Angiotensin II Induces Angiogenesis in the Hypoxic Adult Mouse Heart In Vitro Through an AT$_2$–B2 Receptor Pathway

Veronica C. Munk, Lourdes Sanchez de Miguel, Marco Petrimpol, Nicole Butz, Andrea Banfi, Urs Eriksson, Lutz Hein, Rok Humar, Edouard J. Battegay

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% O$_2$) and hypoxia (1% O$_2$) 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 (AT$_1$) receptor blockade by losartan did not affect angiotensin II–induced sprouting in wild-type mice. Conversely, the angiotensin II type 2 (AT$_2$) receptor antagonist PD 123319 blocked this response. In hearts from AT$_1^{-/-}$ mice, angiotensin II–elicited sprouting was preserved but blocked again by AT$_2$ receptor antagonism. In contrast, no angiotensin II–induced sprouting was found in preparations from hearts of AT$_2^{-/-}$ mice. Angiotensin II–mediated angiogenesis was also abolished by a specific inhibitor of the B2 kinin receptor in both wild-type and AT$_1^{-/-}$ 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 AT$_1^{-/-}$ 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 AT$_2$ 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 (AT$_1$) and Ang II type 2 (AT$_2$) receptors. Most of the Ang II cardiovascular effects, for example, vasoconstriction, are attributed to AT$_1$. AT$_1$ is an ubiquitous receptor that presents 2 subtypes in rodents of a high homology (AT$_1$a and AT$_1$b). On the other hand, the AT$_2$ receptor is highly expressed early in development and at lower levels in the adult. Interestingly, the AT$_2$ receptor is upregulated in response to ischemia and inflammation suggesting a potential role in myocardial angiogenesis. Previous studies have shown that the AT$_2$ 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% O$_2$) and severe hypoxia (1% O$_2$) 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, AT$_1$a$^{-/-}$ (Jackson Laboratories), and B2$^{-/-}$ mice (Jackson Laborato-
ries). FVB/J AT$_2^{-/-}$ mice were a gift from Prof Lutz Hein (Institut für Experimentelle und Klinische Pharmacologie und Toxicologie, Freiburg, Germany) and have been described previously.$^{13}$ The animals were euthanized and the hearts immediately transferred to PBS. Within 30 minutes postmortem, small pieces (1 mm$^3$) of the mouse myocardium (left ventricle) were cut and embedded in fibrin gel. All of the experiments were conducted in accordance with the Swiss Federal Act on Animal Protection (1998) and were approved by the Veterinary Department of the Kanton of Basel (Switzerland). We used between 5 and 9 mice for every experiment. The age of mice ranged from 12 to 14 weeks.

**Angiogenesis In Vitro Assay**

A 3D in vitro assay of heart angiogenesis was established in our laboratory as described in detail previously.$^{14}$ Briefly, 0.5- to 1-mm$^3$ cubes from the left ventricular myocardium 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% O$_2$) or hypoxia (1% O$_2$) 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.$^{13}$ 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]$^{10}$, where total area corresponds with the sprouting area plus tissue area. Sprouting was computed from the area that was actually occupied by endothelial sprouts and not the space between the cells. Sprouting and tissue area were computed by AnalySIS Pro, and 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-alpha-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.$^{14}$

**NO Production Assay**

NO concentrations were measured by the fluorometric nitrite assay$^{15}$ 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 ($P<0.05$). 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 AT$_2$ 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 AT$_1$ and AT$_2$ receptors in Ang II–mediated sprout formation. The selective AT$_2$ 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). AT$_1$ and AT$_2$ receptor inhibitors corroborated these results (Figure 3). Losartan, a specific AT$_1$ inhibitor, did not affect Ang II–induced sprout formation. PD 123319, a selective AT$_2$ 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 AT$_2$ receptor subtype mediates the
angiogenic effect induced by Ang II in the mouse heart under hypoxia.

**Ang II Does Not Induce Sprouting in AT2/−/− Animals**

To confirm these latter findings, we examined hearts from AT1a/−/− and AT2/−/− mice. Ang II could not induce sprouting above control levels in heart explants from adult AT2/−/− mice under hypoxia (Figure 4), either alone or after blocking the AT1 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 AT2 signaling. On the other hand, Ang II induced sprout formation in heart explants from AT1a/−/− 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 AT1a and AT1b receptors, excluding the possibility that the observed angiogenic effect could be mediated by the AT1b receptor still present in the AT1a/−/− mice. On the other hand, PD 123319 completely inhibited sprout formation (\( P<0.05 \)) in the AT1/−/− heart explants. These results clearly demonstrate the exclusive role of the AT2 receptor in Ang II–mediated angiogenesis in adult hypoxic mouse heart explants.

**AT1 and AT2 Receptor Expression Under Hypoxia**

To exclude the possibility that AT2-dependent Ang II–induced sprout formation could be because of the downregulation of AT1 receptor in hypoxia, we determined AT1 and AT2 receptor protein and mRNA expression in wild type mouse heart explants. As shown in figure 5, both AT1 and
Angiotensin II and Heart Angiogenesis In Vitro

AT1 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; AT1−/−: 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 (10−5 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 AT1 and the B2 receptors linked by activation of bradykinin production.

Ang II–Induced Sprouting Requires NO Release

Because stimulation of the AT1 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-N5-(1-iminoethyl)-ornithine, Nα-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, AT1−/−, and B2−/− mice were then incubated with 2 different NO donors, and NO release was measured using NO synthase inhibitors.
Clinical data have shown that blocking the AT1 receptor preserves cardiac function after myocardial infarction. Our precise role of the renin–angiotensin-aldosterone system and system in regulating vascular homeostasis. However, the generation of bradykinin with activation of the B2 receptor leads to NO biosynthesis as the downstream effector.

A type AVT mice were incubated under 21% O2 or 1% O2 during 24 hours, lysed, and protein and mRNA extracted. Western blotting and RT-PCR analysis show that both the AT1 and the AT2 receptor are expressed under normoxia and hypoxia.

S-nitrosothioglutathione (10⁻⁵ mol/L) and PAPA NONOate (10⁻⁵ mol/L; Figure 7B). Both NO donors induced angiogenesis (S-nitrosothioglutathione; 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 receptor 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. 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 both by the AT₁ and the AT₂ receptor in the mesenteric vasculature of Ang II–infused rats or specifically via the AT₂ receptor in tumor angiogenesis in mice. High AT₁ expression was associated with reduced myocardial vessel density in rats. In contrast, others have shown AT₁-dependent angiogenesis in the ischemic hind limb of mice, whereas AT₂ appeared to be antiangiogenic in the same animal model. Tumor angiogenesis was impaired in AT₁⁻⁻⁻ receptor mice. 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.

Ang II can lead to the formation of new vessels in mature tissue, triggering vessel growth by signaling through hypoxia-inducible transcription factor-1. Interestingly, Ang II induces hypoxia-inducible transcription factor-1α. Hypoxia may also modulate the expression of AT₁ and/or AT₂ receptors. 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 AT₁ receptor might exert downstream effects via the B2 receptor. We clearly show that Ang II–induced angiogenesis is abrogated when the B2 receptor is pharmacologically inhibited or knocked out. B2 activation by bradykinin...
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 B\textsubscript{2} receptor.\textsuperscript{33} Importantly, bradykinin was shown to induce angiogenesis via the B\textsubscript{2} receptor\textsuperscript{34} or as shown by using a model of ischemia induced in hind limbs in B\textsubscript{2}\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 B\textsubscript{2} kinin receptor activation.\textsuperscript{36} Ang II–dependent activation of B\textsubscript{2} could be achieved in different ways. A direct interaction between AT\textsubscript{2} and B\textsubscript{2} 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{35,38} In our study, the angiogenic effect of B\textsubscript{2} receptor depended on bradykinin synthesis, because kininogenase inhibition blocked Ang II–induced angiogenesis. Bradykinin induced angiogenesis in hypoxic heart explants only from both wild-type and AT\textsubscript{2}\textsuperscript{-/-} mice. Ang II, however, as mentioned before, failed to induce angiogenesis in hearts from BK\textsubscript{2}\textsuperscript{-/-} mice. We conclude that angiogenesis induced by Ang II requires signaling through 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, B\textsubscript{2}\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 B\textsubscript{2} 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{2}/B\textsubscript{2} 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.

Acknowledgments

We thank Claudia Weiss for secretarial work, Kajia Paris for technical assistance, and Nora Mauermann and Julia Burian for helping with the breeding and genotyping of the mice. Merck Sharp and Dohme-Chibret AG kindly provided us with Losartan. We also thank Hans-Ruedi Brunner and Christian Zaugg for valuable discussions of the article.

Sources of Funding

This work was supported by Swiss National Science Foundation grant 3200-067155, a medical school grant from Merck Sharp & Dohme-Chibret AG (Glattbrugg, Switzerland), and a grant from the Swiss Heart Foundation (to A.B.; 310000–114056); and a grant from the Ministèr de l’Education Nationale (to L.S.d.M.).

Disclosures

None.

References

35. Silvestre JS, Bergaya S, Tamarat R, Duriez M, Boulanger CM, Levy BI. Proangiogenic effect of angiotensin-converting enzyme inhibition is


Angiotensin II Induces Angiogenesis in the Hypoxic Adult Mouse Heart In Vitro Through an AT$_2$-B2 Receptor Pathway

Veronica C. Munk, Lourdes Sanchez de Miguel, Marco Petrimpol, Nicole Butz, Andrea Banfi, Urs Eriksson, Lutz Hein, Rok Humar and Edouard J. Battegay

_Hypertension._ 2007;49:1178-1185; originally published online March 5, 2007;
doi: 10.1161/HYPERTENSIONAHA.106.080242

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/49/5/1178

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/