Angiotensin II Induces Angiogenesis in the Hypoxic Adult Mouse Heart In Vitro Through an AT₂–B2 Receptor Pathway

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Abstract—Angiotensin II is a vasoactive peptide that may affect vascularization of the ischemic heart via angiogenesis. In this study we aimed at studying the mechanisms underlying the angiogenic effects of angiotensin II under hypoxia in the mouse heart in vitro. Endothelial sprout formation from pieces of mouse hearts was assessed under normoxia (21% O₂) and hypoxia (1% O₂) during a 7-day period of in vitro culture. Only under hypoxia did angiotensin II dose-dependently induce endothelial sprout formation, peaking at 10⁻⁷ mol/L of angiotensin II. Angiotensin II type 1 (AT₁) receptor blockade by losartan did not affect angiotensin II–induced sprouting in wild-type mice. Conversely, the angiotensin II type 2 (AT₂) receptor antagonist PD 123319 blocked this response. In hearts from AT₁⁻/⁻ mice, angiotensin II–elicited sprouting was preserved but blocked again by AT₂ receptor antagonism. In contrast, no angiotensin II–induced sprouting was found in preparations from hearts of AT₂⁻/⁻ mice. Angiotensin II–mediated angiogenesis was also abolished by a specific inhibitor of the B2 kinin receptor in both wild-type and AT₁⁻/⁻ mice. Furthermore, angiotensin II failed to induce endothelial sprout formation in hearts from B2⁻/⁻ mice. Finally, NO inhibition completely blunted sprouting in hearts from wild-type mice, whereas NO donors could restore sprouting in AT₁⁻/⁻ and B2⁻/⁻ hearts. This in vitro study suggests the obligatory role of hypoxia in the angiogenic effect of angiotensin II in the mouse heart via the AT₂ receptor through a mechanism that involves bradykinin, its B2 receptor, and NO as a downstream effector. (Hypertension. 2007;49:1178-1185.)

Key Words: heart • angiotensin II • bradykinin • losartan • nitric oxide

Ischemic heart disease and left ventricular hypertrophy are characterized by impaired cardiac function caused by, among others, inadequate blood supply to the myocardium. In order to relieve this condition, blood flow to the myocardium needs to be restored by remodeling of pre-existing unused collateral blood vessels (arteriogenesis) and by the growth of new microvessels (angiogenesis). This process may also prevent the death and promote regeneration of damaged myocardial tissue.

Angiogenic stimuli are generated by hypoxia through activation of endothelial cell signaling and gene transcription of key angiogenic molecules, such as vascular endothelial growth factor (VEGF). In mice, activation of pre-existing collateral vascularization that restores blood flow to the acutely ischemic heart was shown to be induced by angiotensin II (Ang II), a key regulator of blood pressure and the main effector of the renin–angiotensin–aldosterone system. During ischemia or cancer, Ang II was shown to induce angiogenesis. Two major subtypes of Ang II receptors are expressed in the myocardium, Ang II type 1 (AT₁) and Ang II type 2 (AT₂) receptors. Most of the Ang II cardiovascular effects, for example, vasoconstriction, are attributed to AT₁. AT₁ is an ubiquitous receptor that presents 2 subtypes in rodents of a high homology (AT₁a and AT₁b). On the other hand, the AT₂ receptor is highly expressed early in development and at lower levels in the adult. Interestingly, the AT₂ receptor is upregulated in response to ischemia and inflammation suggesting a potential role in myocardial angiogenesis.

Previous studies have shown that the AT₂ receptor may interact with the bradykinin receptor, the B2 kinin receptor (B2), during signaling.

In the present study, we have investigated the mechanism of angiogenesis in response to Ang II in an in vitro model of sprout formation in the mouse heart under conditions of normoxia (21% O₂) and severe hypoxia (1% O₂) by dissecting the role of AT receptor subtypes and identifying the downstream effectors.

Methods

Animals

Experiments were performed with hearts of C57Bl/6 wild-type, AT₁⁻/⁻ (Jackson Laboratories), and B2⁻/⁻ mice (Jackson Laboratories).
Angiogenesis In Vitro Assay

A 3D in vitro assay of heart angiogenesis was established in our laboratory as described in detail previously. Briefly, 0.5- to 1-mm³ cubes from the left ventricle of the mouse heart were placed onto fibrin gels (Sigma-Aldrich) with 500 μL of DMEM plus 5% FCS (Biochrom). Heart explants were incubated under normoxia (21% O₂) or hypoxia (1% O₂) for 7 days. Stimulants/inhibitors were added every other day: hrVEGF164 (R&D systems), HOE140 (Sigma-Aldrich), CGP-42112 (Bachem), PD123319 (Fluka), PKSI-527 (Wako Chemicals), and NO inhibitors and donors (Sigma-Aldrich).

Inhibitors were added fresh 20 minutes before stimulants. After 7 days, endothelial sprouts were photographed digitally (ColorView II-Soft Imaging System) on an inverted light microscope (Olympus IX50). The extent of sprout formation was determined as detailed previously. Briefly, we used octuplicates for each condition, and sprout formation was calculated and averaged by 2 independent investigators by comparison with a standardized scale (angiogenic index). The angiogenic index was defined with the help of an image analysis software (AnalySIS Pro, Soft Imaging System) as [sprouting area/total area]×10. The angiogenic index was rounded to the nearest integer and handled as a scored value.

Characterization of Cells and Tissue

Characterization of outgrowing cells and sprouts was performed by using specific cell markers GSL-IB4 (20 μg/mL; Rectolab) for endothelium, Cy3-conjugated anti-α-smooth muscle actin (1:100; Fluka Chemie) for smooth muscle cells/pericytes, and Hoechst dye (Polysciences Europe) for visualization of cell nuclei as described previously.

NO Production Assay

NO concentrations were measured by the fluorometric nitrite assay with the NO Assay kit (Calbiochem). Briefly, pieces of mouse heart were incubated in phenol-free DMEM. The supernatants were collected, nitrite was detected by fluorescence, and concentration (nanomoles per liter) was calculated according to a calibration curve in each experiment.

Western Blotting

Heart tissue was lysed in radioimmunoprecipitation assay buffer as described before. After SDS-PAGE, proteins were transferred onto polyvinylidene fluoride membrane (Millipore). The membrane was blocked with 4% skim milk powder in Tris-buffered saline–Tween solution and probed with polyclonal anti-AT1 (N-10) and anti-AT2 (C-18) from Santa Cruz Biotechnology. Horseradish peroxidase–conjugated IgGs from Cell Signaling Technology were used to visualize the proteins by a chemiluminescence reaction (Amersham).

RT-PCR

Total RNA was isolated with TRizol Reagent (Invitrogen), quantified, and reverse transcribed with a Moloney-murine leukemia virus reverse transcriptase system (Promega).

The cDNA (1 μL) was amplified in 35 cycles of PCR. The following primer sequences were used: for mouse AT1 receptor sense: 5'-TGGAGAACACCCATACAC TG-3' and antisense: 5'-TTCGTAGACGCTGGTAAG-3'; mouse AT2 receptor sense: 5'-CCTGGTGACTTACTCTT-3' and antisense 5'-GAACTACA-TAAGATGCTTGC-3'; and mouse 18S ribosomal RNA sense: 5'-CCTGATAACCAGCAGCTAAG-3' and antisense 5'-GCGGC-GCAATACGAATGCCC-3'. Specific PCR annealing temperatures were 49°C for AT1, 52°C for AT2, and 57°C for 18S.

Statistical Analysis

All of the depicted results represent experiments repeated using ≥5 different heart explants. Each single condition was performed in octuplicate wells. Data points represent the mean±SEM. Statistical analysis was performed with SPSS for Mac OS X (SPSS Inc). Statistical significance (P<0.05) was computed using nonparametric analysis; Kruskal–Wallis and Mann–Whitney tests were performed accordingly.

Results

Ang II Induces Vascular Sprouting of the Adult Mouse Heart Under Hypoxia

We analyzed the effect of Ang II in an in vitro model of angiogenesis of the heart both under normoxia (21% O₂) and hypoxia (1% O₂). Under normoxia, neither Ang II nor the angiogenic growth factor VEGF was that was used as positive control elicited an angiogenic response (Figure 1A and 1B) in the mouse heart. However, under hypoxia Ang II, bradykinin and VEGF elicited a significant angiogenic response (Figure 1A and 1B) of a similar magnitude (2.2-, 1.9-, and 2.4-fold increase, respectively, compared with negative control P<0.05). Staining with fluorescently labeled antibodies (Figure 1C) revealed that Ang II–induced sprouts typically consist of endothelial cells aligned with smooth muscle cells/pericytes. Because hypoxia was confirmed to be a prerequisite for in vitro angiogenesis of the adult mouse heart (see also Reference 14), all of the following experiments were performed in hypoxia.

Ang II Induces Dose-Dependent Sprouting Through the AT2 Receptor

Stimulation of heart explants with a wide concentrations range of Ang II (10⁻¹⁰ to 10⁻⁶ mol/L) showed that endothelial sprouting induced by Ang II was dose dependent over at least a 1000-fold range of concentrations and was maximal at 10⁻⁷ mol/L (2.2±0.3; n=5; P<0.05; Figure 2).

Next, we evaluated the contribution of AT1 and AT2 receptors in Ang II–mediated sprout formation. The selective AT2 agonist CGP-42112 induced an angiogenic response similar to that observed in Ang II–stimulated hearts (2-fold increase with 10⁻⁷ mol/L CGP-42112; P<0.05 versus control; Figure 2). AT1 and AT2 receptor inhibitors corroborated these results (Figure 3). Losartan, a specific AT1 inhibitor, did not affect Ang II–induced sprout formation. PD 123319, a selective AT2 antagonist, significantly reduced Ang II–induced sprout formation to control levels (P<0.05). The combination of both antagonists elicited a response very similar to that seen with PD 123319 alone (P<0.05). CGP-42112–induced sprout formation was inhibited by PD123319 but not by losartan (data not shown). Taken together, these results suggest that the AT2 receptor subtype mediates the
angiogenic effect induced by Ang II in the mouse heart under hypoxia.

Ang II Does Not Induce Sprouting in AT$_2^{-/-}$ Animals

To confirm these latter findings, we examined hearts from AT$_1$a$^{-/-}$ and AT$_2^{-/-}$ mice. Ang II could not induce sprouting above control levels in heart explants from adult AT$_2^{-/-}$ mice under hypoxia (Figure 4), either alone or after blocking the AT$_1$ receptor with losartan. However, VEGF induced a significant level of sprout formation compared with controls (2.6-fold increase; $P<0.05$), suggesting that VEGF-induced angiogenesis in vitro is independent of AT$_2$ signaling. On the other hand, Ang II induced sprout formation in heart explants from AT$_1$a$^{-/-}$ mice as efficiently as in wild-type hearts (1.9-fold increase; $P<0.05$; Figure 4). In these mice, Ang II also elicited sprouting in the presence of losartan, which inhibits both AT$_1$a and AT$_1$b receptors, excluding the possibility that the observed angiogenic effect could be mediated by the AT$_1$b receptor still present in the AT$_1$a$^{-/-}$ mice. On the other hand, PD 123319 completely inhibited sprout formation ($P<0.05$) in the AT$_1^{-/-}$ heart explants. These results clearly demonstrate the exclusive role of the AT$_2$ receptor in Ang II–mediated angiogenesis in adult hypoxic mouse heart explants.

AT$_1$ and AT$_2$ Receptor Expression Under Hypoxia

To exclude the possibility that AT$_2$-dependent Ang II–induced sprout formation could be because of the downregulation of AT$_1$ receptor in hypoxia, we determined AT$_1$ and AT$_2$ receptor protein and mRNA expression in wild type mouse heart explants. As shown in figure 5, both AT$_1$ and
AT2 were expressed confirming that both pathways are available for signaling.

**Ang II Induces Sprouting via an AT2-B2 Receptor Pathway**

To analyze the role of the B2 receptor, we stimulated hypoxic mouse heart explants with Ang II in both wild-type and AT1-/- animals in the presence of HOE 140, a selective B2 antagonist (Figure 6A). We found an Ang II–induced angiogenic response (wild-type: 3.5-fold increase; AT1-/-: 3.3-fold increase versus control; P<0.05) that was completely abolished by HOE 140 (P<0.05). Bradykinin, per se (10^{-7} mol/L), induced sprout formation both in wild-type and AT1-/- mouse hearts (wild-type: 1.44-fold increase; AT2-/-: 1.5-fold increase versus control; P<0.001). To confirm that Ang II–induced angiogenesis requires the B2 receptor, heart explants from B2-/- mice were assessed. Neither Ang II nor VEGF induced significant sprouting in B2-/- mice (Figure 6B). To clarify whether accumulation of bradykinin was the intermediate step in Ang II–induced sprouting, we treated the heart explants with a specific kininogenase inhibitor, PKSI-527 (10^{-7} mol/L), that blocks the conversion of kinins into bradykinin. PKSI-527 completely inhibited Ang II–induced angiogenesis in the wild-type mouse heart (Figure 6C). Therefore, we conclude that Ang II is angiogenic in the mouse heart under hypoxia via a pathway involving both the AT2 and the B2 receptors linked by activation of bradykinin production.

**Ang II–Induced Sprouting Requires NO Release**

Because stimulation of the AT2 receptor is associated with increased generation of bradykinin, NO, and cGMP, we tested whether the angiogenic effects of Ang II may also require NO. As expected, Ang II (10^{-7} mol/L) and bradykinin (10^{-7} mol/L) significantly increased NO production as measured by nitrite accumulation in the medium after 7 days of incubation (in 10^{-9} mol/L, control: 90±5; bradykinin: 121±5; Ang II: 114±15; n=3; P<0.05; ANOVA). We then inhibited NO generation using NO synthase inhibitors, that is, s-Methylisothiourea, L-N(1-iminoethyl)-ornithine, N^G-nitro-L-arginine methyl ester, and N-(3-aminomethyl)benzyl acetamide. Ang II- and CGP-42112–induced angiogenesis were completely blunted by NO inhibition (Figure 7A). Heart explants derived from wild-type, AT2-/-, and BK2-/- mice were then incubated with 2 different NO donors,

![Figure 2. Ang II induces dose-dependent sprouting in vitro in adult mouse hearts in hypoxia. Pieces of mouse heart were stimulated with Ang II from 10^{-10} to 10^{-6} mol/L (left) or AT2 agonist CGP-42112 from 10^{-7} to 10^{-5} mol/L (right) and incubated under hypoxia for 7 days. Data points represent the mean of 5 independent experiments ± SEM, *P<0.05 vs negative control.](http://hyper.ahajournals.org/)

![Figure 3. AT2 is not required for Ang II–induced sprouting.](http://hyper.ahajournals.org/)

![Figure 4. Ang II–induced angiogenesis is impaired in hearts from AT2-/- but not from AT1-/- mouse. Pieces of mouse heart from AT2-/- (left side of the figure) and AT1-/- mice (right side of the figure) were stimulated with Ang II (10^{-7} mol/L) alone or in combination with losartan (10^{-6} mol/L) or PD 123319 (10^{-6} mol/L) and incubated under hypoxia for 7 days. VEGF (10 ng/mL) was used as a positive control. Data points represent the mean of 5 independent experiments ± SEM, *P<0.05 vs VEGF (left) and *P<0.05 vs negative control (right).](http://hyper.ahajournals.org/)
S-nitrosoglutathione (10^{-5} \text{ mol/L}) and PAPA NONOate (10^{-5} \text{ mol/L}; Figure 7B). Both NO donors induced angiogenesis (S-nitrosoglutathione; wild-type: 1.7-fold increase; AT2^{-/-}: 1.7-fold increase; BK2^{-/-}: 1.6-fold increase versus control; \( P<0.05 \)). These results demonstrate that NO is a key mediator of angiogenesis in the hypoxic mouse heart and is a required downstream effector of Ang II–induced sprout formation.

**Discussion**

Here we show that Ang II induces angiogenesis in the adult mouse heart specifically under hypoxia, signaling through the AT2 but not the AT1 receptor. The mechanism requires generation of bradykinin with activation of the B2 receptor and leads to NO biosynthesis as the downstream effector.

The renin–angiotensin-aldosterone system is an important system in regulating vascular homeostasis. However, the precise role of the renin–angiotensin-aldosterone system and the AT1/AT2 receptor pathway in angiogenesis is unclear. Clinical data have shown that blocking the AT1 receptor preserves cardiac function after myocardial infarction.\(^{19}\) Our results showing that Ang II–induced angiogenesis in the mouse heart under hypoxia is mediated exclusively by the AT2 receptor may explain some beneficial effects of AT1 blockade treatment in the heart. In fact, AT1 blockade may unmask beneficial properties because of preferential AT2 stimulation.

The role of the AT1 and AT2 receptor in angiogenesis is controversial. Ang II–induced angiogenesis was shown to be mediated via both the AT1 and the AT2 receptor in the mesenteric vasculature of Ang II–infused rats\(^ {20} \) or specifically via the AT2 receptor in tumor angiogenesis in mice.\(^ {21} \) High AT1 expression was associated with reduced myocardial vessel density in rats.\(^ {22} \) In contrast, others have shown AT1-dependent angiogenesis in the ischemic hind limb of mice,\(^ {23} \) whereas AT2 appeared to be antiangiogenic in the same animal model.\(^ {24} \) Tumor angiogenesis was impaired in AT1^{-/-} receptor mice.\(^ {25} \) Thus, the role of AT1 and AT2 receptors in angiogenesis is not clear and may vary on model, tissue, and conditions investigated. In particular, the vasculature of the heart has not been investigated in models of controlled hypoxia. Our model of angiogenesis in vitro of the mouse heart provides this possibility and demonstrates the key role of hypoxia in Ang II–induced cardiac angiogenesis.

Hypoxia can lead to the formation of new vessels in mature tissue, triggering vessel growth by signaling through hypoxia-inducible transcription factor-1.\(^ {26} \) Interestingly, Ang II induces hypoxia-inducible transcription factor-1\(^ {27} \). Hypoxia may also modulate the expression of AT1 and/or AT2 receptors.\(^ {28} \) In our experiments, both AT1 and AT2 receptors were present under normoxia and hypoxia. Still, further studies investigating other tissues, receptor expression, and intracellular signaling pathways may reveal whether AT2-dependent angiogenesis is specific for the hypoxic heart.

The AT2 receptor might exert downstream effects via the B2 receptor.\(^ {29} \) We clearly show that Ang II–induced angiogenesis is abrogated when the B2 receptor is pharmacologically inhibited or knocked out. B2 activation by bradykinin

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**Figure 6.** Ang II–induced sprouting in wild-type and AT1 knockout mice requires the B2 receptor. A, Pieces of heart from wild-type or AT1^{-/-} mice stimulated with Ang II (10^{-7} \text{ mol/L}) alone or in combination with the specific B2 receptor antagonist HOE 140 (10^{-7} \text{ mol/L}) and incubated under hypoxia during 7 days. HOE 140 significantly (\( P<0.05 \) vs negative control) decreased Ang II–induced sprout formation in wild-type and AT1^{-/-} mice compared with Ang II–treated heart pieces without the inhibitor. B, Pieces of hearts from B2^{-/-} mice stimulated with Ang II (10^{-7} \text{ mol/L}) alone or in combination with PD123319 (10^{-6} \text{ mol/L}) were incubated in culture medium containing 5% FCS under hypoxia for 7 days. VEGF_{164} (10 \text{ ng/mL}) was used as a positive control. \( P \) value not significant. Data points represent the mean of 5 independent experiments \( \pm \text{SEM} \). C, Pieces of heart from wild-type mice were stimulated with Ang II (10^{-7} \text{ mol/L}) alone or in combination with kininogenase-specific inhibitor PKSI-527 (10^{-5} \text{ mol/L}) were incubated in culture medium containing 5% FCS under hypoxia for 7 days. VEGF_{164} (10 \text{ ng/mL}) was used as a positive control. Data points represent the mean of 3 independent experiments \( \pm \text{SEM} \). *\( P<0.05 \) vs control.
induces vasodilation,\textsuperscript{31} which is also a prerequisite for initiation of angiogenesis.\textsuperscript{32} AT\textsubscript{2}-overexpressing mice blocked Ang II–induced vasopressor effects through the B2 receptor.\textsuperscript{33} Importantly, bradykinin was shown to induce angiogenesis via the B2 receptor\textsuperscript{34} or as shown by using a model of ischemia induced in hind limbs in B2\textsuperscript{−/−} mice.\textsuperscript{35} Collectively, these data suggest a mechanism by which a vasopressor molecule, such as Ang II, can also mediate vasodilator and angiogenic effects specifically by AT\textsubscript{2} receptor–dependent signaling leading to B2 kinin receptor activation.\textsuperscript{36} Ang II–dependent activation of B2 could be achieved in different ways. A direct interaction between AT\textsubscript{2} and B2 leading to NO production has been described recently,\textsuperscript{37} although the precise nature of this interaction has not been fully clarified. Others have pointed out an Ang II–mediated pH increase that may release kininogens to produce bradykinin.\textsuperscript{33,38} In our study, the angiogenic effect of B2 receptor depended on bradykinin production, the AT\textsubscript{2} receptor and is mediated by an increase in bradykinin production.

Endothelium-derived NO synthase is crucial for angiogenesis in vitro and in vivo.\textsuperscript{39} In fact, NO inhibition blocked Ang II–induced endothelial sprout formation in our model of angiogenesis of the heart in vitro. Increased nitrite accumulation in the medium of Ang II–stimulated heart explants was also observed. Accordingly, NO donors directly induced angiogenesis in pieces of heart from wild-type, B2\textsuperscript{−/−}, and AT\textsubscript{2}\textsuperscript{−/−} mice. Our results are in agreement with previous reports, showing that Ang II can induce renal production of bradykinin, NO, and cGMP via the AT\textsubscript{1} receptor.\textsuperscript{40} These data suggest that an increase in NO bioavailability downstream of AT\textsubscript{2} and B2 receptors is the final effector of Ang II–induced angiogenesis in the hypoxic heart.

**Perspectives**

The present study provides evidence for the significant role of the AT\textsubscript{1}/B2 pathway in the Ang II–induced angiogenesis in vitro in the adult mouse heart under hypoxia. In clinical studies, AT\textsubscript{1} blocker treatment of hypertension has revealed additional cardioprotective effects beyond the lowering blood pressure.\textsuperscript{41,42} A potential advantage of AT\textsubscript{1} blockers over
angiotensin-converting enzyme inhibition is the preservation of the AT₁-mediated pathway. Here we describe that Ang II–induced angiogenic effects through AT₁/B2 may provide some explanation for these beneficial effects. Studies on neovascularization of the heart in hypertensive animals and patients after AT₁ treatment are needed to test the clinical relevance of our mechanistic results. This may help us to understand and to uncover novel therapeutic effects of AT₁ receptor blockers for patients with left ventricular hypertrophy, ischemic heart disease, or myocardial infarction.

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Disclosures
None.

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