Spontaneous Stroke in a Genetic Model of Hypertension in Mice

Shinichiro Iida, MD, PhD; Gary L. Baumbach, MD; Julie L. Lavoie, PhD; Frank M. Faraci, PhD; Curt D. Sigmund, PhD; Donald D. Heistad, MD

Background and Purpose—Hypertension is the most common risk factor for hemorrhagic stroke. An experimental model of stroke, the stroke-prone spontaneously hypertensive rat (SHRSP), which has been enormously useful in studies of cerebral circulation, has been used in >1000 papers. However, SHRSP usually have an ischemic or less commonly hemorrhagic stroke in the cortex, not in the brain stem, cerebellum, or basal ganglia, as in patients with hypertension. The goal of this study was to develop a model of hemorrhagic stroke in hypertensive mice.

Methods—A genetic model of hypertensive mice, double transgenic mice (R/H11001) that overexpress both human renin (R+) and human angiotensigen (A+), and nonhypertensive control mice were divided into 3 groups: (1) high-salt diet; (2) Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthases, in drinking water; and (3) high-salt diet and L-NAME.

Results—All R+/A+ mice on high-salt diet and L-NAME died within 10 weeks, with hemorrhage in the brain stem, and several of the mice had hemorrhages in brain stem, cerebellum, and basal ganglia. No control mice on high-salt diet and L-NAME had hemorrhagic stroke. Arterial pressure in R+/A+ mice increased progressively during high-salt diet and L-NAME. In R+/A+ and control mice, high-salt diet or L-NAME alone did not increase arterial pressure.

Conclusions—We now describe the first model of spontaneous hemorrhagic strokes in hypertensive mice. The type and locations of stroke are reasonably similar to those observed in patients with hypertension. (Stroke. 2005;36:1253-1258.)

Key Words: hemorrhage ■ hypertension ■ stroke, acute

Hypertension is the strongest risk factor for primary hemorrhagic stroke.1-3 Hemorrhagic stroke accounts for 15% of all strokes and is associated with a high mortality rate.1-3 Hypertension-induced hemorrhagic stroke primarily affects small blood vessels (50 to 200 μm/L) in the brain stem, cerebellum, and basal ganglia, but not in the cerebral cortex, which is the major site of ischemic stroke.1-3 The causes of hemorrhagic strokes are poorly understood. It is widely recognized that a stroke-prone mouse is needed to facilitate understanding of causes and treatment of stroke.4

A strain of spontaneously hypertensive rats was bred to be stroke-prone (SHRSP). SHRSPs have a high incidence of ischemic and hemorrhagic stroke in the cerebral, but not in brain stem, cerebellum, or basal ganglia.5 There is no experimental model for spontaneous intraparenchymal hemorrhage in hypertensive mice. Previous studies of experimental stroke in mice have focused on ischemic stroke6 or need surgical intervention to produce hemorrhagic stroke.7,8 The goal of this study was to develop and characterize a model of hemorrhagic stroke in hypertensive mice that closely resembles stroke in hypertensive humans.

Transgenic mice containing both the human renin and angiotensigen genes (R+/A+) are chronically hypertensive, with mean arterial pressure (MAP) ≈ 150 mm Hg.9 Despite life-long increase in blood pressure, survival of R+/A+ is normal.10 We therefore used a treatment to increase blood pressure, with the goal of inducing hemorrhage in the brain. Because SHRSP that were fed high-salt diet11 or Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthases,12 had severe hypertension with ischemic and hemorrhagic strokes in the cortex, we gave R+/A+ and control mice high-salt diet and L-NAME in drinking water.

Materials and Methods

Experimental Animals

The experimental protocol was approved by the institutional animal care and use committee of the University of Iowa. Breeding and genotyping were performed in the transgenic animal facility (directed by C.D.S.). Mice were housed in individual cages and kept on a 12-hour light/dark cycle (6:00 AM to 6:00 PM light).

Double transgenic mice (R+/A+) were generated by crossing breeding human renin (R) transgenic mice with human angiotensigen (A+) transgenic mice.5 The presence of the transgenes was assessed by gene-specific and species-specific polymerase chain reaction of DNA isolated from tail biopsy samples.9 R+/A+ and single transgenic mice (R+/A- or R-/A+) were used for controls in this study. R+/A-, R+/A- and R+/A- mice do not have...

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hypothesis because strict species specificity in the enzymatic reaction exists between the mice and human renin-angiotensinogen systems.9

Experimental Protocols
Female mice (n=48, 4 to 5 months old) were given an 8% high-salt diet and/or 100 to 120 mg/kg per day L-NAME in drinking water to increase arterial pressure.13 We divided the R+/A- and control mice into 3 groups (n=8 each): (1) high-salt diet and normal drinking water; (2) normal diet and L-NAME in drinking water; and (3) high-salt diet and L-NAME in drinking water. In a preliminary study, mice on high-salt diet drank 3-times more water than mice on normal chow. We therefore gave 3-times higher concentration of L-NAME in the drinking water to mice that received L-NAME alone than to mice that were fed a high-salt diet.

Clinical signs of stroke were assessed by periodic neurological examinations, including contralateral forelimb extension, circling behavior, or other motor dysfunction. Mice were euthanized if these signs developed. Mice were observed for 24 weeks if they did not die prematurely, and the brains were excised after an overdose of pentobarbital (150 mg/kg intraperitoneal).

Histopathology of the Brain
The brain of each mouse was fixed in 7% formaldehyde, cut into 8 to 10 coronal sections of equal thickness, and examined grossly for evidence of hemorrhage.14 Paraffin-embedded tissue was sectioned at 5 to 7 μm and stained with hematoxylin and eosin. The brain was examined with light microscopy and the size of hemorrhages was quantified with Image J from National Institutes of Health by an experienced neuropathologist (G.L.B.).

Measurements of Systolic Blood Pressure
Systolic blood pressure (SBP) in conscious mice was determined by tail cuff (Visitech Systems BP-2000).15 R+/A- mice and control mice on treatment were trained for 3 consecutive days in the prewarmed tail-cuff device, and then SBP was measured for 2 days. On each day, 30 measurements were obtained and averaged for each mouse. Averaged values of SBP were measured at baseline and once monthly for 24 weeks.

Measurements of MAP
Arterial pressure in the other R+/A- mice (n=14) on each treatment was measured by radiotelemetry.15 A pressure-sensing catheter was implanted in the left carotid artery, and a transmitter was placed in the flank. After 1 week, arterial pressure was measured. MAP was recorded every 5 minutes for 24 hours on alternate days for 1 week and averaged for the values of MAP. MAP after treatment was recorded every 3 days for up to 1 month, and values were averaged weekly. Arterial pressure was divided into day (6:00 AM to 6:00 PM) and night (6:00 PM to 6:00 AM).

Statistics
Results are expressed as mean±SEM. Data for SBP and MAP for 24 hours were analyzed with repeated-measures ANOVA. One-way ANOVA followed by Scheffe test was used for multiple comparisons. P<0.05 was considered significant.

Results
Survival
All R+/A- mice fed high-salt diet and L-NAME (8 of 8) died within 10 weeks (Figure 1). Three of 8 R+/A- mice on L-NAME alone died during 18 to 23 weeks of treatment. Two of 8 R+/A- mice on high-salt alone died (1 died 2 weeks and the other died 19 weeks after beginning treatment). No control mice, irrespective of treatment, died during 24 weeks of the study.

Pathology of the Brain
The brain of each mouse was excised when they were either found to have clinical signs of stroke or after 24 weeks of study. All R+/A- mice on high-salt diet and L-NAME (8 of 8) had multiple acute hemorrhages in the brain stem (pons) (Figure 2a), 4 of 8 in the cerebellum (Figure 2b), and 2 of 8 in basal ganglia (Figure 2c). The area of hemorrhages was 22±9 μm² in the brain stem, 47±25 μm² in the cerebrum, and 110 μm² in basal ganglia (Table 1). No control mice, irrespective of treatment, had hemorrhages in the brain stem, cerebrum, cerebellum, or basal ganglia (Figure 2d, 2e, and 2f). In contrast, among the 5 of 16 R+/A- mice treated with either high-salt diet or L-NAME alone that died during 24 weeks of the study, 3 mice had hemorrhage in the brain stem and 2 others had a subarachnoid hematoma either in the cerebrum (Figure 2g) or base of brain (Figure 2h). None of the R+/A- mice on either high-salt diet or L-NAME alone that survived 24 weeks had hemorrhages in the brain.

SBP
SBP at baseline (4 to 5 months of age) was higher in R+/A+ mice than in control mice (R+/A+ mice 117±7 mm Hg versus control mice 98±2 mm Hg; P<0.05) (Figure 3). In R+/A- mice on high-salt diet and L-NAME, SBP increased progressively during 8 weeks of treatment (Figure 3). In R+/A- mice on L-NAME alone, SBP increased modestly during 24 weeks of study, whereas high-salt diet had no effect of SBP in R+/A- mice (Figure 3). In control mice on high-salt diet and L-NAME or L-NAME alone, SBP increased modestly during 24 weeks of study, whereas high-salt diet had no effect on SBP in control mice (Figure 3).

MAP
MAP measured continuously by radiotelemetry in R+/A- was 149±2 mm Hg at baseline and increased progressively to 198±6 mm Hg during 3 weeks of treatment with both high-salt diet and L-NAME (Figure 4a). Treatment with high-salt diet or L-NAME alone did not affect MAP in R+/A+ mice. MAP was paradoxically higher during the day than at night in R+/A+ mice treated with high-salt diet and L-NAME, whereas MAP was lower during the day (which is normal) in
the other groups (Figure 4b and 4d). There was no significant difference in MAP at night in any group (Figure 4c).

Cardiac Hypertrophy, Renal Function, and Aortic Aneurysm

Heart/body weight was greater in R'/A+ mice than in control mice. R'/A+ mice on high-salt diet and L-NAME were heaviest in all groups (Table 2).

R'/A+ mice on high-salt diet and L-NAME were heaviest in all groups (18.2±1.9 mg/g).

Plasma creatinine was between 0.16±0.02 mg/dL and 0.26±0.08 mg/dL in all groups of mice, with normal plasma creatinine (by Jeffe method16) of 0.4±0.1 mg/dL.

Two of 8 R'/A+ mice on high-salt diet and L-NAME had dissecting aortic aneurysm in addition to hemorrhagic strokes. No control mice or R'/A+ mice on either high-salt diet or L-NAME had aortic aneurysm.

TABLE 1. Hemorrhages in the Brain of R'/A+ Mice on High Salt and L-NAME

<table>
<thead>
<tr>
<th>Region</th>
<th>No.</th>
<th>Area, μm²</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain Stem (Pons)</td>
<td>8</td>
<td>2±9</td>
<td>Intraparenchymal</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>4</td>
<td>47±25</td>
<td>Intraparenchymal</td>
</tr>
<tr>
<td>Basal Ganglia</td>
<td>2</td>
<td>111</td>
<td>Intraparenchymal</td>
</tr>
</tbody>
</table>

No. indicates number of mice.
Values are mean±SEM.

Figure 2. Brain sections in R'/A+ and control mice. There were hemorrhages in the brain stem (pons) (a), cerebellum (b), and basal ganglia (c) in R'/A+ mice on high-salt diet and L-NAME. No hemorrhage was observed in the brain stem (pons) (d), cerebellum (e), and basal ganglia (f) in control mice (hematoxylin and eosin staining). Bar is 50 μmol/L (a), (c), and (f); 100 μm in (b), (d), and (e). Acute subarachnoid hematomas were observed in cerebrum (g) and base of brain (h) of R'/A+ mice on single treatment. Bar is 250 μm (g) and 100 μm (h).

Figure 3. SBP in R'/A+ and control mice by tail cuff (n=8 in each group). Values are mean±SEM. *P<0.05 R'/A+ mice on high-salt diet and L-NAME vs R'/A+ mice on high-salt diet or L-NAME alone, or control mice on any treatment.

Discussion

This is the first report to our knowledge of a model of hypertensive mice with hemorrhagic stroke. The strokes occur in the brain stem, cerebellum, and basal ganglia, which is reasonably similar in location to those that occur in hypertensive patients.
Characterization of R/H11545 Mice

R/H11545 mice are transgenic mice that were generated by expression of human renin and angiotensinogen genes in many tissues, including brain, kidney, and liver. The mice have 4-fold higher plasma angiotensin II than normal mice and are chronically hypertensive, with MAP 150 to 160 mm Hg. We observed that R/H11545 mice on high-salt diet and L-NAME had augmented hypertension and hemorrhagic stroke.

Single transgenic (R/H11002 or R/H11545) mice and nontransgenic (R/H11002) mice are normotensive and have normal angiotensin II in plasma, because mouse renin does not cleave human angiotensinogen and human renin does not cleave mouse angiotensinogen. Thus, we used normotensive nontransgenic or single transgenic mice as controls.

Tsukuba-hypertensive mice comprise another model of transgenic mice that overexpress human renin and human angiotensinogen and have hypertension and aortic aneurysm. Most Tsukuba-hypertensive mice on 1% sodium diet die from rupture of aortic aneurysm. To our knowledge, there have been no studies that have demonstrated strokes in Tsukuba-hypertensive mice. Our findings suggest that R/H11545 mice on high-salt diet and l-NAME have hemorrhagic stroke and lesser frequency of dissecting aneurysm of the aorta.

The cause of death in R/H11545 mice on high-salt diet and l-NAME is not entirely clear. The mice had cardiac hypertrophy and glomerulosclerosis, but we did not see pleural effusions or other evidence of heart failure, and plasma creatinine was normal. Six of 8 R/H11545 mice on high-salt diet and l-NAME had bradykinesia and tremor, and thus had

<table>
<thead>
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<th>Treatment</th>
<th>Heart Wt, g</th>
<th>Heart Wt/Body Wt, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>R/H11545 mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High salt and l-NAME</td>
<td>0.33±0.03†*</td>
<td>18.2±1.9†*</td>
</tr>
<tr>
<td>High salt</td>
<td>0.25±0.04*</td>
<td>11.5±2.1†</td>
</tr>
<tr>
<td>l-NAME</td>
<td>0.22±0.03*</td>
<td>11.3±1.6†</td>
</tr>
<tr>
<td>Control mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High salt and l-NAME</td>
<td>0.15±0.01</td>
<td>6.5±0.2</td>
</tr>
<tr>
<td>High salt</td>
<td>0.15±0.01</td>
<td>6.2±0.2</td>
</tr>
<tr>
<td>l-NAME</td>
<td>0.13±0.01</td>
<td>6.0±0.5</td>
</tr>
</tbody>
</table>

Values are mean±SEM.  
*P<0.05 mice on high salt and L-NAME vs mice on high salt or L-NAME alone.  
†P<0.05 R/H11545 mice vs Control mice.
signs of stroke. Two of 8 R\(^+\)/A\(^+\) mice on high-salt diet and L-NAME also had an aortic aneurysm.

**Experimental Stroke-Prone Hypertensive Model**

In an experimental model of stroke in rats, SHRSP typically have hypertension and ischemic stroke in the neocortex.\(^5\) Thus, the stroke is different from the typical stroke in patients with hypertension.\(^1\)–\(^3\)

In mice, currently available models of stroke require a surgical intervention, such as ligation of a middle cerebral artery\(^6\) to produce ischemic stroke, injection of bacterial collagenase\(^7\) or blood and cerebrospinal fluid\(^8\) to produce or simulate hemorrhagic stroke, or exhibit hemorrhages in cerebrum, in a model of cerebral amyloid angiopathy.\(^19\) This is the first report to our knowledge of a model of hypertensive mice with hemorrhagic stroke. The stroke occurs in the brain stem, cerebellum, and basal ganglia, which is reasonably similar in location to those that occur in hypertensive patients.

R\(^+\)/A\(^+\) mice on both high-salt diet and L-NAME had bradykinesia and tremor after strokes, whereas SHRSPs\(^20\) and mice with surgical intervention\(^21\) sometimes have unilateral hemiparesis. Our observations of neurological changes in R\(^+\)/A\(^+\) mice are compatible with the location of hemorrhages, which occurred mainly in brain stem and cerebellum, whereas impaired motor function in SHRSP and mice with surgical intervention is concordant with location of strokes in motor regions of the cortex.

**Mechanisms of Stroke in R\(^+\)/A\(^+\) Mice Receiving High-Salt Diet and L-NAME**

SHRSP on high-salt diet have severe hypertension and rapid onset of stroke.\(^11\) Despite high-salt diet intake, SHRSP maintain paradoxically high renin activity when fed a high-salt diet, although high-salt diet suppresses plasma renin activity in normal rats.\(^22\) This finding suggests that activation of the renin-angiotensin system may be associated with the stroke in SHRSP. High renin activity is a hallmark of the R\(^+\)/A\(^+\) mice that we have studied.

We speculate that the primary mechanism by which L-NAME in combination with high-salt diet increases hemorrhagic stroke in our model relates to augmentation of the increase in arterial pressure. A variety of other mechanisms may also contribute to hemorrhagic stroke in these mice. First, stroke may be related to inhibition of nitric oxide synthases or effects of salt on cerebral blood vessels. Second, R\(^+\)/A\(^+\) mice might have platelet dysfunction from uremia. But R\(^+\)/A\(^+\) mice on high-salt diet and L-NAME had normal plasma creatinine. Third, levels of superoxide in vessels of R\(^+\)/A\(^+\) mice increase and contribute to vasomotor dysfunction.\(^23\)

Angiotensin-induced superoxide impairs endothelial function in cerebral arteries.\(^24\) Thus, we speculate that increases in superoxide induced by angiotensin may contribute to development of hemorrhagic stroke in this model. Fourth, long-term administration of L-NAME, which stimulates angiotensin-converting enzyme,\(^25\) might increase angiotensin II in R\(^+\)/A\(^+\) mice, and it is possible that increased levels of angiotensin II are a risk factor of stroke.

**Arterial Pressure and Circadian Rhythm**

We used the tail-cuff method to measure SBP,\(^15\) because the tail-cuff method is noninvasive and is useful for long-term measurement of SBP. We also used the radiotelemetry method to provide an accurate measurements of MAP in R\(^+\)/A\(^+\) mice.\(^15\) MAP by the radiotelemetry method is more reliable than SBP by tail-cuff method.\(^15\) Moreover, the radiotelemetry method allows measurement of circadian rhythm in mice because parameters are sampled continuously.\(^15\) Our findings related to effects of treatment on arterial pressure were similar with the tail-cuff and radiotelemetry methods.

In normal humans, arterial pressure decreases 10% to 20% during sleep. Humans with this response are termed “dippers.”\(^26\) Hypertensive patients who have a decrease in nocturnal arterial pressure >20 mm Hg (“extreme dippers”) have increased risk of ischemic strokes,\(^27\) and those who have an increase in nocturnal arterial pressure (“reverse dippers”) have twice the risk of stroke, especially hemorrhagic stroke.\(^27\) All R\(^+\)/A\(^+\) mice exhibited the normal pattern (for rodents) of activity at night and sleep during the day, and also a normal circadian rhythm, with night MAP 156±2 mm Hg and day MAP 143±2 mm Hg. Remarkably, however, arterial pressure was higher during the day than night in R\(^+\)/A\(^+\) mice on high-salt diet and L-NAME, which suggests that model high-risk “reverse dippers” in humans. In contrast, R\(^+\)/A\(^+\) mice with or without high-salt diet or L-NAME alone had normal circadian rhythms (“dippers”). These data provide strong evidence that this experimental model imitates one of the risk factors observed in humans (reverse dipping) and exhibits stroke with similar features to humans.

To our knowledge, no other strains of hypertensive mice have hemorrhagic stroke. It will be of great interest in future studies to examine mechanisms that lead to, and protect against, stroke in these mice. It also will be of interest to determine whether mice with comparable levels of hypertension that do not have activation of the renin-angiotensin system also are susceptible to development of hemorrhagic stroke.

**Acknowledgments**

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