Transgenic CuZn-Superoxide Dismutase Inhibits NO Synthase Induction in Experimental Subarachnoid Hemorrhage

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**Background and Purpose**—The expression of inducible NO synthase (iNOS) after experimental subarachnoid hemorrhage (SAH) has been postulated to play a critical role in the pathogenesis of SAH and subsequent cerebral vasospasm. The inhibitory effect of CuZn-superoxide dismutase (CuZn-SOD) on the induction of iNOS after SAH was examined by using transgenic mice overexpressing CuZn-SOD.

**Methods**—SOD-transgenic mice and nontransgenic littermates were subjected to SAH by endovascular perforation of the left anterior cerebral artery. The iNOS mRNA expression after SAH was determined by reverse transcription–polymerase chain reaction, and the distribution of iNOS-positive cells was immunohistochemically examined. The nuclear expression of activated nuclear factor-κB, a major transcription factor of iNOS gene, was also immunohistochemically examined.

**Results**—In nontransgenic mice, SAH-induced iNOS protein and mRNA expressions in the arteries of basal cistern as well as in the cerebral cortex were demonstrated by immunohistochemistry and reverse transcription–polymerase chain reaction. SAH-induced iNOS protein and mRNA expressions in those tissues were much reduced in SOD-transgenic mice compared with nontransgenic mice. Moreover, the nuclear expression of the activated form of nuclear factor-κB was immunohistochemically detected in the cerebral cortices of nontransgenic mice but not in those of SOD-transgenic mice.

**Conclusions**—These results indicate that oxygen-derived free radicals, particularly superoxide, play an important role in the iNOS gene expression after SAH and provide a molecular basis for the protective role of SOD against vasospasm after SAH. (Stroke. 2001;32:1652-1657.)

**Key Words:** cerebral ischemia, transient nitric oxide subarachnoid hemorrhage superoxide dismutase

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Cerebral vasospasm after subarachnoid hemorrhage (SAH) has the features of a typical free radical–mediated disease.1,2 Shishido et al3 have demonstrated that intrathecal injection of superoxide dismutase (SOD) prevents morphological endothelial injury and attenuates the occurrence of vasospasm. Moreover, in our previous study,4 we showed that transgenic CuZn-SOD was effective in reducing vasospasm after SAH. Thus, oxygen free radicals, particularly superoxide, appear to play an important role in the pathogenesis of vasospasm after SAH.4 However, the molecular basis for the protective role of SOD against vasospasm has not yet been clarified.

Recently, inducible NO synthase (iNOS) expression in vascular and neuronal tissues has been observed in experimental SAH models.5–7 iNOS is an enzyme that can produce toxic levels of NO.8 The iNOS-derived NO can exert injurious effects on endothelial cells, smooth muscle cells, and adventitial cells7 by several mechanisms, such as increased lipid peroxidation, DNA strand breaks, the inhibition of mitochondrial enzymes, and disruption of gene transcription,9–12 ultimately leading to vasoconstrictive changes. Sayama et al6 reported that aminoguanidine, a selective inhibitor of iNOS, ameliorates the vasoconstrictive changes after SAH. These results suggest that iNOS expression after SAH plays a critical role in the occurrence and progression of vasospasm after SAH.5–7

It has been reported that iNOS protein expression, mRNA levels, and NO synthesis are all increased by the generation of superoxide.13,14 Analysis of the 5′ region of the mouse iNOS
gene revealed that it contains redox-sensitive elements, such as an antioxidant-responsive element and nuclear factor-κB (NF-κB)-responsive element. In fact, oxidative stress mediated by 1,2,3-benzenetriol (BZT), a superoxide generator, enhanced iNOS gene expression, whereas the addition of SOD eliminated the BZT-mediated augmentation of iNOS promoter activity. NF-κB is also an oxidative stress-responsive transcription factor that can be activated by reactive oxygen species. From these results, it appeared that transgenic SOD could inhibit iNOS gene expression after SAH and thereby ameliorate vasospasm. In the present study, by using SOD transgenic mice as a model system, the effect of transgenic SOD on the induction of iNOS after SAH was investigated.

Materials and Methods

Generation of Transgenic Mice

SOD-transgenic heterozygotes and nontransgenic controls were generated by crossing TgHS-SF218/10 mice with CD1 (Charles River Laboratories, Wilmington, Mass). Animals were maintained under specific pathogen-free conditions and fed standard mouse chow ad libitum. Transgenic and nontransgenic mice were segregated at 4 weeks of age by polymerase chain reaction (PCR) analysis of the genomic DNA. A 3-fold increase in CuZn-SOD activity was observed in all brain regions, including the cerebral cortex in the transgenic mice; the CuZn-SOD levels were 7.9 and 22.7 U/mg protein in nontransgenic and SOD-transgenic mice, respectively. Neurons, astroglia, and cerebral vessels were all stained immunocytochemically for a polyclonal antibody against human SOD-1.

Gel Electrophoresis

For polyacrylamide gel analysis, cerebral vascular tissues as well as the cerebral cortex adjacent to the basal cistern were homogenized in 50 mmol/L Tris-HCl (pH 7.0). Homogenates were centrifuged at 12,000g for 15 minutes at 4°C. Each tissue extract (30 μg protein) was loaded onto 10% acrylamide gel and electrophoresed for 3 hours at 160 V. To perform staining for SOD enzymatic activity, the gels were soaked in nitro blue tetrazolium, tetramethyl-ethylenediamine, and riboflavin in potassium phosphate for 50 minutes.

Induction of SAH

Adult mice weighing 35 to 40 g were used in the present study. Anesthesia was induced by an intraperitoneal injection of xylazine (0.2 mL, 2 mg/kg) and maintained by inhalation of a mixture of 0.75% halothane, 70% N2O, and 30% O2. Mice were placed in the supine position on an operating table, and rectal temperature of the animals was maintained at 37°C by a Homeothermic Blanket Control Unit (Harvard Apparatus). The left common carotid artery was exposed, and the external carotid artery and its branches were isolated and coagulated. A 5-0 monofilament nylon suture, blunted at the tip, was introduced into the internal carotid artery through the external carotid artery stump up to the left anterior cerebral artery near the anterior communicating artery, where resistance was encountered, as in a mouse ischemia model used in our previous studies. Then the suture was advanced 5 mm further to perforate the artery and was immediately withdrawn through the internal carotid artery into the external carotid artery, allowing reperfusion and producing SAH. Some mice died before reaching end points. The mortality rate within 72 hours was 20.2% in nontransgenic mice and 19.0% in SOD-transgenic mice, which was not significantly different between nontransgenic and SOD-transgenic mice. At 1, 3, and 7 days after SAH, the mice were anesthetized with an intraperitoneal injection of 20 mg/kg pentobarbital and perfused through the left ventricle with 4% paraformaldehyde for immunohistochemistry or with PBS for reverse transcription (RT)-PCR.

Results

We first determined the expression of human CuZn-SOD in cerebral vascular tissues as well as the basal cortex in the transgenic mice (Figure 1). Because CuZn-SOD is a dimer composed of 2 identical subunits, in transgenic tissues the enzyme forms a heterodimer (mouse plus human), which is seen in Figure 1 as the middle band. In cerebral vascular tissues (Figure 1, lanes C and D) and in basal cortex (Figure 1, lanes G and H), the predominant form of SOD is human/SOD homodimer; mouse/human, mouse and human SOD heterodimer; and human/human, human SOD homodimer.

Immunohistochemistry

SOD-transgenic and nontransgenic mouse brains were embedded in paraffin. Coronal 5-μm-thick sections near the internal carotid artery bifurcation were cut and mounted on slides. The sections were quenched with hydrogen peroxide, washed, and incubated with normal goat serum for 20 minutes. The sections were incubated for 24 hours at 4°C with rabbit polyclonal antibody to iNOS (1:200, Transduction Laboratories) or with rabbit polyclonal antibody to the NF-κB p50 subunit (1:200, Santa Cruz) and were subjected to the streptavidin-biotin technique for visualization by using biotin-complexed secondary antibody (Vector Laboratory.). Sections immunostained by iNOS antibody were counterstained with methyl green. The iNOS and NF-κB expressions were not detected in the absence of primary antibody.

RT-PCR Analysis

We first obtained whole cerebrum by dissecting the skull bone after perfusion fixation. Then we separated the cerebral vascular tissues and arachnoid membrane (tissues of basal cistern) from the basal side of the cerebral cortex by cautiously using microforceps under a stereoscopic microscope. Even in mice, this can be routinely achieved under a microscope. Briefly, the vascular RNA was prepared by homogenizing the cerebral vascular tissues in the basal cistern. The basal cortex RNA was prepared by homogenizing the cerebral cortex separated from the cerebral vascular tissue and arachnoid membrane. First-strand cDNA was synthesized by using oligo(dT) primer as described. The reverse-transcribed sample (1 μL) was used for PCR amplifications. The sequences of the primers for iNOS cDNA amplification were 5’-CGGGCTCCATGACTCCCGAGCAAAAG3’ and 5’-TCCACCTGCTCTCCGCCTCAAGTTC-3’ (nucleotides 2968 to 2992 and 3314 to 3290 in Nunokawa et al24); for GAPDH cDNA amplification, they were as described. PCR was performed in a thermal cycler (Perkin-Elmer) for 35 and 25 cycles for iNOS and GAPDH, respectively, with the following parameters: denaturation at 95°C for 30 seconds, annealing at 69°C for 30 seconds, and extension at 72°C for 30 seconds for iNOS; for GAPDH, they were as described. The PCR products were resolved on 1.5% to 2.0% agarose gel and visualized by ethidium bromide staining.

Figure 1. Polyacrylamide gel analysis of CuZn-SOD enzymatic activity in mouse tissues. Analysis of CuZn-SOD activities in cerebral vascular tissues (A through D) and cerebral cortex (E through H) of nontransgenic mice (A, B, E, and F) and transgenic mice (C, D, G, and H). Mouse/mouse indicates mouse SOD homodimer; mouse/human, mouse and human SOD heterodimer; and human/human, human SOD homodimer.
wall, especially in endothelial cells and the adventitial layer. In contrast, the iNOS immunoreactivity was negative in untreated mice (Figure 2A). In SOD-transgenic mice (Figure 2C), the iNOS immunoreactivity in the vascular wall was much reduced compared with that in nontransgenic mice. In the cerebral cortex of nontransgenic mice (Figure 3B), at 24 hours after SAH, the iNOS immunoreactivity was mainly found in the neuronal nuclei. In contrast, the NF-κB immunoreactivity was negative in untreated mice (Figure 3D). In SOD-transgenic mice (Figure 3F), the NF-κB immunoreactivity in the neuronal cells was much reduced compared with that in nontransgenic mice.

We next determined the iNOS mRNA levels in the vascular tissues as well as in the cerebral cortex by RT-PCR. We could not use Northern blotting to clarify the iNOS mRNA expression; however, we could examine iNOS mRNA apparently by RT-PCR. The iNOS mRNA was expressed in the vascular cells exposed to SAH but not in those from untreated mice (Figure 4, lanes A and B). The densitometric analysis indicated that the iNOS mRNA was reduced in vascular cells from SOD-transgenic mice compared with nontransgenic mice (Figure 4, lane C).

We then determined the iNOS mRNA levels in the cerebral cortex. The iNOS mRNA was abundantly expressed in the cerebral cortex exposed to SAH (Figure 4, lanes F and G) but not in those from untreated mice (Figure 4, lanes D and E). The densitometric analysis indicated that the iNOS mRNA levels were much reduced in the cerebral cortices from SOD-transgenic mice (Figure 4, lanes H and I) compared with those from nontransgenic mice. The GAPDH mRNA, an internal control, was equally expressed under the respective conditions (Figures 4, lanes A through I); this suggests that SAH itself does not affect general mRNA expression in the vascular tissues and the cerebral cortex.

**Discussion**

The involvement of iNOS expression in the development of central nervous system disorders, such as cerebral ischemia, multiple sclerosis, Parkinson’s disease, Alzheimer’s disease, tumors, and encephalitis, has been demonstrated previously. Recently, the vascular expression of iNOS after SAH in rats has been reported. In the present study, by using an endovascular perforating method in mice, intense iNOS expression at the vascular wall was demonstrated after SAH. Because aminoguanidine, a selective inhibitor of iNOS, ameliorated the vasoconstrictive change after SAH, iNOS expression after SAH appears to play a critical role in the occurrence and progression of vasospasm after SAH. Therefore, the mechanism and inhibition of iNOS expression after SAH are now a focus of interest.

We next evaluated the nuclear expression of the NF-κB p50 subunit by using a polyclonal antibody that specifically recognizes its activated form. In the cerebral cortex of nontransgenic mice (Figure 3E), at 24 hours after SAH, the NF-κB immunoreactivity was mainly found in the neuronal nuclei. In contrast, the NF-κB immunoreactivity was negative in untreated mice (Figure 3D). In SOD-transgenic mice (Figure 3F), the NF-κB immunoreactivity in the neuronal cells was much reduced compared with that in nontransgenic mice.

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**Figure 2.** Immunohistochemical demonstration of iNOS in the arteries of basal cistern. iNOS immunoreactivity was negative in untreated mice (A). iNOS immunoreactivity was observed in endothelial cells, the adventitial layer, and the infiltrated cells in nontransgenic mice after SAH (B). iNOS immunoreactivity after SAH was much reduced in SOD-transgenic mice (C).

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Analysis of the 5′ region of the iNOS gene in the mouse revealed features relevant to the redox modulation of iNOS gene expression. These include elements with known redox-sensitive functions: 2 copies each of an antioxidant-responsive element and an NF-κB–responsive element. Kuo et al reported that oxidative stress mediated by BZT, a superoxide generator, enhanced iNOS gene expression, whereas the addition of SOD eliminated the BZT-mediated augmentation of iNOS promoter activity, indicating that antioxidant-responsive elements present in the 5′ region of iNOS gene are functional. NF-κB is also an oxidative stress–responsive transcription factor that can be activated by reactive oxygen species.
In the present study, at 24 hours after SAH, iNOS expression was much reduced in the vascular wall of the SOD-transgenic mice compared with that of nontransgenic mice. The only biochemical difference between SOD-transgenic mice and nontransgenic mice is whether they possess transgenic SOD or not, so the phenotypic differences between transgenic and nontransgenic mice are logically thought to come from the presence or absence of transgenic SOD. Together with the results of in vitro studies showing that exogenously added SOD prevented iNOS gene expression, it is reasonable to assume that scavenging oxygen-derived free radicals, particularly superoxide, inhibits iNOS gene expression after SAH, and this can account for the protective role of SOD against vasospasm after SAH.

In experimental cerebral ischemia, iNOS mRNA expression in the postischemic brain began 24 hours after middle cerebral artery occlusion. The iNOS expression after cerebral ischemia is thought to be one of the factors contributing to the expansion of the brain damage that occurs in the postischemic period. In our SAH model, intense iNOS staining and mRNA expression were observed at 24 hours in the cerebral cortex of nontransgenic mice. NO produced by iNOS after SAH might affect the survival of potentially viable neurons exposed to SAH insults, as in cerebral ischemia. The transgenic SOD inhibited the iNOS expression in the cerebral cortex, indicating that oxygen-derived free radicals, particularly superoxide, play a critical role in the iNOS expression after SAH not only in vascular tissues but also in the cerebral cortex.

The NF-κB p50 subunit is present in the cytoplasm as an inactive multisubunit complex, but when the cell is stimulated, NF-κB dissociates from IκB and translocates into the nucleoplasm in an activated form. NF-κB activation occurs by a variety of stimuli, such as focal ischemia, glutamate, tumor necrosis factor-α, hypoxia, interleukin-1α, and reactive oxygen species. NF-κB activation is thought to play a central role in iNOS mRNA expression. In the present study, the nuclear expression of the activated form of NF-κB was immunohistochemically demonstrated in the cerebral cortices of nontransgenic mice but not in those of SOD-transgenic mice. It appears that the transgenic SOD prevents iNOS gene expression by inhibiting NF-κB activation after SAH; however, further experiments are needed to determine whether it is the transgenic SOD itself that inhibits NF-κB activation.

Several mechanisms by which NO produced by iNOS contributes to vasoconstriction changes after SAH have been proposed. These include NO-mediated oxidative damage, DNA damage, and energy failure resulting from inhibition of energy-producing enzymes and from poly(ADP-ribose) synthase activation. The present results indicate that superoxides play an early role in the development of the pathological series of events after SAH, including iNOS expression at the vascular wall as well as in the cerebral cortex. To prevent vasospasm, scavenging superoxides by SOD should be extremely important, because SOD can inhibit the cascade of free radical production after SAH.

### References


![Figure 4. Expressions of iNOS mRNA (A through I, upper bands) and GAPDH mRNA (A through I, lower bands) in the vascular tissues (A, B, and C) and basal cortex (D through I) analyzed by RT-PCR. A, D, and E, Untreated mice. B, F, and G, Nontransgenic mice 24 hours after SAH. C, H, and I, SOD-transgenic mice 24 hours after SAH.](image-url)

**Editorial Comment**

The authors have provided a very interesting assessment of the role of superoxide dismutase (SOD) in vasospasm after experimental subarachnoid hemorrhage (SAH). Wild-type mice and the same strain that differed only in that it overexpressed human copper-zinc SOD underwent creation of SAH by endovascular perforation of the internal carotid artery bifurcation or anterior cerebral artery. It had been shown previously that transgenic mice overexpressing SOD developed less vasospasm 3 days after SAH. The degree of vasospasm was not different on days 1, 7, and 14 after SAH. In this series of experiments, vasospasm was not measured, so it has to be assumed, with reasonable certainty I think, that the transgenics would have had less vasospasm. That transgenic mice overexpressing SOD develop less vasospasm after SAH, however, is not without controversy, since a recent abstract reported no difference in vasospasm between these and wild-type mice. In any case, the present study noted that SAH increased inducible nitric oxide synthase (iNOS) mes-
senger ribonucleic acid (mRNA) and immunoreactivity in cerebral vessels and brain tissue and that this increase was inhibited in SOD transgenic mice. Similarly, NF-κB immunoreactivity was reduced in brain neurons in transgenic mice compared with the induction that occurred after SAH in wild-type mice. That superoxide anion radical contributes in some way to vasospasm is supported. Whether the changes in iNOS and NF-κB are related to vasospasm or secondary are less certain. It would be interesting to conduct further studies to assess the effect of SOD overexpression on the inflammatory response to SAH.

These results lend strong support to the hypothesis that free radicals contribute to the pathogenesis of vasospasm after SAH. Prior studies of the effect of administration of exogenous SOD have been conflicting, possibly due to the difficulty of delivering adequate amounts of functional protein to the appropriate sites in the arterial wall, which may be intracellular.1,2 The effectiveness of iron chelators, which might remove iron as a stimulator of free radical reactions,3,4 and tirilazad and other free radical scavengers, at least in experimental studies,5–7 also supports a role for free radical mechanisms as contributors to vasospasm. The time course of generation of superoxide in the subarachnoid space correlates well with vasospasm.8 The mouse model used here could be a great tool for advancing our knowledge of the pathogenesis of vasospasm. Data that would help validate it as a reasonable model of vasospasm akin to that occurring after SAH in humans would be a detailed examination of the time course of the spasm, associated histopathological changes, and development of the characteristic delayed phase that is resistance to reversal with papaverine. This assumes, of course, that these delayed changes are due to some fundamental mechanism that is in addition to or different from that which initiates the narrowing in the first days after SAH.

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