Angiotensin II Relaxes Microvessels Via the AT_2 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, which may then activate AT_2 receptors. This receptor bears only 34% sequence homology to the AT_1 receptor and mediates growth inhibition and apoptosis 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. Nonetheless, evidence suggests that AT_2 receptors modulate vascular contractility. AT_2 receptors mediate endothelium-dependent vasodilation of renal arterioles. Furthermore, AT_2-deficient mice exhibit an increased pressor response to Ang II, whereas overexpression of AT_2 receptors enhances Ang II–induced endothelium-dependent vasodilation. 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 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.
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 isopropanol alcohol. The amount of RNA was determined spectrophotometrically at A_{260}. Total RNA (1 to 1.5 μg) was treated with DNase I (GIBCO BRL) for 15 minutes at room temperature and subsequently with RNase-free DNase I (GIBCO BRL) for 15 minutes at room temperature and then amplified for 35 or 38 cycles of 94°C for 40 seconds, 65°C for 1 minute, and 72°C for 1 minute. An aliquot of 10 μL of the RT-PCR amplified sample was fractionated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide. Arrows denote the DNA size marker. The sizes for the AT1{B} and AT2 receptors and the human β-actin (internal control) were 346, 446, and 276 bp, respectively. The gel is representative of 3 separate RNA samples from mesenteric arterial SMCs.

**Results**

**Ang II Dilates Microvessels Via the AT2 Receptor**

Baseline intraluminal diameter of microvessels (intraluminal pressure=40 mm Hg) was 226±12 μm (n>30). Vessels were preconstricted to ~45% of the original baseline diameter with ET-1 (diameter reduced from 229±14 to 126±8 μm). 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 nmol/L) induced a concentration-dependent relaxation with a maximum of 72±5% (Figure 1B; n=19). Pretreating microvessels with N-nitro-l-arginine (0.1 mmol/L; 30 minutes) had no effect.
effect on 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–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.11,14,18 To investigate whether AT2 receptor–mediated vasodilation is dependent on K+ efflux, microvessels were preconstricted with 80 mmol/L K1. 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

### Intraluminal Diameters of Mesenteric Microvessels

<table>
<thead>
<tr>
<th>Condition</th>
<th>Diameter, μm</th>
<th>Control</th>
<th>PD123,319</th>
<th>Losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>229±14</td>
<td>219±18</td>
<td>240±15</td>
<td></td>
</tr>
<tr>
<td>Endothelin</td>
<td>126±8*</td>
<td>109±10*</td>
<td>126±14*</td>
<td></td>
</tr>
</tbody>
</table>

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).

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,16,17 whereas mRNA for β-actin was well detected in liver by RT-PCR.

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 −50 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 on the same cell is also plotted.
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.
Blockade of AT1 receptors with losartan did not affect BKCa channel activity. Channel openings (----) by arrow. Third tracing, Recordings from the same cell-attached patch after subsequent addition of 100 nmol/L Ang II, as indicated by arrow. Bottom tracing, Recordings from the same cell-attached 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.

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 ± 0.02, 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. In the presence of PD123,319 (100 nmol/L; 30 minutes), 100 nmol/L Ang II had no effect on BKCa channel activity (NPo PD123,319, < 0.001; PD123,319 + Ang II, < 0.001; n = 4; bottom panel).

Discussion

We have demonstrated that Ang II relaxes mesenteric microvessels via a mechanism involving the AT2 receptor and BKCa channel activity, and this mechanism of Ang II action on intact tissues was confirmed by molecular studies on isolated microvascular myocytes demonstrating (1) expression of AT2 receptor mRNA and (2) AT2 receptor–mediated stimulation of BKCa channel activity in cell-attached patches. These findings provide evidence for a novel endothelium-independent effect of Ang II on the microvasculature. Previous studies suggest that Ang II relaxes cerebral microvessels or renal afferent arterioles by an endothelium-dependent mechanism, probably involving release of bradykinin and endothelium-derived dilators (eg, NO). In the present study, however, pretreating mesenteric microvessels with N-nitro-L-arginine did not affect Ang II–induced relaxation, suggesting that endothelium-derived NO does not play a role in the response of this vascular bed to Ang II. More directly, in the absence of endothelium Ang II clearly stimulated activity of BKCa channels in isolated myocytes.

Previous studies indicated that Ang II stimulates both whole-cell and single-channel potassium currents in cultured neurons via the AT1 receptor; however, this is the first study on the effects of Ang II on K+ channels in smooth muscle cells. Our studies demonstrate that Ang II increases both whole-cell and single-channel BKCa currents in mesenteric myocytes. Instead of traditional whole-cell recordings that require dialysis of the cytoplasm with exogenous calcium buffers, we used the perforated patch configuration to obtain whole-cell currents from “metabolically intact” myocytes. These findings indicated that Ang II increases a TEA-sensitive outward current in these cells, and subsequent single-channel studies revealed that this channel had a high conductance (>150 picoSiemens) and was stimulated by increasing “intracellular” calcium levels in inside-out patches. Therefore, we have identified this protein as the BKCa channel that is highly expressed in vascular smooth muscle and other cell types. Like other vascular smooth muscle cells, myocytes from mesenteric microvessels possess substantial outward K+ currents composed primarily of K+ efflux through BKCa channels. Because of their large conductance and high density of expression, these channels help to set and maintain the resting membrane potential of vascular smooth muscle. When intracellular [Ca2+] levels increase during contraction, BKCa channels provide an important repolarizing negative feedback mechanism that helps to reverse active contraction. Given the importance of BKCa channels in regulating vascular tone, these proteins constitute a powerful effector mechanism that mediates microvascular relaxation induced by a variety of vasodilatory agents, eg, nitrovasodilators. Our studies indicated that microvascular relaxation required physiological gradients of [K+] suitable for potassium efflux, suggesting involvement of potassium channels. Although several species of K+ channels are also expressed in vascular smooth muscle, we did not observe significant effects of Ang II on other channel species at the single-channel level. Moreover, neither glibenclamide (KATP channel antagonist) nor 4-aminopyridine (delayed rectifier channel antagonist) affected Ang II–stimulated whole-cell
currents. In contrast, selective blockade of BK_{Ca} 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_{Ca} channel. IBTx, on the other hand, is a highly selective BK_{Ca} channel antagonist. Moreover, our single-channel patch-clamp data clearly identified the BK_{Ca} 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_{Ca} channel as an effector molecule for AT_{2} 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_{Ca} channels expressed in microvascular smooth muscle cells are the critical effector molecules involved in AT_{2} receptor–mediated relaxation of microvessels.

The signaling process linking AT_{2} receptors to BK_{Ca} channels in vascular smooth muscle is poorly understood. For example, metabolites of arachidonic acid might play a role in the response of BK_{Ca} channels to Ang II in mesangial cells; however, it is unclear whether the response of BK_{Ca} channels in these cells is mediated via AT_{1} or AT_{2} receptors. 24 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_{Ca} channels in other arteries, 25 a potential role of arachidonic acid in mediating the response of mesenteric microvessels to Ang II remains a possibility. Alternatively, BK_{Ca} 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_{Ca} 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^{2+}]_{i} shifts the sensitivity of BK_{Ca} 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_{Ca} channels in vascular smooth muscle. 15 In the present study, however, inhibitors of either the cAMP- or the cGMP-dependent protein kinase had no effect on Ang II–stimulated BK_{Ca} 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_{2} receptors to BK_{Ca} channels.

It is clear that AT_{2}-induced vasodilation affects blood pressure. Animals lacking AT_{2} receptors exhibit an enhanced pressor response to Ang II, 26 whereas overexpression of AT_{2} receptors antagonizes AT_{1} receptor–mediated pressor effects. 27 AT_{2} receptor–mediated vasodilation, particularly of microvessels, may serve as a negative feedback mechanism to counterbalance the potent vasoconstrictor effect of AT_{1} receptor activation. For example, Ang II levels are increased during exercise, 27 and AT_{2} receptor–mediated dilation of precapillary vessels might help to offset potentially dangerous effects of diminished capillary perfusion in face of excessive AT_{1} receptor stimulation. Therapeutically, it seems clear that AT_{2} receptors mediate salutary responses. For example, AT_{2} receptor stimulation may play a part in the antihypertensive effects of AT_{1} 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_{2} receptors reduces infarct size. 19 The present findings are the first to provide direct experimental evidence for a novel molecular effector (the BK_{Ca} channel) that can mediate endothelium-independent relaxation of vascular smooth muscle via Ang II stimulation of the AT_{2} receptor. Future studies will identify and characterize the postreceptor signal transduction cascade stimulated by Ang II in these microvessels.

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