Roles of Hypertension in the Rupture of Intracranial Aneurysms

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Background and Purpose—Systemic hypertension has long been considered a risk factor of aneurysmal rupture. However, a causal link between systemic hypertension and the development of aneurysmal rupture has not been established. In this study, using a mouse model of intracranial aneurysm rupture, we examined the roles of systemic hypertension in the development of aneurysmal rupture.

Methods—Aneurysms were induced by a combination of deoxycorticosterone acetate (DOCA)-salt and a single injection of elastase into the cerebrospinal fluid in mice. Antihypertensive treatment was started 6 days after aneurysm induction. Aneurysmal rupture was detected by neurological symptoms and confirmed by the presence of intracranial aneurysm with subarachnoid hemorrhage. Hydralazine (direct vasodilator) or discontinuation of DOCA-salt treatment was used to assess the roles of systemic hypertension. Captopril (angiotensin-converting enzyme inhibitor) or losartan (angiotensin II type 1 receptor antagonist) was used to assess the roles of the local renin–angiotensin system in the vascular wall.

Results—Normalization of blood pressure by hydralazine significantly reduced the incidence of ruptured aneurysms and the rupture rate. There was a dose-dependent relationship between reduction of blood pressure and prevention of aneurysmal rupture. Captopril and losartan were able to reduce rupture rate without affecting systemic hypertension induced by DOCA-salt treatment.

Conclusions—Normalization of blood pressure after aneurysm formation prevented aneurysmal rupture in mice. In addition, we found that the inhibition of the local renin–angiotensin system independent from the reduction of blood pressure can prevent aneurysmal rupture. (Stroke. 2014;45:579-586.)

Key Words: angiotensins ■ hypertension ■ intracranial aneurysm ■ models, animal ■ subarachnoid hemorrhage

Systemic hypertension has long been considered a risk factor of aneurysmal rupture.1,2 However, findings from clinical studies are conflicting, presumably because of the fact that the majority of patients with a diagnosis of hypertension are treated with antihypertensive agents,3,4 and as a result, these patients tend to have normal blood pressure at the time of diagnosis of intracranial aneurysm.5 Although experimental studies showed a link between the formation of intracranial aneurysms and systemic hypertension,6–10 a causal link between systemic hypertension and development of subarachnoid hemorrhage—aneurysmal rupture—has not been fully established in either experimental or clinical setting.

In patients with systemic hypertension, different types of antihypertensive agents with different molecular targets are chosen based on the types of end organ damages and underlying pathophysiology.2 However, it is not clear which type of antihypertensive agents may be suitable for patients with unruptured aneurysms, or which type of antihypertensive agents can reduce aneurysmal subarachnoid hemorrhage.

Hypertension may directly or indirectly contribute to aneurysmal rupture. Hypertension may weaken the aneurysmal wall by directly increasing mechanical stresses. In addition, activation of the local renin–angiotensin system by systemic hypertension can cause vascular inflammation and remodeling11 and may contribute to aneurysmal rupture. Certain polymorphisms in the genes related to the renin–angiotensin system are reported to be associated with aneurysmal rupture.12–14

Recently, we have developed a mouse model of intracranial aneurysm that morphologically and histologically resembles human intracranial aneurysms.9,15 In this model, aneurysmal rupture causes neurological symptoms that can be easily detected by a simple neurological examination.16,17 This model provides a unique opportunity to conduct preclinical studies.
studies for identifying therapeutic targets for the prevention of aneurysmal rupture. Using this mouse model of intracranial aneurysm, we examined the roles of systemic hypertension and the local renin–angiotensin system in the mechanisms for the rupture of intracranial aneurysms.

Methods
Experiments were conducted in accordance with the guidelines approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. Intracranial aneurysms were induced in 8- to 10-week-old male mice (C57BL/6J; Jackson Laboratory) as previously described.17,18 We combined induced systemic hypertension and a single injection of elastase into the cerebrospinal fluid at the right basal cistern.9,17,18 Detailed Methods are presented in the online-only Data Supplement.

To induce systemic hypertension, we used deoxycorticosterone acetate (DOCA)-salt.19 Mice underwent unilateral nephrectomy followed by an implantation of DOCA pellet 1 week later; 1% NaCl drinking water was started on the same day as DOCA pellet implantation.19,20 Mice received a single injection of elastase (0.035 U) into the cerebrospinal fluid at the right basal cistern on the same day as DOCA pellet implantation. Aneurysm was defined as a localized outward bulging of the vascular wall, whose diameter was greater than the parent artery diameter.9,18

To detect aneurysmal rupture, 2 blinded observers performed daily neurological examination as previously described.17 Neurological symptoms were scored as follows: 0—normal function; 1—reduced eating or drinking activity demonstrated by a weight loss >2 g of body weight (≈10% weight loss) >24 hours; 2—flexion of the torso and forelimbs on lifting of the whole animal by the tail; 3—circling to 1 side with a normal posture at rest; 4—leaning to 1 side at rest; 5—no spontaneous activity. We have shown that this neurological testing system is sensitive and specific for detecting aneurysmal rupture in this model.17 Mice were euthanized when they developed neurological symptoms (score 1–5). All asymptomatic mice were euthanized 21 days after aneurysm induction. The brain samples were perfused with PBS, followed by a gelatin containing blue dye to visualize cerebral arteries. Two blinded observers assessed aneurysm formation and subarachnoid hemorrhage. Aneurysm rate was defined as the total number of mice with ruptured aneurysms divided by the number of mice with any aneurysms.17 Figure 1 shows a mouse with normal cerebral arteries, an unruptured aneurysm from a mouse that was asymptomatic throughout the experimental period, and a ruptured aneurysm with subarachnoid hemorrhage from a mouse that became symptomatic 12 days after aneurysm induction.

Our previous study found that aneurysm formation happens during the first 6 days after aneurysm induction, and aneurysmal rupture start occurring ≥7 days after aneurysm induction in this model.17 We found that by treating the mice with an experimental agent starting from 6 days after aneurysm induction, we could test whether the experimental agent can reduce the rupture rate.17 Therefore, in this study, treatments with antihypertensive agents were started 6 days after aneurysm induction and continued for 2 weeks.

Statistical Analysis
Primary outcomes were the incidence of ruptured intracranial aneurysms and the rupture rate (number of mice with ruptured aneurysms/number of mice with ruptured or unruptured aneurysms). We used Fisher exact test for the analysis of primary outcomes. As an exploratory analysis, the survival analysis was performed using the log-rank test. Mice that did not develop aneurysms were excluded from the survival analysis. Blood pressure and body weight were analyzed by ANOVA, followed by the Tukey–Kramer post hoc test. All the results were expressed as mean±SD. Statistical significance was considered at P<0.05.

Results
Effect of Normalization of Blood Pressure on the Development of Aneurysmal Rupture
As a first step to examine the contributions of systemic hypertension to aneurysmal rupture, we tested the effects of normalization of systemic hypertension after aneurysmal formation on the development of aneurysmal rupture. We used hydralazine to normalize blood pressure. Hydralazine, a direct vasodilator, can normalize systemic hypertension induced by DOCA-salt hypertension without directly affecting the renin–angiotensin system.19 Hydralazine treatment (25 or 50 mg/kg per day in drinking water)19,20 was started 6 days after aneurysm induction and continued for 2 weeks (Figure 2A). The control group received drinking water without hydralazine.

A No aneurysm

B Unruptured aneurysm

C Ruptured aneurysm

Figure 1. Representative intracranial aneurysms. A, Normal cerebral arteries. B, Unruptured aneurysm in the posterior cerebral artery. C, Ruptured aneurysm with subarachnoid hemorrhage. Cerebral arteries were visualized by blue dyes dissolved in gelatin. Arrows indicate aneurysms.
As shown in Figure 2A, systemic hypertension was successfully induced in the mice receiving DOCA-salt treatment. Hydralazine at 50 mg/kg per day started 6 days after aneurysm induction effectively normalized blood pressure. A lower dose of hydralazine (25 mg/kg per day) partially normalized blood pressure. Normalization of blood pressure by hydralazine did not significantly affect the formation of aneurysms, as demonstrated by no difference in the total incidence of aneurysms (ie, incidence of both ruptured and unruptured aneurysms) between 2 groups (75 versus 60%; P=0.46; Figure 2B). However, normalization of blood pressure by hydralazine (50 mg/kg per day) significantly reduced the incidence of ruptured aneurysms and the rupture rate (incidence of ruptured aneurysms: 56% versus 13%; P<0.05; rupture rate: 75% versus 22%; P<0.05; Figure 2B and 2C). Partial normalization of blood pressure by a lower dose of hydralazine (25 mg/kg per day) showed a trend of reduction in rupture rate (incidence of ruptured aneurysms: 56% versus 30%; P=0.25; rupture rate: 75% versus 38%; P=0.17). Taken together, there was a dose-dependent effect of blood pressure reduction on the development of aneurysmal rupture. For the purpose of exploratory analysis, a symptom-free curve (Kaplan–Meier analysis curve) was plotted after excluding mice that did not have aneurysms (Figure 2D). A log-rank test revealed a significant reduction of aneurysmal rupture with the normalization of blood pressure by hydralazine (P<0.05).

To further confirm the critical role of hypertension in the development of aneurysmal rupture, we tested whether a reduction of blood pressure by the discontinuation of DOCA-salt treatment would reduce aneurysmal rupture. To this end, we removed the DOCA pellet and switched to drinking water without salt at 6 days after aneurysm induction (Figure 3A). The control group received standard DOCA-salt treatment throughout the experimental period. As shown in Figure 3A, in mice for which DOCA-salt treatment was discontinued 6 days after aneurysm induction, there was a gradual reduction in blood pressure. However, the discontinuation of DOCA-salt treatment did not completely normalize the blood pressure during the course of 2 weeks, possibly reflecting the residual effects of DOCA-salt treatment. There was no difference in the total incidence of aneurysms (72 versus 58%; P=0.38; Figure 3B). The discontinuation of DOCA-salt treatment significantly reduced the incidence of ruptured aneurysms (P<0.05; Figure 3B). There was a trend for the discontinuation of the DOCA-salt treatment to reduce the rupture rate (P=0.07; Figure 3C), but the difference was not statistically significant. This result is probably reflecting the partial normalization of blood pressure.
pressure in this group. The improvement of survival by the discontinuation of DOCA-salt treatment was not statistically significant (Figure 3D).

Local Renin–Angiotensin II System Was Upregulated in Aneurysmal Walls
Because systemic hypertension can affect the local renin–angiotensin system in the vascular wall,19 we examined the expression of angiotensin II and angiotensin type 1 (AT1) receptor in the normal cerebral artery and intracranial aneurysms in mice. Three representative samples were used. Although there was a weak expression of angiotensin II and AT1 receptor in the normal cerebral artery, both angiotensin II and AT1 receptor were abundant in the mouse model of intracranial aneurysm (Figure 4).

Roles of the Local Renin–Angiotensin System in the Development of Aneurysmal Rupture
To test the roles of local renin–angiotensin system in the development of aneurysmal rupture, we used captopril (angiotensin-converting enzyme inhibitor) and losartan (AT1 antagonist) in mice with DOCA-salt hypertension. We took advantage of the ability of these agents to block the local renin–angiotensin system without affecting systemic hypertension induced by DOCA-salt treatment.15,19 Captopril and losartan doses were chosen based on previous studies.15,19,21–23 Treatment with captopril (6 mg/kg per day in PBS; AK Scientific) or a vehicle through an implanted osmotic pump was started 6 days after aneurysm induction and continued for 2 weeks (Figure 5A). As was expected, captopril did not affect systemic hypertension induced by DOCA-salt treatment. Although captopril treatment started 6 days after aneurysm induction did not affect the overall incidence of aneurysms, it significantly reduced the incidence of ruptured aneurysms and the rupture rate (incidence of ruptured aneurysms: 63% versus 24%; \( P<0.05 \); rupture rate: 91% versus 36%; \( P<0.05 \); Figure 5B and 5C). Survival analysis revealed that mice that had aneurysms showed significant improvement in survival by captopril treatment (\( P<0.05 \); Figure 5D).

As a next step, we tested the effect of losartan in blocking AT1, which is downstream of captopril’s target. Treatment with losartan (30 mg/kg per day; AK Scientific) or a vehicle through an implanted osmotic pump was started 6 days after aneurysm induction. Losartan did not affect systemic hypertension induced by DOCA-salt treatment (Figure 6A). Similar to captopril treatment, losartan treatment significantly reduced the incidence of ruptured aneurysms and the rupture rate (incidence of ruptured aneurysms: 54% versus 24%; \( P<0.05 \); rupture rate: 71% versus 33%; \( P<0.05 \)) without affecting the total incidence of aneurysms (Figure 6B).
Survival analysis revealed that mice that had aneurysms showed a significant improvement in survival by losartan treatment ($P<0.05$; Figure 6D).

The inhibition of the local renin–angiotensin system by captopril or losartan without affecting systemic hypertension reduced aneurysmal rupture, suggesting a potential contribution of the activation of the local renin–angiotensin system in the development of aneurysmal rupture.

Discussion

In this study, we found that normalization of blood pressure after aneurysm formation could prevent aneurysmal rupture in mice and that there is a dose-dependent relationship between blood pressure and aneurysmal rupture, establishing, for the first time to our knowledge, the causal relationship between normalization of blood pressure and prevention of aneurysmal rupture. In addition, we found that the inhibition of the local renin–angiotensin system, independent from the reduction of blood pressure, could prevent aneurysmal rupture. These observations suggest that the activation of the local renin–angiotensin system in the aneurysmal wall presumably by systemic hypertension may induce aneurysmal rupture, at least, in a mouse model of intracranial aneurysm.

In patients with hypertension, normalization of blood pressure has been clinically proved to be effective in preventing ischemic stroke, intracerebral hemorrhage, and cardiac events. Although antihypertensive treatment is generally recommended for hypertensive patients with unruptured intracranial aneurysm, whether normalization of blood pressure confers protection against the development of aneurysmal rupture is not known. It would be practically impossible to test such a question in clinical setting, because not treating hypertension is not an ethical option. However, it may be possible to test which class of antihypertensive agent is suitable for hypertensive patients with unruptured aneurysms.

In recent years, unruptured intracranial aneurysms have increasingly been diagnosed, primarily because of the widespread use of noninvasive brain imaging techniques. Unruptured aneurysms are asymptomatic until they rupture. The 30-day mortality rate after aneurysmal subarachnoid hemorrhage is as high as 45%. Surgical clipping or endovascular coiling can be offered to patients with unruptured aneurysms for the prevention of aneurysmal rupture. Significant technical advancements and refinements have been made in these invasive treatments. However, mortality and morbidity resulting from the clipping and coiling of unruptured aneurysms are not negligible. The 1-year adverse outcome rate, including mortality and significant morbidity, can be as high as 20%. In addition, these invasive therapies are technically intensive and costly. Therefore, the pharmacological prevention of aneurysmal rupture is an attractive alternative approach in patients with unruptured aneurysms.

Previous studies performed by our group and others have focused on the mechanisms involved in the formation of aneurysms. These efforts were based on the assumption that the processes of aneurysmal formation, growth, and rupture share similar mechanisms. However, the mechanisms of aneurysmal rupture may be fundamentally different from those of their formation and growth. In the mouse model used in this study, spontaneous aneurysmal rupture occurs with a predictable time course, and aneurysmal rupture can be easily detected by assessing neurological symptoms, as presented in our recently published article. This model provides us with a unique opportunity to study the mechanisms of aneurysmal rupture as well as pharmacological prevention.

In patients with hypertension, normalization of blood pressure has been clinically proved to be effective in preventing ischemic stroke, intracerebral hemorrhage, and cardiac events. Although antihypertensive treatment is generally recommended for hypertensive patients with unruptured intracranial aneurysm, whether normalization of blood pressure confers protection against the development of aneurysmal rupture is not known. It would be practically impossible to test such a question in clinical setting, because not treating hypertension is not an ethical option. However, it may be possible to test which class of antihypertensive agent is suitable for hypertensive patients with unruptured aneurysms.

Systemic hypertension can affect tissue remodeling and inflammation of the aneurysmal wall. In addition to exerting abnormal hemodynamic stresses, hypertension can activate...
the local renin–angiotensin system in the vascular wall.\textsuperscript{19} Local renin–angiotensin system can control vascular remodeling by affecting smooth muscle migration and proliferation,\textsuperscript{31} processes that could potentially lead to destabilization of the aneurysmal wall. In addition, it can mediate vascular inflammation through the activation of NF-κB, which can further promote inflammation inside the aneurysmal wall.\textsuperscript{30} Gene polymorphisms in angiotensin-converting enzymes are associated with the rupture of intracranial aneurysms,\textsuperscript{12–14} suggesting a link between renin–angiotensin system and aneurysmal rupture. Taken together, the local renin–angiotensin system can be a therapeutic target for the prevention of aneurysmal rupture. There are many clinically available antihypertensive agents that can inhibit different steps of the local renin–angiotensin system. Antihypertensive agents that possess inhibitory effects on the local renin–angiotensin system may contribute to the prevention of aneurysmal rupture not only through normalization of blood pressure, but also through the inhibition of the local renin–angiotensin system.

There are several limitations in this study. First, the study design depended on the pharmacological approach. Although the use of clinically available antihypertensive agents makes our findings readily translatable, the antihypertensive agents chosen for our study may not be specific to their designed targets (ie, vascular calcium channel, angiotensin II type 1 receptor, angiotensin-converting enzyme, etc). These antihypertensive agents possess nonhemodynamic effects that can potentially affect the processes that lead to aneurysmal rupture. Another major limitation of this study is that we did not directly assess the activity of the local renin–angiotensin system. Although our immunohistochemistry data suggested the activation of the renin–angiotensin system, we were not able to measure its activity directly. As previously recognized by others, currently there is no reliable way to directly quantify the activation of the local renin–angiotensin system.\textsuperscript{19}

Conclusions
Using the mouse model of intracranial aneurysms, we revealed the critical roles of systemic hypertension and the local renin–angiotensin system in the development of aneurysmal rupture. Our study may become a basis for the clinical study to find the optimal choice of antihypertensive agents for patients with unruptured intracranial aneurysms.

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Figure 5. Inhibition of the local renin–angiotensin system by captopril prevented aneurysmal rupture without affecting systemic hypertension. \textbf{A}, Blood pressure. \textbf{B}, Incidence of ruptured and unruptured aneurysms. \textbf{C}, Rupture rate. \textbf{D}, Survival curve.
represent the official views of NINDS, NIH, the American Heart Association, or the Brain Aneurysm Foundation.

Disclosures
None.

References
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Supplemental Material

Roles of hypertension in the rupture of intracranial aneurysms

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Short title: Hypertension and intracranial aneurysm rupture

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**Supplemental Methods**

Experiments were conducted in accordance with the guidelines approved by the University of California, San Francisco’s Institutional Animal Care and Use Committee. We used 10- to 12-week-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine). All of the surgical procedures were performed under general anesthesia with isoflurane or a combination of ketamine and xylazine.

Intracranial aneurysms were induced by combining induced systemic hypertension and a single injection of elastase into the cerebrospinal fluid at the right basal cistern using the previously described method.1-4

To induce systemic hypertension, we used deoxycorticosterone acetate-salt hypertension (DOCA-salt hypertension).2 The mice underwent unilateral nephrectomy followed one week later by the implantation of a DOCA pellet (66 mg, 28-day release); 1% sodium chloride drinking water was started on the same day as the pellet implantation.5 The mice received a single injection of elastase (35 milli-units) into the cerebrospinal fluid at the right basal cistern on the same day as the DOCA pellet implantation.3, 4

Using the tail cuff method, systolic blood pressure was measured in the mice before the treatment and two and three weeks after the elastase injection.5 Three weeks after the aneurysm induction, we euthanized the mice and perfused the animals with bromophenol blue dye. Three blinded observers assessed the formation of intracranial aneurysms by examining the Circle of Willis and its major branches under a dissecting microscope (10X). Intracranial aneurysms were operationally defined as a localized outward bulging of the vascular wall in the Circle of Willis or in its major primary branches, as previously described.4

Two blinded observers performed daily neurological examination using a previously described method with minor modifications.6-9 Neurological signs were scored as followings; 0: normal function; 1: reduced eating or drinking activity demonstrated by the weight loss greater than 2 grams of body weight (approximately 10% weight loss) over 24 hours; 2: flexion of torso and forelimb upon lifting of the whole animal by the tail; 3: circling to one side but normal posture at rest; 4: leaning to one side at rest; and 5, no spontaneous activity. Mice were euthanized when the neurological score was 1-5. All asymptomatic mice were euthanized 21 days after aneurysm induction. After euthanasia, mice were perfused with phosphate-buffered saline, then with gelatin containing blue dyes to visualize the cerebral arteries. Two blinded investigators assessed brains for aneurysm formation and subarachnoid hemorrhage.

Immunohistochemistry was performed as previously described.4, 5 The mice were dissected and perfused transcardially with saline followed by perfusion with a mixture of bromophenol blue dye and gelatin mixture. After the brain tissues were fixed 4% paraformaldehyde, the tissues were frozen in OCT compound (Tissue-Tek), and cut into 6-um sections. Sections were stained with hematoxylin and eosin (H&E). In all staining procedures we referred to positive controls. Visualization was under a microscope (BIOREVO, Keyence, California, USA). The primary antibodies we used were goat polyclonal anti-angiotensin II (Santa-Cruz), and rabbit polyclonal anti-angiotensin II type 1 receptor (Santa Cruz).
References