Critical Role for Copper/Zinc–Superoxide Dismutase in Preventing Spontaneous Intracerebral Hemorrhage During Acute and Chronic Hypertension in Mice

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**Backgrounds and Purpose**—Superoxide is associated with spontaneous intracerebral hemorrhage (ICH) during hypertension. The goal of this study was to test the hypothesis that changes in superoxide, in genetically altered mice with deletion and overexpression of copper/zinc–superoxide dismutase (SOD1), modulate susceptibility to ICH.

**Methods**—Chronic hypertension was produced by infusion of angiotensin II and an inhibitor of nitric oxide synthase in drinking water in SOD1 transgenic (SOD1Tg) mice, SOD1-deficient (SOD1\(^{-/-}\)) mice, and their respective wild-type littermates. Acute hypertension was produced by daily injections of angiotensin II in some mice with chronic hypertension to produce ICH. We evaluated susceptibility to ICH, oxidative stress (superoxide, NAD[P]H oxidase activity, SOD activity), gene expression, and activity of matrix metalloproteinases.

**Results**—Incidence, size, and number of ICHs were reduced in SOD1Tg mice and were increased in SOD1\(^{-/-}\) mice compared with their wild-type littermates. Levels of superoxide increased in the brain even before developing ICH in wild-type littermates, whereas levels of superoxide remained low in SOD1Tg mice. Changes in level of matrix metalloproteinase-9 paralleled oxidative stress in SOD1Tg mice and wild-type littermates. Moreover, levels of superoxide and matrix metalloproteinase-9 were greater in SOD1\(^{-/-}\) mice than wild-type littermates after induction of ICH. Active matrix metalloproteinases colocalized on cerebral vessels that appeared to lead toward regions with ICH.

**Conclusions**—These results suggest that superoxide contributes to the pathogenesis of spontaneous ICH, possibly through activation of matrix metalloproteinase-9, and that SOD1 protects against spontaneous ICH during hypertension.

**Key Words:** MMP-9 and brain hemorrhage ■ oxidative stress ■ SOD1

Several years ago, we developed the first model of spontaneous intracerebral hemorrhage (ICH) in chronically hypertensive mice. A limitation of the model is that it is difficult to breed the mice with other genetically altered mice, because the mice are double transgenic. Therefore, we recently developed another experimental model of ICH in hypertensive C57BL/6 mice.

Mice with acute hypertension, induced by daily injection of angiotensin II (AngII), superimposed on chronic hypertension, have a high incidence of spontaneous ICH. There was an association of increases in AngII-mediated oxidative stress with spontaneous ICH during hypertension. An association of oxidative stress and ICH, however, clearly does not provide direct evidence for a causal relationship.

Copper/zinc–superoxide dismutase (CuZn-SOD1) is a crucial antioxidant enzyme. Deficiency of SOD1 increases superoxide and produces vascular dysfunction in large arteries and microvessels, augments vascular dysfunction produced by AngII, and increases expression and activation of matrix metalloproteinase-9 (MMP-9). Overexpression of SOD1 decreases oxidative stress, attenuates induction and activation of MMP-9, and protects against vascular dysfunction. In this study, we tested the hypothesis that decreases in superoxide by overexpression of SOD1 protect against development of spontaneous ICH and that increases in superoxide by deficiency in SOD1 increase susceptibility to ICH.

**Materials and Methods**

**Experimental Animals**

Studies were conducted in 8-month-old male hemizygous CuZnSOD-transgenic (SOD1Tg) mice (n=40) and wild-type (WT) littermates (n=44) and homozygous CuZnSOD-deficient (SOD1\(^{-/-}\)) mice (n=15) and WT littermates (n=14). SOD1Tg mice and WT littermates were derived from breeding male hemizygous CuZnSOD (human)-transgenic (C57BL/6-TgN[SOD1]10Cje) mice.

Received October 8, 2009; final revision received November 20, 2009; accepted November 30, 2009.

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**Stroke** is available at http://stroke.ahajournals.org

DOI: 10.1161/STROKEAHA.109.569616
with female C57BL/6J mice (Jackson Laboratories; Bar Harbor, Maine, USA). SOD1 \(^{-/-}\) and WT littermates were derived from breeding pairs of heterozygous CuZn-SOD–deficient (B6;129S-SODtm1Leb) mice (Jackson Laboratories). Breeding and genotyping were performed in a virus- and pathogen-free barrier facility at the University of Iowa. The genotype of each mouse was ascertained by polymerase chain reaction of DNA isolated from tail biopsy samples as described previously. All experimental protocols and procedures conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

There were 2 cohorts of mice in studies using SOD1 \(^{-/-}\) mice and WT littermates. The first cohort was used to measure systolic blood pressure (SBP), incidence of stroke, and size of ICH. Mice in this cohort were euthanized with an injection of Nembutal (150 mg/kg, intraperitoneally) when a mouse developed signs of stroke or at Day 28 if the mouse did not have neurological signs. The second cohort was used to evaluate oxidative stress, expression of mRNA, and MMPs. In the second cohort, when a mouse developed signs of stroke, we euthanized the mouse with signs of stroke and one mouse without neurological signs from each of the other groups to collect tissue samples after the same duration of treatment.

Only one cohort of SOD1 \(^{-/-}\) mice and WT littermates was studied, because of limited availability of these mice; SBP and incidence of sign of stroke were estimated and mice were euthanized when a mouse developed signs of stroke. Brains were used to evaluate size and number of ICH, oxidative stress, and MMPs.

**Model of Spontaneous ICH**

The methods to produce spontaneous ICH and to assess signs of stroke in mice were described recently in detail. Briefly, mice were treated with an AngII infusion (1000 ng/kg/min; Sigma-Aldrich, St Louis, Mo) and L-NAME (100 mg/kg per day; Sigma-Aldrich) in drinking water to produce chronic hypertension (HT; chronically hypertensive group). One week later, transient acute hypertension was produced by daily AngII injection (0.5 \(\mu g/g\), twice per day, subcutaneously; chronic/acute HT group). SBP was measured daily in conscious mice using tail cuff plethysmography. Clinical signs of stroke were assessed by daily neurological examinations at least 3 times per day, including contralateral forelimb extension, circling behavior, or other motor dysfunction, as described previously. Mice without any treatments were used as control mice.

Mice were euthanized and perfused transcardially with phosphate-buffered saline. Paraffin-embedded brain was serially sectioned at
5 μm, resulting in approximately 2000 to 2400 sections of the entire brain of each mouse, for determination of size and number of ICHs. The first and second sections of every set of 5 serial sections were stained with hematoxylin and eosin and with diaminobenzidine (DAB), respectively.2,7 Diaminobenzidine reacts with peroxidases in red blood cells and facilitates precise identification of ICH, because DAB highlights hemorrhages and nonhemorrhagic areas unstained.2,7 All section stained with hematoxylin and eosin and DAB were screened, and images of ICH were captured and analyzed with Image J software (National Institutes of Health) to quantify the size and number of hemorrhages. The size of ICH was estimated as follows: (area [mm²] of ICH on each section) / (distance between successive DAB-stained sections).

Brain Preparation for Oxidative Stress, Gene Expression, and MMPs

Brains of the second cohort of SOD1Tg mice and WT littermates were collected 12 ± 1 (mean ± SE) days after the start of treatment. During this period, mice in the chronic/acute HT group of WT littermates developed signs of stroke, and SOD1Tg mice did not develop any neurological signs.

In SOD1−/− mice and WT littermates, brains were collected when the mice developed neurological signs, at 10 ± 1 day in SOD1−/− mice and 14 ± 2 days in WT littermates.

Brains were perfused transcardially with phosphate-buffered saline and cut sagittally in half. Half of each mouse brain was fixed and used for histological detection of ICH. The middle third of the other half of the brain (4 mm thick) was not fixed and was used to evaluate oxidative stress (superoxide and NAD(P)H oxidase activity, SOD activity), expression of mRNA, and MMPs. This slice of brain contains cerebral cortex, basal ganglia, thalamus, hippocampus, and the upper part of the brain stem.2

Oxidative Stress

Superoxide and NAD(P)H oxidase activity in brain homogenates were quantified with lucigenin-enhanced chemiluminescence.2,7 NAD(P)H oxidase activity was estimated as levels of superoxide after adding NADPH (100 μmol/L) to brain homogenates.

Total SOD activity of brain homogenates was determined using the WST SOD assay kit (Sigma-Aldrich) as reported previously.2 Expression levels of pro-oxidant/NAD(P)H oxidase subunits (Nox1, Nox2, Nox4, and p47phox), antioxidants (SOD1, SOD2, SOD3, and catalase), and nuclear factor-erythroid factor 2 were measured by quantitative real-time reverse transcription–polymerase chain reaction using the TaqMan method as described previously.2,8

Matrix Metalloproteinases

Levels of MMP-2 and MMP-9 in brain homogenates were evaluated using gelatin zymography as described previously.2 The organomercurial compound 4-aminophenylmercuric acetate (Sigma-Aldrich) was used to activate the murine MMP-9 standard.

In situ gelatinolytic activity was assessed on frozen sections of half brains of 3 SOD1−/− mice as described previously.2 Briefly, coronal sections (10 μm thick) were cut for the entire brain of each mouse. One of every 5 serial sections was stained with hematoxylin and eosin and DAB for detection of ICH. The remaining sections were used for MMP in situ zymography and immunohistochemistry as described previously.2 We also examined vessels that appeared to lead to ICH on serial sections of entire brains. Despite intensive examination of the tissue around ICH in >1200 specimens/mouse in 3 SOD1−/− mice, we detected only a small number of vessels that appeared to lead to ICH and thus may be the “culprit” vessels.

Statistics

Results are expressed as mean ± SE. Analysis of variance followed by Bonferroni test was used for comparison of multiple groups. Mann-Whitney U test was used for comparison of 2 groups. A probability value of <0.05 was considered significant. Cumulative incidence of signs of stroke was evaluated using a Kaplan-Meier test and the difference among groups was analyzed by log rank test.
Results

CuZnSOD-Transgenic Mice and WT Littermates

Systolic Blood Pressure

SBP increased from 96±3 (mean±SE) to 160±2 mm Hg and from 99±3 to 160±3 mm Hg in SOD1Tg mice and WT littermates, respectively, 7 days after initiation of chronic hypertension (Figure 1A). Basal levels of SBP (Figure 1A) were similar in SOD1Tg mice and WT littermates during chronic hypertension. SBP increased for approximately 20 minutes after injection of AngII and was similar in the chronic/acute HT group of SOD1Tg mice and WT littermates (Figure 1B–C).

Signs of Stroke

In the chronic/acute HT group, SOD1Tg mice had a lower incidence (40% versus 82%) and later onset (20±2 versus 14±2 days after the start of AngII infusion and L-NAME) of signs of stroke compared with WT littermates (P<0.05; Figure 1D). No SOD1Tg mice or WT littermates in the chronically hypertensive group, without acute HT, showed signs of stroke (Figure 1D).

Histological Analysis

All SOD1Tg mice and WT littermates that showed signs of stroke in the chronic/acute HT group had multiple ICHs, which were distributed widely in the brain (Figure 1E–F). The number and volume of ICH in the cerebral cortex, basal
ganglia, thalamus, and brain stem were less in SOD1Tg mice than WT littermates (Figure 1E–F). No mice without neurological signs had histologically detectable ICH.

**Oxidative Stress**

In WT littermates, the level of superoxide in the brain was low in control mice, tended to increase (not significant) in chronically hypertensive mice, increased significantly in the chronic/acute HT group without ICH, and was highest in the chronic/acute HT group with ICH (Figure 2A). In contrast, the level of superoxide did not differ among groups in SOD1Tg mice and was less in SOD1Tg mice than WT littermates in the chronic/acute HT group without ICH (Figure 2A). NAD(P)H oxidase activity increased in parallel with increases in basal levels of superoxide in WT littermates (Figure 2B). In SOD1Tg mice with chronic/acute HT without ICH, NAD(P)H oxidase activity also was higher than the control group (Figure 2B). Total SOD activity was higher in SOD1Tg mice than in WT littermates (Figure 2C).

**Gene Expression (mRNA)**

In WT littermates, expression of subunits of NAD(P)H oxidase did not increase in chronically hypertensive mice (Figure 3A). Expression of Nox1 and Nox4 increased significantly in the chronic/acute HT group without ICH, and increased further in the chronic/acute HT group with ICH (Figure 3A). Expression of Nox2 and p47phox increased only in the chronic/acute HT group with ICH. In SOD1Tg mice in the chronic/acute HT group without ICH, expression of Nox2 and Nox4 also increased.

As expected, expression of SOD1 increased greatly in SOD1Tg mice (Figure 3B). Expression of SOD2 and catalase decreased significantly in WT littermates in the chronic/acute HT group without and with ICH (Figure 3B). Expression of other antioxidant enzymes was not different among groups in SOD1Tg mice. In WT littermates, expression of nuclear factor-erythroid factor 2 (Figure 3C), a redox-sensitive transcription factor that appears to upregulate antioxidant genes, was decreased significantly in mice with chronic/acute HT without and with ICH; in SOD1Tg mice, expression of nuclear factor-erythroid factor 2 was not different among groups.

**Matrix Metalloproteinases**

In WT littermates, gelatinolytic activity of MMP-9 was barely detectable in the control group (Figure 4A–B). Levels of MMP-9 (98 kDa) tended to increase (not significant) in chronically hypertensive mice, increased significantly in chronic/acute HT mice without ICH, and increased further in chronic/acute HT mice with ICH (Figure 4B). In contrast to WT littermates, levels of MMP-9 (98 kDa) in SOD1Tg mice remained low in all groups; levels of MMP-9 (98 kDa) in chronic/acute HT mice without ICH were significantly less in SOD1Tg mice than in WT littermates. Levels of MMP-2 (68 kDa) were detectable but were not different among SOD1Tg mice and WT littermates. No detectable levels of the cleaved form of MMP-9 and MMP-2 were observed in any group, except in WT littermates with ICH.

**CuZnSOD-Deficient Mice and WT Littermates**

**Systolic Blood Pressure**

Basal levels of SBP were not different in SOD1−/− and WT littermates (Figure 5A). Acute increases in SBP after injection of AngII were not different (P=0.08) between chronically hypertensive SOD1−/− mice (65±3 mm Hg) and WT littermates (57±3 mm Hg).
Signs of Stroke
All SOD1<sup>−/−</sup> mice and 6 of 8 WT littermates (75%) with acute, superimposed on chronic HT developed signs of stroke 10±1 days and 14±2 days after the start of AngII infusion and l-NAME, respectively (log rank test, \( P<0.01 \); Figure 5B).

Histological Analysis
All SOD1<sup>−/−</sup> mice and WT littermates that showed signs of stroke had multiple ICHs, which were distributed widely in the brain (Figure 5C–D). There were more ICHs in the thalamus and brain stem in SOD1<sup>−/−</sup> mice than in WT littermates (Figure 5C). Volume of ICH in all regions of brain of SOD1<sup>−/−</sup> mice was larger than that of WT littermates (Figure 5D). WT littermates without neurological signs did not have histologically detectable ICH.

Oxidative Stress
Total SOD activity in normal SOD1<sup>−/−</sup> mice (15±1 U/mg brain protein) was significantly less than in WT littermates (110±9; \( P<0.05 \)). After development of ICH, basal levels of superoxide (Figure 5E) and NAD(P)H oxidase activity (Figure 5F) in the brain were significantly higher in SOD1<sup>−/−</sup> mice than WT littermates.

Matrix Metalloproteinases
Levels of MMP-9 (98 kDa) were higher in SOD1<sup>−/−</sup> mice with ICH than WT littermates with ICH (Figure 5G–H). Levels of MMP-2 (68 kDa) were not different between the 2 groups (Figure 5G–H).

Intense in situ gelatinolytic activity on cerebral vessels was found diffusely on endothelial cells and extracellular matrix of vessels that led toward regions with ICH (Figure 6A–E). In situ
gelatinolytic activity was also occasionally detected on cerebral vessels that were not close to an ICH lesion (Figure 6F).

Discussion

There are 2 major new findings in this study. First, overexpression of SOD1 protects against increases in levels of superoxide and MMP-9 and prevents the development of spontaneous ICH during hypertension. Increases in levels of superoxide and MMP-9 preceded the development of ICH in WT mice, and these levels remained low in SOD1Tg mice. Second, deficiency of SOD1 increased susceptibility to ICH. ICH occurred earlier in SOD1-deficient mice than in WT littermates, and the number and size of ICHs were greater in SOD1-deficient mice than in WT littermates. These findings provide strong evidence that oxidative stress, and perhaps activation of MMP-9, contribute to spontaneous ICH.

Oxidative Stress, MMP-9, and Spontaneous ICH

We recently developed a new experimental model of spontaneous ICH in C57BL/6 mice based on the hypothesis that acute, superimposed on chronic, HT may increase oxidative stress and levels of MMPs and lead to spontaneous ICH. AngII-mediated oxidative stress was associated with concomitant upregulation of Nox1 and Nox4 and downregulation of antioxidant enzymes as in the previous study. We also confirmed that superoxide and NAD(P)H oxidase activity increased in the brain of WT littermates of SOD1Tg mice during acute and chronic HT, even before development of spontaneous ICH.

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Figure 6. ICH in thalamus of SOD1−/− mouse in chronic/acute HT group. A–C, DAB staining followed by hematoxylin and eosin staining showing ICH lesion in thalamus. Boxes in (A) and (B) were magnified in panels (B) and (C), respectively. Arrow indicates a cerebral vessel that appeared to lead toward a region with ICH. D–F, In situ zymography combined with immunohistochemistry on cerebral vessel in thalamus. Box in (E, merge) was magnified in the panel at far right (merge, magnified). F, Vessel that is distant from an ICH region in the thalamus. vWF, endothelial cell; GFAP, astrocytes; collagen type IV, extracellular matrix. Images of (C) to (E) were derived from serial sections. Bars: 100 μm.
SOD1Tg mice. These findings support our previous observation in C57BL/6 mice and demonstrate an association of increases in oxidative stress and activation of MMP-9 with spontaneous ICH in mice.

CuZnSOD and Spontaneous ICH

To more directly determine whether there is a causal relationship between superoxide and spontaneous ICH, we studied SOD1-transgenic and -deficient mice. SOD1 is the most abundant of the 3 isoforms of SOD in terms of total SOD expression and activity within the vascular wall, and SOD1 reduces superoxide levels in cerebral blood vessels.3,9,10

We now report that overexpression of SOD1 protects against ICH, because the incidence, number, and size of ICH were decreased in SOD1Tg mice compared with WT littermates. We also found that deficiency of SOD1 increases the incidence, number, and size of ICHs. These changes in susceptibility to spontaneous ICH were concordant with changes in levels of superoxide and MMP-9.

Changes in blood pressure do not account for effects of SOD1 on susceptibility to ICH. Basal SBP and changes in SBP after induction of acute hypertension were not different in SOD1Tg mice or SOD1−/− mice and their WT littermates. These findings are concordant with previous findings that blood pressure of SOD1-deficient and -transgenic mice was similar to blood pressure in WT littermates.3,11

Instead, it is likely that effects of SOD1 on susceptibility to ICH are mediated by direct effects of superoxide on blood vessels. Overexpression of SOD1 in transgenic rats and mice decreases vascular oxidative stress and attenuates induction and activation of MMP-9-mediated disruption of the blood–brain barrier after focal cerebral ischemia.6,12 Overexpression of SOD1 also protects against increases in superoxide and endothelial dysfunction in response to AngII.5 In contrast, deficiency of SOD1 increases superoxide levels in blood vessels and enhances AngII-induced vascular dysfunction.4,5 SOD1-deficient mice also are susceptible to focal cerebral ischemia–reperfusion with upregulation of MMP-9, disruption of the blood–brain barrier, and a higher mortality than WT littermates.3,13

We found in situ gelatinolytic activity on vessels that lead toward ICH and occasionally on vessels that were not close to an ICH lesion. This finding corresponds to our previous finding that in situ gelatinolytic activity on cerebral vessels increased even before development of ICH.2

Thus, our findings in toto provide direct support for the hypothesis that increases in oxidative stress contribute to spontaneous ICH, possibly through activation of MMP-9, in hypertensive mice and that SOD1 plays a critical role in protection against spontaneous ICH during hypertension.

Limitations

In this study, we have not provided direct evidence for a role of MMPs in spontaneous ICH. We speculate, however, that because induction and activation of MMPs are redox-sensitive,3 SOD1 may contribute to development of spontaneous ICH by modulating the activation of MMPs and thereby affecting degradation of cerebral vessels.

Conclusions

Overexpression of SOD1 decreased basal levels of superoxide and the incidence, size, and number of spontaneous ICHs, whereas deficiency of SOD1 increased the susceptibility to ICH. Furthermore, spontaneous ICH occurred later in mice with overexpression of SOD1 and earlier in mice with deficiency in SOD1 than respective WT littermates. Changes in level of MMP-9 paralleled the basal levels of superoxide. These findings suggest that SOD1 may protect against spontaneous ICH during hypertension by reducing levels of superoxide.

Acknowledgments

We thank Drs Frank M. Faraci and Gary L. Baumbach for discussions regarding the data and study design and Dr Yi Yang for technical assistance.

Sources of Funding

This study was supported by National Institutes of Health grants NS24621 and HL62984 and funds from a Carver College of Medicine Program of Excellence.

Disclosures

None.

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Stroke. 2010;41:790-797; originally published online February 11, 2010;
doi: 10.1161/STROKEAHA.109.569616
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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