Angiotensin 1–7 Reduces Mortality and Rupture of Intracranial Aneurysms in Mice

Ricardo A. Peña Silva, David K. Kung, Ian J. Mitchell, Natalia Alenina, Michael Bader, Robson A.S. Santos, Frank M. Faraci, Donald D. Heistad, David M. Hasan

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Abstract—Angiotensin II (Ang II) stimulates vascular inflammation, oxidative stress, and formation and rupture of intracranial aneurysms in mice. Because Ang 1–7 acts on Mas receptors and generally counteracts deleterious effects of Ang II, we tested the hypothesis that Ang 1–7 attenuates formation and rupture of intracranial aneurysms. Intracranial aneurysms were induced in wild-type and Mas receptor–deficient mice using a combination of Ang II–induced hypertension and intracranial injection of elastase in the basal cistern. Mice received elastase+Ang II alone or a combination of elastase+Ang II+Ang 1–7. Aneurysm formation, prevalence of subarachnoid hemorrhage, mortality, and expression of molecules involved in vascular injury were assessed. Systolic blood pressure was similar in mice receiving elastase+Ang II (mean±SE, 148±5 mm Hg) or elastase+Ang II+Ang 1–7 (144±5 mm Hg). Aneurysm formation was also similar in mice receiving elastase+Ang II (89%) or elastase+Ang II+Ang 1–7 (84%). However, mice that received elastase+Ang II+Ang 1–7 had reduced mortality (from 64% to 36%; P<0.05) and prevalence of subarachnoid hemorrhage (from 75% to 48%; P<0.05). In cerebral arteries, expression of the inflammatory markers, Nox2 and catalase increased similarly in elastase+Ang II or elastase+Ang II+Ang 1–7 groups. Ang 1–7 increased the expression of cyclooxygenase-2 and decreased the expression of matrix metalloproteinase-9 induced by elastase+Ang II (P<0.05). In Mas receptor–deficient mice, systolic blood pressure, mortality, and prevalence of subarachnoid hemorrhage were similar (P>0.05) in groups treated with elastase+Ang II or elastase+Ang II+Ang 1–7. The expression of Mas receptor was detected by immunohistochemistry in samples of human intracranial arteries and aneurysms. In conclusion, without attenuating Ang II–induced hypertension, Ang 1–7 decreased mortality and rupture of intracranial aneurysms in mice through a Mas receptor–dependent pathway. (Hypertension. 2014;64:362-368.) ● Online Data Supplement

Key Words: angioten (1–7) ■ angiotensin (1–7) receptor Mas, human ■ hypertension ■ intracranial aneurysm ■ subarachnoid hemorrhage

With the exception of surgical interventions, treatment options for intracranial aneurysms are limited, thus greater insight into molecular mechanisms that control formation and rupture of intracranial aneurysms may lead to new treatment options. The wall of human intracranial aneurysms is rich in inflammatory cells and molecules. Inflammation may contribute to formation of cerebral aneurysms, with disruption of the elastic membrane, which ultimately may contribute to aneurysm rupture. Angiotensin II (Ang II) increases the expression of proinflammatory cytokines and oxidative stress in blood vessels and stimulates remodeling of the extracellular matrix in blood vessels. Although Ang II plays a critical role in the formation and rupture of abdominal aortic aneurysms, its role in the formation and rupture of intracranial aneurysms is not clear.

Ang 1–7 acts as a functional antagonist of Ang II.6–9 Ang 1–7 is a product of the metabolism of Ang II by the angiotensin-converting enzyme type 2.7,10,11 When bound to the Mas receptor,12 Ang 1–7 reduces inflammation and oxidative stress in peripheral vessels, articular, and adipose tissue.7,11,14 In the current study, we tested the hypothesis that Ang 1–7 decreases the rupture of intracranial aneurysms.
Methods

Experimental Animals
Studies were performed in adult (11±1 months) wild-type (WT) and Mas receptor–deficient (Mas KO) mice. The mice were bred on the C57BL/6 background, as described previously. All experimental protocols and procedures conform to the National Institute of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Aneurysms were induced in mice according to published methods, using the combination of stereotactic injection of elastase in the basal cistern and hypertension induced by systemic administration of Ang II (or Ang II+Ang 1–7) using osmotic minipumps. Systolic blood pressure was measured using the tail cuff method. Animals were monitored daily and euthanized immediately if signs of neurological deficit were apparent or after 3 weeks. Cerebral arteries isolated from mice with aneurysms and shams were used for gene expression analysis by real-time quantitative polymerase chain reaction.

Human Intracranial Aneurysms
Studies were approved by the University of Iowa Internal Review Board. Samples of intracranial aneurysms and arteries were collected from patients who underwent microsurgical clipping. The expression of Mas receptor was examined using immunostaining.

Drugs
Ang 1–7 and Ang II were obtained from Bachem (Torrance, CA). All other reagents were obtained from Sigma (St Louis, MO).

Statistical Analysis
Analysis was performed using Prism 6 (Graphpad, La Jolla, CA). Categorical data (incidence of aneurysms and subarachnoid hemorrhage) were compared between mice treated with Ang II or Ang II+Ang 1–7 using 1-tailed Fisher exact test. Survival was analyzed with log-rank (Mantel–Cox) test. Gene expression in cerebral arteries from sham mice with aneurysms and shams was used for gene expression analysis by real-time quantitative polymerase chain reaction.

Results

Effect of Ang 1–7 in the Formation and Rupture of Intracranial Aneurysms
Systolic pressure increased significantly after intracranial stereotactic injection of elastase and implantation of osmotic pumps containing Ang II (mean±SE, 148±5 mm Hg) or Ang II+Ang 1–7 (144±5 mm Hg; P<0.05; Figure 1A) versus baseline. Ang II–induced hypertension was not attenuated by Ang 1–7 after 1, 2, or 3 weeks of treatment.

When compared with control mice (Figure 2A), ≥80% of hypertensive mice that received an intracranial injection of elastase displayed evidence of fusiform and/or saccular intracranial aneurysms during necropsy (Figure 2B). Most aneurysms were saccular or a mix of saccular and fusiform aneurysms; <20% were fusiform aneurysms. In some mice, ruptured aneurysms were identified near areas of subarachnoid hemorrhage (Figure 2B, left).

Mortality was higher in mice treated with Ang II (64% [18/28]) than in mice treated with Ang II+Ang 1–7 (36% [9/25]; P<0.05; Figure 1B). Ang 1–7 did not attenuate formation of aneurysms (89% [25/28] Ang II versus 84% [21/25] Ang II+Ang 1–7; Figure 1C). Incidence of subarachnoid hemorrhage was lower (48% [12/25]) in Ang II+Ang 1–7 than in Ang II–treated mice (75% [21/28]; P<0.05; Figure 1D).

Formation and Rupture of Intracranial Aneurysms in Mas KO Mice
Similar studies were performed in Mas KO mice. Increase in systolic pressure was similar in Mas KO mice after intracranial stereotactic injection of elastase and infusion of Ang II or Ang II+Ang 1–7 (136±4 versus 136±8 mm Hg), respectively (Figure 3A). Mortality of Mas KO mice treated...
Expression of Genes Involved in Vascular Injury

The expression of several genes involved in vascular inflammation, oxidative stress, and extracellular matrix remodeling was examined in cerebral arteries. In WT mice, intracranial injection of elastase and infusion of Ang II increased the expression of the proinflammatory cytokine tumor necrosis factor-α, integrin alpha M (Itgam; a marker of macrophage infiltration), and the proinflammatory enzyme microsomal prostaglandin E2 synthase-1 (P<0.05; Figure 4). Elastase+Ang II also increased

Figure 2. A, Cerebral blood vessels in a control mouse (scale bar, 1 mm; left). Section of anterior communicating artery (Art; right). B, Cerebral arteries in situ (left) and after excision (middle) from a mouse with several intracranial aneurysms (Ans) and acute subarachnoid hemorrhage and histological sections of intracranial Ans (right). Sections were stained with Masson trichrome. Scale bar, 100 μm.

Figure 3. Angiotensin 1–7 (Ang 1–7) does not attenuate Ang II–induced hypertension (A) in Mas receptor–deficient mice (Mas KO) mice. Ang 1–7 does not decrease mortality (B), aneurysm formation (C), or prevalence of subarachnoid hemorrhage (SAH; D) in Mas KO mice (P>0.05). n=19 Mas KO mice treated with elastase+Ang II and 15 Mas KO mice treated with elastase+Ang II+Ang 1–7.
the expression of Nox2, catalase, and the wound repair factor, hepatocyte growth factor. Coinfusion of Ang 1–7 did not attenuate the expression of inflammation mediators/markers or enzymes associated with oxidative stress in cerebral arteries, but notably increased the expression of cyclooxygenase-2. Elastase+Ang II increased the expression of matrix metalloproteinase (MMP)-9, MMP-2, and tissue inhibitor of metalloproteinases-1. Coinfusion of Ang 1–7 markedly attenuated the increase in MMP-9 in cerebral arteries (Figure 4).

In Mas KO, combination of intracranial injection of elastase and Ang II also increased the expression of molecules associated with inflammation, oxidative stress, and vascular remodeling. In these mice, coinfusion of Ang 1–7 did not alter the changes in gene expression induced by elastase+Ang II (Figure 5). In Mas KO mice, coinfusion of Ang 1–7 did not attenuate the increased expression of MMP-9 or the increased expression of cyclooxygenase-2 induced by elastase+Ang II.

Expression of Mas Receptor in Human Aneurysms
Mas receptor expression was demonstrated in media and intima of control human arteries (meningeal and superficial temporal arteries). Immunostaining for Mas was also positive in sections of unruptured and ruptured human intracranial aneurysms (Figure 6).

Discussion
In the current study, we replicated a model of intracranial aneurysms in mice. As first demonstrated by Nuki et al, cerebral aneurysms are produced in ≈80% of mice treated with Ang II and intracranial injections of elastase. Using this model, we observed that Ang 1–7 decreased mortality and frequency of rupture of intracranial aneurysms in mice. Moreover, protective effects of Ang 1–7 on aneurysm rupture were absent in Mas KO. Finally, Ang 1–7 decreased Ang II–induced increases in the expression of MMP-9 in cerebral arteries.

Ang 1–7 has several protective effects in models of stroke. Ang 1–7 decreased oxidative stress, apoptosis, and autophagosome formation in spontaneously hypertensive rats. Ang 1–7 decreased infarct size and neurological deficit after middle cerebral artery occlusion in rats. Moreover, Ang 1–7 increased survival of stroke-prone spontaneously hypertensive rats. In our study, we focused on effects of Ang 1–7 in cerebral arteries in a model of intracranial aneurysms.

Because Ang 1–7 counteracts some of the deleterious effects of Ang II, we anticipated that Ang 1–7 might reduce susceptibility to cerebral aneurysms in this model. However, it was not clear whether Ang 1–7 would be sufficiently potent, especially against key mechanisms, to have a detectable effect on aneurysms. A broader implication of our findings is that hypertension, which is often associated with activation of the renin/angiotensin system, is a major risk factor for rupture of aneurysms, and Ang 1–7 may be effective in protection against rupture of aneurysms.

Ang 1–7 attenuated aneurysm rupture but did not reduce the hypertensive effect of Ang II in our study. Antihypertensive effects of Ang 1–7 are not clear. Although Ang 1–7 decreased blood pressure in spontaneously hypertensive rats, it failed to reduce blood pressure in other models of hypertension. Our results agree with studies in which Ang 1–7 did not attenuate the increase in systolic blood pressure induced by Ang II or deoxycorticosterone acetate-salt. Similarly, delivery of Ang 1–7 to the cerebral ventricles of spontaneously hypertensive rats...
decreased brain damage but did not attenuate hypertension.\textsuperscript{19,23} Thus, attenuation of aneurysm rupture by Ang 1–7 is not the result of an antihypertensive action of Ang 1–7.

Inflammation seems to play an important role in rupture of intracranial aneurysms. Proinflammatory enzymes, such as cyclooxygenase-2 and microsomal prostaglandin E2 synthase-1, are increased in the wall of ruptured cerebral aneurysms in humans.\textsuperscript{1} Infiltration of leukocytes into the cerebral aneurysmal wall has also been found in humans.\textsuperscript{2} Macrophage depletion, or decreased vascular macrophage infiltration in cerebral arteries from monocyte chemoattractant protein–deficient mice, is associated with decreased aneurysm formation and rupture in mice.\textsuperscript{1,28} We found that Ang II increased the expression of tumor necrosis factor-\(\alpha\) and microsomal prostaglandin E2 synthase-1 in cerebral arteries and increased macrophage infiltration assessed by the specific macrophage marker integrin alpha M.\textsuperscript{7}

Ang 1–7 has multiple beneficial actions in blood vessels.\textsuperscript{7} Ang 1–7 dilates cerebral arteries\textsuperscript{29} and seems to attenuate neurological damage in stroke.\textsuperscript{20–22} Ang 1–7 also increases survival and decreases the number of subcortical hemorrhages in stroke-prone hypertensive rats.\textsuperscript{32} Part of the protective effect of Ang 1–7 in stroke seems to be related to its modulatory effects on nuclear factor-\(\kappa\)B and inflammation.\textsuperscript{22,23} Therefore, it was of interest that Ang 1–7 decreased aneurysm rupture and mortality without decreasing Ang II–induced infiltration of macrophages or overexpression of tumor necrosis factor-\(\alpha\) and microsomal prostaglandin E2 synthase-1 in cerebral arteries.

Ang 1–7 increased cyclooxygenase-2 expression in cerebral arteries. Although cyclooxygenase-2 is generally associated with inflammatory responses, it is also responsible for the synthesis of prostacyclin, which is vasoprotective.\textsuperscript{30} Protective effects of Ang 1–7 in the heart are attenuated by the cyclooxygenase inhibitor, indomethacin.\textsuperscript{31} Thus, although Ang 1–7 did not seem to attenuate inflammation in our study, it is possible that some of the protective effects of Ang 1–7 may be mediated by increased synthesis of prostacyclin through the cyclooxygenase-2 pathway.

Expression and activation of MMPs play a critical role in aneurysm rupture. Increased expression of MMP-2 and MMP-9 is seen in patients with ruptured cerebral aneurysms.\textsuperscript{32} Increased expression of MMP-2 and MMP-9 is associated with progression of cerebral aneurysms in rats.\textsuperscript{33} Pharmacological inhibition of MMPs decreases aneurysm rupture in mice.\textsuperscript{34} We found that Ang 1–7 attenuated Ang II–induced increase in the expression of MMP-9. Pathways by which Ang 1–7 or the Mas receptor regulate MMP expression are not known.

Ang 1–7 did not attenuate effects of intracranial injection of elastase and Ang II in Mas KO. Increased expression of cyclooxygenase-2 by Ang 1–7 was not observed in Mas KO mice. Increased expression of MMP-2 and MMP-9 is seen in patients with ruptured cerebral aneurysms.\textsuperscript{32} Moreover, in contrast to findings in WT mice, Ang 1–7 tended to increase the levels of MMP-2 and MMP-9 in cerebral arteries of Mas KO mice with intracranial aneurysms. Because Ang 1–7 is a weak agonist of Ang II receptors,\textsuperscript{35} we speculate that, in the absence of Mas receptors, Ang 1–7 may activate angiotensin type 1 receptors for Ang II and may induce further vascular damage. Our studies in Mas KO mice indicate that mice deficient in the receptor Mas had a lower mortality. This finding is puzzling because most literature suggests that
the activation of Mas receptors generally plays a protective role in disease, thus its deletion would be expected to exacerbate vascular damage. However, there are exceptions to this generalization because Mas activation is associated with aggravation of renal\(^1\) and cardiac\(^2\) disease and liver steatosis.\(^3\) In these experimental models, genetic deletion of Mas is associated with better outcomes.\(^3\)–\(^5\) Little is known about intracellular signaling pathways activated by Mas receptors or regulation of other receptors, such as angiotensin type 1 by Mas. Thus, we speculate that when Ang 1–7 levels are low, Mas receptors may not signal or may not regulate the activation of pathways, such as those activated by angiotensin type 1 receptors. In contrast, in conditions in which Ang 1–7 levels increase, Mas is activated and can physiologically antagonize other pathways, including the Ang II pathway.

**Perspective**

We demonstrated that the Mas receptor is expressed in the wall of human arteries and intracranial aneurysms. In addition, the infusion of Ang 1–7 attenuated aneurysm rupture and mortality in a mouse model of intracranial aneurysms. Ang 1–7 did not decrease the expression of markers of inflammation but regulated the expression of MMP-9 and cyclooxygenase-2. In conclusion, this study implies a potential novel therapeutic strategy for medical management of intracranial aneurysms. Additional studies may explore pharmacological strategies to modulate Ang 1–7 signaling in human intracranial aneurysms via agonists of the Mas receptor.

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**Disclosures**

None.

**References**


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Expanded Methods

Induction of Aneurysms

Intracranial aneurysms were induced according to previously published methods. Following anesthesia (ketamine-xylazine i.p.) and analgesia (buprenorphine 0.2mg/kg i.p), an incision was made in the scalp, a 1 mm hole was drilled in the skull and a needle was inserted stereotactically using the following coordinates: 2.7 mm posterior to the bregma, 1 mm to the right of the midline, depth of 6.2 mm from the skull. Bovine elastase (35 mU in 2.5 μl) was injected and an osmotic minipump was implanted subcutaneously to deliver a pressor dose of Ang-II (1000 ng/kg/min), or Ang-II + Ang-1-7 (400 ng/kg/min). In control mice (shams), physiologic 0.9% saline was used for the intracranial injection and in the osmotic pump.

After mice recovered, food and water were provided ad libitum. Mice that showed a full recovery 3 days after surgery were included in the studies. Mice were monitored daily for 3 weeks, and were immediately euthanized if signs of neurological deficit (suggesting subarachnoid hemorrhage) or weight loss (>20% baseline) were evident. A survival curve was made, and deaths include animals that were found dead or were euthanized because of signs of neurological deficit.

Tissue collection and aneurysm analysis

Immediately after euthanasia, mice were perfused transcardially with 10-15 ml of ice-cold physiologic saline containing papaverine (100 μM) to produce vasodilation, followed by infusion of 2 mg/ml of bromophenol blue dye in 8% gelatin/saline to facilitate visualization of arteries and aneurysms. The brain was then removed and inspected for the presence of intracranial aneurysms and/or subarachnoid hemorrhage. Intracranial aneurysms were defined as enlargement greater than 150% the diameter of the parent artery. Both saccular and fusiform enlargements were included, because both may rupture to produce subarachnoid hemorrhage. The arteries from mice found with signs of neurological deficit or euthanized at the end of the study were harvested for analysis of gene expression. Brains from mice found dead were photographed but their arteries were not harvested.

Gene expression

Arteries of the circle of Willis, including the basilar artery and middle cerebral arteries, were harvested, rapidly frozen in liquid nitrogen, and stored at -80°C. RNA was harvested in trizol and reverse transcribed as described previously. Real time polymerase chain reaction was performed using primer assays from Life technologies (Table S1). Ct levels of several mediators of inflammation and oxidative stress were obtained including, Nox2 (a catalytic subunit of NADPH oxidase), catalase, tumor necrosis factor α (TNFα), microsomal prostaglandin E2 synthase type 1 (mPGES-1), cyclooxygenase-2 (Cox2), regulator of calcineurin-1 (Rcan1), hepatocyte growth factor (HGF), integrin alpha M chain (Itgam), metalloproteinase 2 (MMP-2) and metalloproteinase 9 (MMP-9), and tissue inhibitor of metalloproteinases 1 (TIMP-1).
Histological analysis

In some mice, immediately after euthanasia, samples containing cerebral aneurysms or control arteries were rapidly dissected, fixed with buffered zinc formalin, and mounted in paraffin blocks. Masson’s trichrome staining was performed in 6 µm thick sections. Images were collected in an Olympus BX-61 motorized microscope.

Expression of Mas receptor in human intracranial aneurysm samples

Studies were approved by the University of Iowa Internal Review Board. Samples of superficial temporal and meningeal arteries and aneurysm tissue were obtained from patients that underwent microsurgical clipping. Tissues were fixed in formalin and embedded in paraffin blocks. Individual slides (6 µm thick) were stained using a previously validated rabbit anti Mas antibody 1/100 (AAR-013, Alomone Labs Ltd, Jerusalem, Israel)⁴. Chromogenic detection was performed using EnVision Plus (K-4008, Dako, Carpinteria, CA), and Chromogen-DAB kit (Dako, Carpinteria, CA). Images were collected using a 20x objective lens in an Olympus BX-61 motorized microscope.

References

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