the bath solution, as indicated by arrow. Third tracing, Recordings from the same cell-attached patch. Cell was pretreated with losartan (10 μmol/L; 30 minutes), and recordings were then taken before and 15 minutes after subsequent addition of 100 nmol/L Ang II, as indicated by arrow. Bottom tracing, Recordings from the same cell-attached patch. Cell was pretreated with PD123,319 (100 nmol/L; 30 minutes). Recordings were then taken before and 15 minutes after subsequent addition of 100 nmol/L Ang II, as indicated by arrow.
currents. In contrast, selective blockade of BK<sub>Ca</sub> channels (with either 1 mmol/L TEA or 100 nmol/L IBTx; Figure 2) abolished Ang II–induced relaxation. Although TEA can block several species of potassium channels, at this low concentration it exhibits selectivity for the BK<sub>Ca</sub> channel. IBTx, on the other hand, is a highly selective BK<sub>Ca</sub> channel antagonist. Moreover, our single-channel patch-clamp data clearly identified the BK<sub>Ca</sub> channel as a primary target of Ang II action in microvascular myocytes, and our findings that PD123,319 (but not losartan) inhibited this stimulatory effect of Ang II further implicate the BK<sub>Ca</sub> channel as an effector molecule for AT<sub>2</sub> receptor–mediated vasodilation. Although our findings clearly demonstrate an endothelium-independent effect of Ang II on microvascular myocytes, the vasodilatory response to Ang II in vivo would probably involve both endothelium-dependent and -independent mechanisms. In either case, our data obtained from intact tissues or single myocytes indicate that BK<sub>Ca</sub> channels expressed in microvascular smooth muscle cells are the critical effector molecules involved in AT<sub>2</sub> receptor–mediated relaxation of microvessels.

The signaling process linking AT<sub>2</sub> receptors to BK<sub>Ca</sub> channels in vascular smooth muscle is poorly understood. For example, metabolites of arachidonic acid might play a role in the response of BK<sub>Ca</sub> channels to Ang II in mesangial cells; however, it is unclear whether the response of BK<sub>Ca</sub> channels in these cells is mediated via AT<sub>1</sub> and/or AT<sub>2</sub> receptors. Therefore, it is difficult to draw strict comparisons between these 2 studies on different cell types and species. Nonetheless, because arachidonic acid metabolites open BK<sub>Ca</sub> channels in other arteries, a potential role of arachidonic acid in mediating the response of mesenteric microvessels to Ang II remains a possibility. Alternatively, BK<sub>Ca</sub> channels could be opened indirectly by Ang II–stimulated intracellular calcium. Although we have not tested this hypothesis conclusively, we do not believe that direct activation by calcium plays a major role because of the following: (1) Ang II induces vasodilation; therefore, the putative increase in calcium would have to be minimal and probably localized to the more peripheral regions of the cytoplasm near the BK<sub>Ca</sub> channels. Such a “calcium-spark” model is possible, but we are unaware of any studies suggesting that Ang II stimulates such a mechanism in microvessels. (2) Ang II produced no obvious shift in the voltage sensitivity of outward currents, whereas increased Ca<sup>2+</sup> shifts the sensitivity of BK<sub>Ca</sub> channels to more negative potentials. In contrast, the effect of Ang II appears to be due mainly to increased current amplitude rather than increased voltage sensitivity. In contrast to the aforementioned mechanisms, it is clear that cyclic nucleotide–dependent vasodilators open BK<sub>Ca</sub> channels in vascular smooth muscle. In the present study, however, inhibitors of either the cAMP- or the cGMP-dependent protein kinase had no effect on Ang II–stimulated BK<sub>Ca</sub> channel activity (data not shown). These data suggest that a more novel mechanism of action underlies the effect of Ang II in microvessels; however, further experiments are necessary to elucidate the transduction mechanism coupling AT<sub>2</sub> receptors to BK<sub>Ca</sub> channels.

It is clear that AT<sub>2</sub>-induced vasodilation affects blood pressure. Animals lacking AT<sub>2</sub> receptors exhibit an enhanced pressor response to Ang II, whereas overexpression of AT<sub>2</sub> receptors antagonizes AT<sub>1</sub> receptor–mediated pressor effects. AT<sub>1</sub> receptor–mediated vasodilation, particularly of microvessels, may serve as a negative feedback mechanism to counterbalance the potent vasoconstrictor effect of AT<sub>1</sub> receptor activation. For example, Ang II levels are increased during exercise, and AT<sub>2</sub> receptor–mediated dilation of precapillary vessels might help to offset potentially dangerous effects of diminished capillary perfusion in face of excessive AT<sub>1</sub> receptor stimulation. Therapeutically, it seems clear that AT<sub>2</sub> receptors mediate salutary responses. For example, AT<sub>2</sub> receptor stimulation may play a part in the antihypertensive effects of AT<sub>1</sub> receptor antagonists, which increase plasma Ang II levels. In addition, our proposed “endothelium-independent” vasodilatory effect of Ang II could constitute a protective vasodilatory mechanism to preserve tissue perfusion when the endothelium is damaged as a result of atherosclerosis and/or hemodynamic stress. Interestingly, activation of AT<sub>2</sub> receptors reduces infarct size. The present findings are the first to provide direct experimental evidence for a novel molecular effector (the BK<sub>Ca</sub> channel) that can mediate endothelium-independent relaxation of vascular smooth muscle via Ang II stimulation of the AT<sub>2</sub> receptor. Future studies will identify and characterize the postreceptor signal transduction cascade stimulated by Ang II in these microvessels.

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