

Manganese Superoxide Dismutase Deficiency Exacerbates Cerebral Infarction After Focal Cerebral Ischemia/Reperfusion in Mice

Implications for the Production and Role of Superoxide Radicals

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Background and Purpose—Superoxide anion radicals ($O_2^{\cdot-}$) are implicated in ischemia/reperfusion injury, although a direct relationship has not been elucidated. Recently, a specific method of hydroethidine (HEt) oxidation by $O_2^{\cdot-}$ was developed to detect $O_2^{\cdot-}$ production in a variety of experimental brain injury models. To clarify the role of $O_2^{\cdot-}$ in the mechanism of ischemia/reperfusion, we investigated $O_2^{\cdot-}$ production after ischemia/reperfusion and ischemia/reperfusion injury in mutant mice deficient in mitochondrial manganese superoxide dismutase (MnSOD) and in wild-type littermates.

Methods—Ischemia/reperfusion was performed for 60 minutes using intraluminal suture blockade of the middle cerebral artery in the mutant or wild-type mice. We evaluated fluorescent kinetics of HEt or ethidium, the oxidized form of HEt, in brains after an intravenous injection of HEt, followed by measurement of cellular $O_2^{\cdot-}$ production using specific HEt oxidation by $O_2^{\cdot-}$ before and after ischemia/reperfusion. Furthermore, we compared $O_2^{\cdot-}$ production and subsequent infarct volume in the mice using triphenyltetrazolium chloride after ischemia/reperfusion.

Results—HEt oxidation to ethidium is primarily a result of mitochondrially produced $O_2^{\cdot-}$ under physiological conditions. Cerebral ischemia/reperfusion produced $O_2^{\cdot-}$ prominently in neurons shortly after reperfusion, followed by a delayed increase in endothelial cells. A deficiency in MnSOD in mutant mice increased mitochondrial $O_2^{\cdot-}$ production and exacerbated cerebral infarction, worsening neurological deficits after ischemia/reperfusion.

Conclusion—These results suggest that mitochondrial $O_2^{\cdot-}$ production may be a critical step underlying the mechanism of ischemia/reperfusion injury and that MnSOD may protect against ongoing oxidative cell death after ischemia/reperfusion. (*Stroke*. 2002;33:809-815.)

Key Words: cerebral ischemia, transient ■ oxidative stress ■ superoxide dismutase ■ mice, transgenic

Reactive oxygen species (ROS) are believed to be involved in the pathogenesis of a variety of central nervous system disorders, including cerebral ischemia/reperfusion injury.^{1,2} During cerebral ischemia, a number of events may occur that predispose the brain to the formation of oxygen-free radicals.³ After reperfusion, these events can set off a cascade of other biochemical and molecular sequelae, such as the xanthine-xanthine oxidase reaction and phospholipase activation, leading to free-radical production.³ Among these oxygen-free radicals, superoxide anion ($O_2^{\cdot-}$) in particular, may directly affect mitochondrial function by inhibiting aconitase activity or by initiating a free radical-mediated chain reaction causing additional central nervous system damage.^{4,5}

ROS are scavenged by antioxidant enzymes, such as superoxide dismutases (SODs), glutathione peroxidase, and

catalase. Previous reports have demonstrated that copper-zinc SOD (CuZnSOD, Sod1), a cytosolic antioxidant isoenzyme, attenuates infarct volume after transient focal or global cerebral ischemia,^{6,7} suggesting that $O_2^{\cdot-}$ is an important factor for developing ischemia/reperfusion injury. Another isoenzyme of SOD, manganese SOD (MnSOD, Sod2), localized to the mitochondria, provides a first line of defense against $O_2^{\cdot-}$ overproduced from mitochondria.² Studies using mutant mice with genetically modified MnSOD activity showed that reduced MnSOD activity exacerbates glutamate toxicity in cortical cell cultures in vitro,⁸ whereas an increased MnSOD level prevented $O_2^{\cdot-}$ production and subsequent apoptosis in cells treated with an excitotoxin.⁹ MnSOD-deficient mice (Sod2 $-/+$) have shown increased $O_2^{\cdot-}$, subsequent increased infarct volume, or the involvement of an apoptotic pathway after permanent focal cerebral ische-

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mia,^{10,11} suggesting that MnSOD may play a crucial role against excitotoxic damage linked to O₂⁻ production.

Ischemia/reperfusion generates an increased amount of ROS, particularly O₂⁻, from mitochondria in the brain and heart.^{2,12} However, aspects of cellular O₂⁻ production and the role of MnSOD in the cell death pathway after cerebral ischemia/reperfusion have not been thoroughly elucidated. In the present study, we investigated cellular and subcellular localization of O₂⁻ using the method of selective hydroethidine (HET) oxidation by O₂⁻^{13,14} before and after cerebral ischemia/reperfusion, and quantified the sequential change of O₂⁻ production after reperfusion. Furthermore, we compared O₂⁻ production and infarct volume between Sod2 ^{-/+} and wild-type (Wt) mice after ischemia/reperfusion.

Materials and Methods

Animals

Sod2 ^{-/+} mice with a CD-1 background, which were backcrossed with CD-1 mice for at least 10 generations,¹⁰ and Wt mice (CD-1) with an identical genetic background as the Sod2 ^{-/+} mice (3-month-old males, 35 to 40 g) were subjected to each experiment. To investigate the source of O₂⁻ production, another homozygous CuZnSOD (Sod1 ^{-/-}) knockout mutant was used from the same CD-1 background pool as previously reported.¹⁵ Age-matched Wt mice were used as controls.

Focal Cerebral Ischemia

Male Wt or mutant mice (35 to 40 g, 3 months old) were subjected to transient focal ischemia by intraluminal middle cerebral artery (MCA) blockade with a nylon suture, as previously described.⁶ The mice were anesthetized with 2.0% isoflurane in 30% oxygen and 70% nitrous oxide with the use of a face mask. The rectal temperature was controlled at 37±0.5°C with a homeothermic blanket. Cannulation of a femoral artery allowed monitoring of blood pressure and arterial blood gases, with samples for analysis taken immediately after cannulation, 10 minutes after occlusion, and 10 minutes after reperfusion. After the midline skin incision, the left external carotid artery was exposed and its branches were electrocoagulated. An 11-mm 5-0 surgical monofilament nylon suture, blunted by heating at the end, was introduced to the origin of the MCA through the external carotid artery stump. After 60 minutes of MCA occlusion, blood flow was restored by withdrawing the suture. Neurological deficit scores were examined by a previously reported method.¹⁰ All procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by Stanford's Administrative Panel on Laboratory Animal Care.

Detection and Quantitation of Cellular Superoxide

HET (Molecular Probes) was prepared as a 100-mg/mL stock in dimethylsulfoxide. The solution was made by a 1:100 dilution of stock solution with PBS. The ethidium (Et) solution (Molecular Probes) was prepared by a 1:10 dilution of ethidium bromide solution (Sigma) with PBS. Each mutant or Wt mouse received a 200-μL intravenous injection of the solution under isoflurane anesthesia 1 hour before they were killed by transcardiac perfusion of heparinized saline. The brains were then removed. For the tissue extract assay, the brains were separated into the ischemic and nonischemic hemispheres and homogenized in Tris-EDTA buffer (pH 8.0) and ultrasonicated. Protein concentration was adjusted to 10 mg/mL, and fluorescent activity was scanned with a Fluorolog excitation (Ex) – emission (Em) meter (Spex; JY Inc.). For microscopic study, the brains (n=5 each) were fixed with 3.7% formaldehyde in PBS and then cut into a 20-μm thickness using a vibratome. The brain sections were mounted, dried, and then observed with an Axioplan fluorescent microscope (Zeiss) with illumination from an HBO 100W/2 light (Zeiss). Analysis of HET or

Et was performed immediately after preparation of samples. To quantify HET or Et, the ischemic cortex and corresponding contralateral cortex were respectively sampled and treated as described above. Et fluorescent activity was measured at Ex=495 nm, Em=595 nm. Fluorescent activity in the ischemic cortex was compared with the contralateral cortex as a basal value in individual animals. Cryoprotected frozen brain sections were freshly prepared (n=5 each) as previously described.¹⁰ The frozen brain sections were incubated with rhodamine 123 (Rh123), a cell-permeant cationic fluorescent probe selectively accumulated by transmembrane potential in viable mitochondria (10 ng/mL; Molecular Probes) or 10 N-nonyl acridine orange (NAO), one of the mitochondria-specific probes (10 μg/mL; Molecular Probes), in PBS for 15 minutes. Sections were fixed with 3.7% formaldehyde in PBS, washed, and counterstained with Hoechst 33258. The specimens were examined and photographed with a fluorescent microscope.

Determination of Infarction

Four or 16 hours after reperfusion, the mice were anesthetized with an overdose of isoflurane and killed without perfusion. The head was then removed and the brain carefully dissected en bloc. The brain was sliced coronally at 2-mm intervals. Individual slices were freed from the dura mater and vascular tissue and soaked for 10 minutes in a solution of 2% 2,3,5-triphenyltetrazolium chloride (TTC) in 0.1 mol/L PBS (pH adjusted to 7.4) and warmed to 37°C in a water bath as reported previously.¹⁶ Gentle stirring of the slices ensured even exposure of the surfaces to staining. Samples were washed in PBS, and slices were refrigerated in 10% formalin until use. The infarct area was quantified by an image analysis system (Bio-Rad Laboratories). The total infarct volume was calculated using a previously reported method.¹⁷

Statistical Analysis

Data are expressed as mean±SD. The statistical comparisons among multiple groups were made using an analysis of variance followed by Fisher's post-hoc protected least significant difference test. Comparisons between two groups were performed using the unpaired *t* test or Mann-Whitney *U* test according to parameters (StatView, version 5. 01; SAS Institute Inc.).

Results

Neurological Outcome and Physiological Parameters

Higher mortality was seen in the Sod2 ^{-/+} mice compared with the Wt mice (12.5% in Wt mice and 56.25% in Sod2 ^{-/+} mice). Autopsies were conducted to determine the cause of death; then the dead mice that were confirmed to have had no surgical complications, such as subarachnoid hemorrhage, were assigned a score of 5 in the evaluation of neurological deficits. The dead mice had severe brain swelling, consistent with our previous reports.^{10,15} The median scores of the Wt and Sod2 ^{-/+} mice were 2.0 (n=8) and 4.4 (n=16), respectively, and neurological outcome was significantly exacerbated in the Sod2 ^{-/+} mice (*P*<0.001, Figure 1). Among the surviving Wt and Sod2 ^{-/+} mice, a significant difference was seen in neurological outcome at 16 hours of ischemia (*P*<0.01). Physiological parameters showed no significant difference in mean arterial blood pressure and arterial blood gas analysis between the groups. The preischemic physiological values were as follows: pH, 7.31±0.02 and 7.32±0.04; PaCO₂, 41.5±1.1 and 36.1±1.8 mm Hg; Pao₂, 151.1±12.7 and 164.4± 6.6 mm Hg; mean arterial blood pressure, 83.0±4.3 and 87.2±6.5 mm Hg (Wt and Sod2 ^{-/+} mice, respectively; mean±SD of each value; n=4, each group). There was no deviation from these values over the period of assessment.

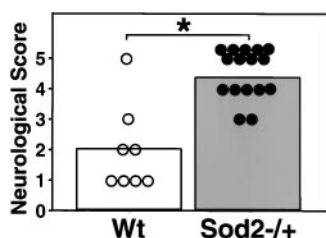


Figure 1. Neurological deficit scores in Wt and knockout mutant mice (Sod2 $-/+$) 16 hours after ischemia/reperfusion. Circles and bars are the scores of each animal and mean scores, respectively. $*P < 0.001$, Mann-Whitney U test. 0=No observable neurological deficit, 1=failed to extend right forepaw, 2=circled to the right, 3=fell to the right, 4=could not walk spontaneously, and 5=dead. Mean scores of the Sod2 $-/+$ mice were significantly higher than those of the Wt mice.

HEt and Et Fluorescent Kinetics and Cellular Localization After Intravenous Injection of HEt

Fluorescent kinetics showed that HEt rapidly disappeared from the serum and penetrated into the brain shortly after intravenous injection (Figure 2A), whereas Et remained largely in the serum (Figure 2B) and did not penetrate into the brain (Figure 2C) up to 60 minutes after intravenous injection. Sixty minutes after HEt injection, the Et signal was detected in brain tissue (Figure 2C) but not in the serum (Figure 2B). These results indicate that HEt was freely permeable through the brain parenchyma and oxidized in the brain to Et in this *in vivo* system. To investigate the aspect or source of cellular superoxide production under normal physiological conditions in the brains of animals that received intravenous HEt injection, fluorescence was microscopically assessed at $Ex = 355$ nm, $Em > 415$ for detection of HEt (Spectra 1) or at $Ex = 510$ to 550 nm, $Em > 580$ nm for Et detection (Spectra 2) (Figure 3A through 3C). HEt injection resulted in nuclear and diffuse cytosolic HEt signals in neurons, suggesting HEt uptake into the cells (Figure 3A). HEt injection also resulted in vesicular Et signals in the cytosolic compartment, suggesting HEt oxidation to Et in the vesicles (Figure 3B and 3C). Similar vesicular staining was obtained with Rh123 and NAO staining (Figure 3D and 3E), which corresponded, respectively, to selective mitochondrial localization of cardiolipin.¹⁷ These results indicate that HEt penetrates into cells and is oxidized to Et in mitochondria under normal physiological conditions. To determine the source of HEt oxidation of Et in this *in vivo* system, we used two different kinds of SOD gene knockout mutant mice. The Sod2 $-/+$ mice showed increased vesicular Et signals (Figure 3G) compared with normal Wt mice (Figure 3F) after HEt injection under normal physiological conditions. The Sod1 $-/-$ mice did not show any obvious difference in Et signals (Figure 3H) compared with normal Wt mice (Figure 3F) under normal physiological conditions.

Sequential Changes in Cellular Localization of Et Signals After Cerebral Ischemia

MCA occlusion moderately increased Et signals in some neuronal subpopulations during ischemia (Figure 4A) compared with neurons in the contralateral hemisphere or under

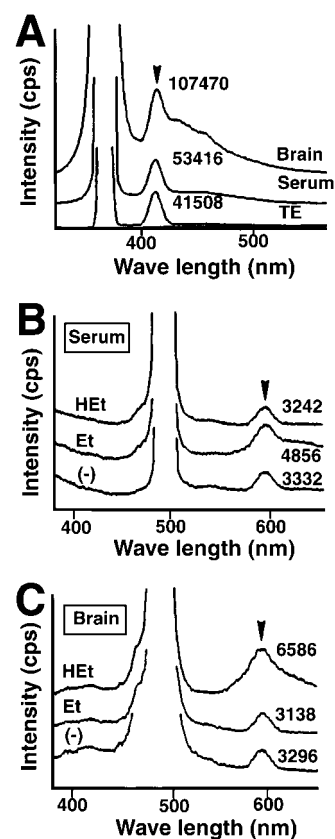


Figure 2. HEt and Et fluorescent kinetics after intravenous injection of HEt. A, Em spectra of HEt ($Ex = 365$ nm) in brain tissue extract (upper curve) and serum (middle curve) 5 minutes after HEt injection. HEt signal (arrowhead, $Em_{max} = 415$ nm) in brain tissue extract was significantly higher than the value in serum, which was almost the same as the basal value of Tris-EDTA buffer (lower curve, TE). B, Em spectra of Et ($Ex = 495$ nm) in serum 60 minutes after HEt (upper curve) or Et (middle curve) injection. After HEt injection, the Et signal (arrowhead, $Em_{max} = 595$ nm) in serum was the same as the basal value (lower curve; 200 mL PBS injection), although it was persistent in serum after Et injection. C, Em spectra of Et ($Ex = 495$ nm) in brain tissue extract 60 minutes after HEt (upper curve) or Et (middle curve) injection. The Et signal (arrowhead, $Em_{max} = 595$ nm) in the brain increased after HEt injection but was the same as the basal value (lower curve; 200 mL PBS injection) after Et injection. Data were obtained from a sample mixture of three independent animals. Em indicates emission; Ex, excitation; HEt, hydroethidine; Et, ethidium.

normal physiological conditions in Wt mice (Figure 3). The Et signals increased remarkably and extended to the entire neuronal population at 1 hour of reperfusion after ischemia (Figure 4B). Four hours after reperfusion, Et signals were mostly detected in endothelial cells, which were morphologically identified (Figure 4C, arrows). As shown in Figure 4D and 4E (high magnification), Et signals were slightly enhanced in the cytosol as well as in mitochondria using double exposure of Spectra 1 (HEt) and 2 (Et) (Figure 4D) during ischemia. Et signals were highly induced in the cytosol as well as in mitochondria 1 hour after reperfusion (Figure 4E). The endothelial Et signals showed a pattern different from the neuronal expression, as indicated by the relatively larger size of the vesicles occupying the cytosolic compartment (Figure 4F). Endothelial expression continued up to 16 hours of

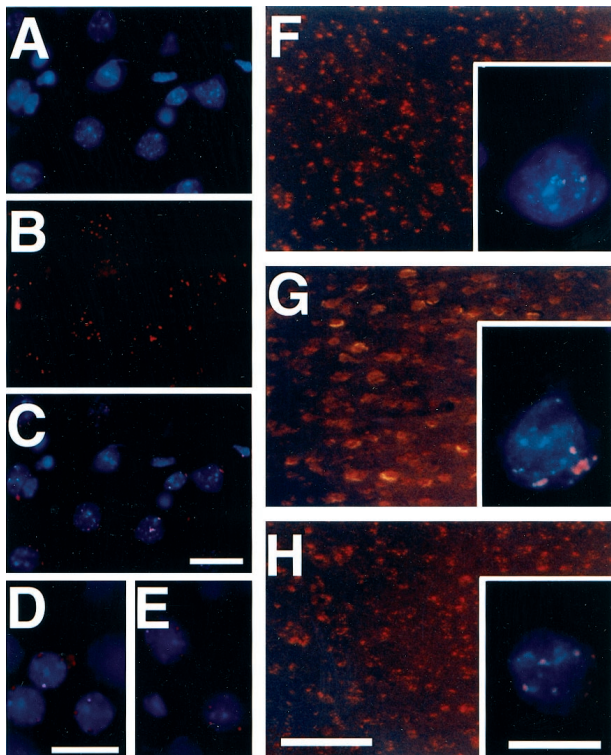


Figure 3. Cellular localization of HET and Et in normal cortical neurons 1 hour after HET injection. Brain specimens after HET injection, observed with a fluorescent microscope at Spectra 1 (HET) and/or Spectra 2 (Et). HET signals are detected in the nucleus, as well as diffusely in the cytosol by Spectra 1 (A). Spectra 2 shows scattered vesicular expression of Et signals in the same cells (B). Double exposure of Spectra 1 and Spectra 2 shows the Et-positive vesicles (red) in the cytosolic compartment (C). Double-exposed fluorescent photomicroscopy of either rhodamine 123 (D) or *N*-nonyl acridine orange (E) vesicles (red) and Hoechst 33258 nuclear staining (blue) are shown. Et signals in Wt mice (F), *Sod2*^{-/+} mice (G), and *Sod1*^{-/-} mice (H) under low magnification with fluorescent microscopy. Mitochondrial basal expression is shown as a background of red fluorescence in Wt mice (F). It is increased in the *Sod2*^{-/+} mice (G) but not changed in the *Sod1*^{-/-} mice (H). High magnification with double exposure of Spectra 1 and 2 shows an increase in both number and magnitude of vesicular signals in *Sod2*^{-/+} mice (inset in G) but not in *Sod1*^{-/-} mice (inset in H), compared with Wt mice (inset in F). Results are representative of *n*=5. Bars=5 μ m in A through E, 5 μ m in F through H, 20 μ m in insets in F through H.

reperfusion (data not shown). To quantify superoxide production after ischemia/reperfusion, Et fluorescent activity was measured in the ischemic or nonischemic brains. Quantitative assay showed that Et signals were significantly increased to $114.0 \pm 4.0\%$ in the ischemic hemisphere at 1 hour of reperfusion compared with the contralateral nonischemic hemisphere (Figure 4G; *n*=5 each, *P*<0.01). The increase in Et signals was moderate at 4 hours and returned to a normal level at 16 hours of reperfusion after ischemia (Figure 4G). These results suggest that $O_2^{\cdot -}$ is mainly produced at an early period of reperfusion.

Et Signals and Infarct Volume Were Increased in *Sod2*^{-/+} Mice After Ischemia/Reperfusion

Deficiency in MnSOD activity in the *Sod2*^{-/+} mice significantly increased Et signals compared with Wt mice

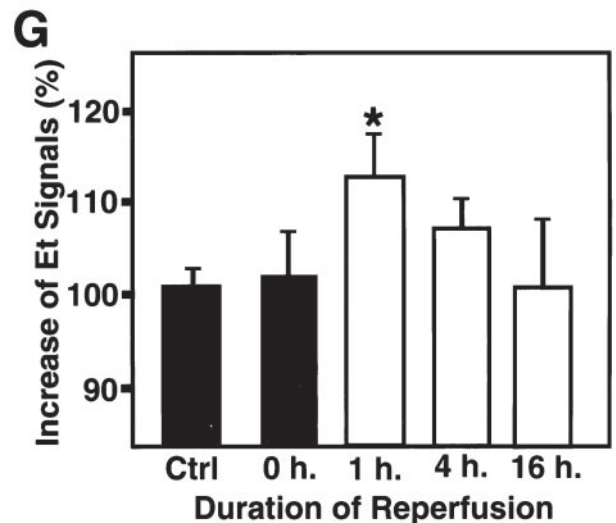
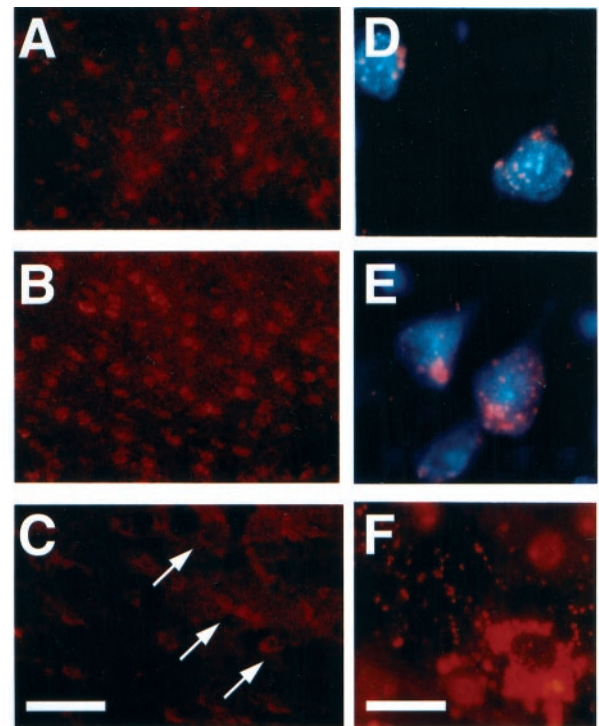


Figure 4. Sequential changes in Et signals after cerebral ischemia/reperfusion in Wt mice. Low magnification of fluorescent photomicroscopy at Spectra 2 revealed characteristic changes in Et signals (red in A through C). Arrows in C indicate endothelial Et signals. High magnification fluorescent photomicroscopy of neurons with double exposure of Spectra 1 and Spectra 2 at 0 hours (D) and 1 hour (E) after reperfusion. F, High magnification fluorescent photomicroscopy of endothelial cell with single exposure of Spectra 2 at 4 hours after reperfusion. Relatively larger vesicular signals of Et (red) around the nucleus (dark area) are shown. Bars=20 μ m in A through C, 5 μ m in D through F. G, Quantitative assay of Et in the ischemic hemisphere after 1 hour of MCA occlusion (see Materials and Methods). Ratio (mean \pm SD) of the Et value in the ischemic hemisphere is compared with the contralateral hemispheric value at 0, 1, 4, and 16 hours after reperfusion (*n*=5 each). Results are representative of *n*=5. Asterisk indicates a significant increase compared with control group; *P*<0.01 by Fisher's post-hoc protected least-significant difference test.

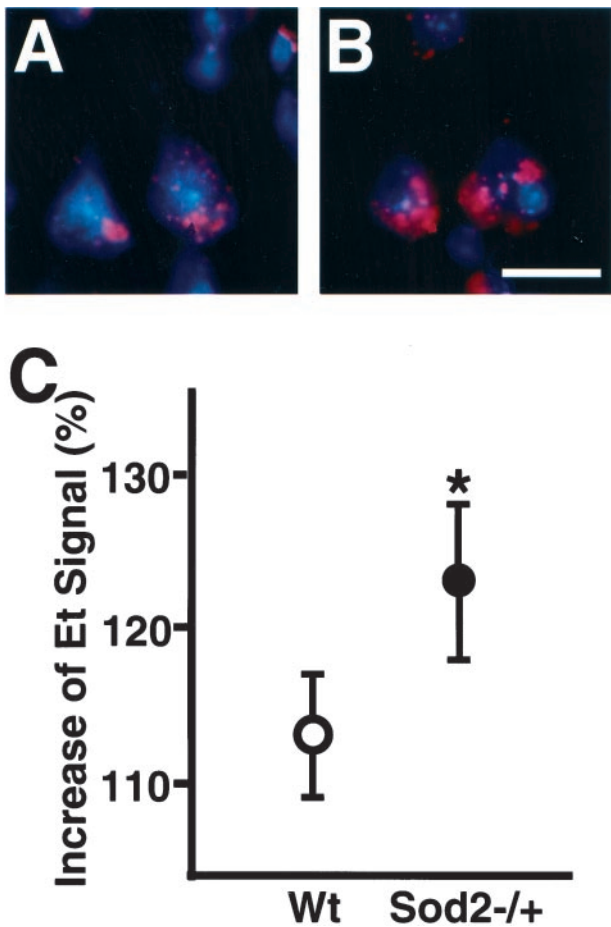


Figure 5. Comparison of Et signals 1 hour after ischemia/reperfusion between Wt and Sod2 ^{-/+} mice. A and B, High magnification fluorescent photomicroscopy of neurons with double exposure of Spectra 1 and Spectra 2. Vesicular signals of Et (red) are highly observed in Sod2 ^{-/+} mice (B) compared with Wt mice (A). Bar=5 μm in A and B. Results are representative of n=5. C, Quantitative assay of Et in the ischemic hemisphere in Sod2 ^{-/+} and Wt mice 1 hour after reperfusion. Ratio (mean±SD) of the Et value in the ischemic hemisphere is compared with the contralateral hemispheric value in 5 individual animals. Asterisk indicates a significant increase compared with Wt mice by unpaired *t* test. **P*<0.01.

after ischemia/reperfusion (Figure 5A and 5B). The quantitative assay confirmed significantly increased Et signals (123±5.4%) 1 hour after ischemia/reperfusion in the Sod2 ^{-/+} mice compared with Wt mice (114.0±4.0%) (Figure 5C; n=5 each, *P*<0.01). To investigate ischemic cell death after ischemia/reperfusion, TTC staining was performed and infarct volume was compared in the Wt and Sod2 ^{-/+} mice (Figure 6A and 6B). An ischemic lesion was detected in the Sod2 ^{-/+} mice 4 hours after reperfusion, whereas an infarct was barely observed in the Wt mice (Figure 6A). The infarct size was increased in the entire MCA territory cortex and caudate/striatum in the Sod2 ^{-/+} mice, but the Wt mice showed an infarct in the caudate putamen 16 hours after reperfusion (Figure 6A). A quantitative assay of infarct volume using TTC staining showed a significantly increased infarct volume in the Sod2 ^{-/+} mice compared with the Wt mice 4 or 16 hours after reperfusion (Figure 6B; Wt,

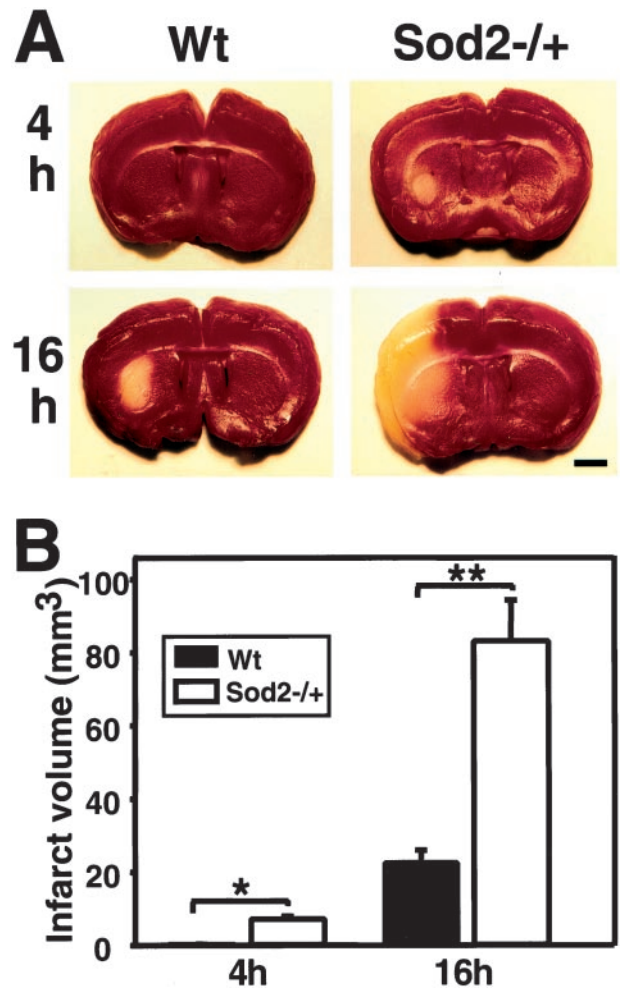


Figure 6. Comparison of cerebral infarction after ischemia/reperfusion in Wt and Sod2 ^{-/+} mice. Photomicrographic findings of cerebral infarction using TTC staining 4 or 16 hours after ischemia/reperfusion in both groups (A). Bar=1 mm. B, Quantitative assay of infarct volume using TTC staining 4 or 16 hours after ischemia/reperfusion. Data represent mean±SD (n=7 to 8 each). Asterisks indicate a significant increase compared with Wt mice by unpaired *t* test. **P*<0.01, ***P*<0.001.

0.63±0.15; Sod2 ^{-/+}, 7.25±0.68 mm³ 4 hours after reperfusion; Wt, 22.53±1.20; Sod2 ^{-/+}, 83.16±11.13 mm³ 16 hours after reperfusion; *P*<0.001).

Discussion

Our study demonstrates that HET may be oxidized to Et in the brain primarily due to mitochondrially produced O₂⁻ under normal physiological conditions in this *in vivo* system. The increased Et signals in mitochondria are largely a result of their binding to mitochondrial DNA. The present data show that MnSOD deficiency increased oxidized HET as early as 1 hour after reperfusion and that subsequent cerebral infarction compared with Wt mice suggests that overproduced O₂⁻ may be one critical step underlying the mechanism of ischemia/reperfusion injury. These results are consistent with the oxidative hypothesis that CuZnSOD may play a role in preventing neuronal cell death by reducing O₂⁻ levels after cerebral ischemia/reperfusion.^{6,7,15}

O₂⁻ production in the cytosol was significantly low and insufficient to oxidize HET, even in the Sod1 ^{-/-} mice,

under normal physiological conditions. These data are consistent with a previous report showing that mitochondrial respiration is the main source of basal $O_2^{\cdot-}$ production.¹² Furthermore, hypotheses of exclusive compartmentalization in SODs and the fact that MnSOD is located and functions only in mitochondria, while CuZnSOD functions in the cytoplasmic matrix,^{18,19} suggest that $O_2^{\cdot-}$ levels are dependent on the level of MnSOD under normal physiological conditions. However, the situation after ischemia/reperfusion might be different from normal physiological conditions, because an excitotoxic cascade could be induced. In fact, recent studies have linked excitotoxicity and ROS production to mitochondrial dysfunction.^{20,21} A failure in energy production from mitochondria after ischemic insult may lead to ROS production as well as to membrane depolarization, removal of the voltage-dependent Mg^{2+} block of the NMDA receptor, and subsequent activation of this receptor. During these episodes, increased intracellular Ca^{2+} levels can initiate a number of deteriorative processes such as proteinase activation and free radical generation.^{1,2} In Ca^{2+} -dependent enzyme activation, $O_2^{\cdot-}$ production additionally occurs in the cytosol of neurons through the arachidonic acid cascade²¹ or the xanthine oxidase pathway in the early period of reperfusion.²² Thus, the present results with enhanced Et signals in both the mitochondria and cytosol of neurons after ischemia/reperfusion are most likely to be explained by neuronal oxidative stress-linked excitotoxicity. In addition, our data demonstrate that Et signals were mostly detected in endothelial cells 4 hours after reperfusion, suggesting that endothelial cells may be important in $O_2^{\cdot-}$ production. This is consistent with a previous report that endothelial cells are a significant source of $O_2^{\cdot-}$ production after in vitro hypoxia reoxygenation through their high levels of xanthine oxidase activity.²³

Numerous available methods have been reported to detect extracellular $O_2^{\cdot-}$ production in vivo, such as calorimetric or luminescence assays.^{24,25} Recently, cellular $O_2^{\cdot-}$ production using a HET in situ detection method was reported in a variety of experimental models such as in vitro excitotoxin-treated cell culture,¹⁰ in vivo permanent focal cerebral ischemia,^{10,11} permanent cortical infarction,²⁶ transient global or focal ischemia,^{7,27} or mitochondrial toxin injury.^{28,29} There is evidence of HET-specific oxidation by $O_2^{\cdot-}$, that HET is oxidized to Et by $O_2^{\cdot-}$ produced by activated leukocytes,¹³ and that HET is selectively oxidized to Et by $O_2^{\cdot-}$ but not by other ROS in cultured hippocampal neurons.¹⁴ Benov et al³⁰ suggest that HET conversion to Et might be a useful tool for detecting $O_2^{\cdot-}$ production, because HET is rapidly oxidized to Et by $O_2^{\cdot-}$. In the present study, we provide clear basic information and a possible quantitation method to detect $O_2^{\cdot-}$ production using selective HET oxidation by $O_2^{\cdot-}$ after ischemia/reperfusion. However, there should be some caution in using the quantitation method: low fluorescent quantum efficacy of Et in the cytosol may occur, because Et binds to DNA in mitochondria and RNA in the cytosol, and quantum efficacy in RNA is lower than in DNA,³¹ or HET might catalyze the dismutation of $O_2^{\cdot-}$.¹⁰

The direct relationship between $O_2^{\cdot-}$ production and neuronal cell death after ischemia/reperfusion is unknown. Our data demonstrate that a deficiency in MnSOD activity in

Sod2 $-/+$ mice exacerbates cerebral infarction after ischemia/reperfusion using TTC staining, suggesting that increased $O_2^{\cdot-}$ production in mitochondria after ischemia/reperfusion may cause severe mitochondrial damage and early energy failure in cells, eventually leading to the enhanced cell death process including an excitotoxic cascade in Sod2 $-/+$ mice. These findings are in agreement with a previous report showing that increased mitochondrial derangement and subsequent infarct volume were detected in Sod2 $-/+$ mice compared with Wt mice after permanent focal cerebral ischemia.¹⁰ Thus, we conclude that MnSOD may play a key role in mitochondrial protection and the subsequent cell death process after ischemia/reperfusion.

Acknowledgments

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