Hypertrophy of Cerebral Arterioles in Mice Deficient in Expression of the Gene for CuZn Superoxide Dismutase

Gary L. Baumbach, MD; Sean P. Didion, PhD; Frank M. Faraci, PhD

Background and Purpose—Reactive oxygen species are believed to be an important determinant of vascular growth. We examined effects of genetic deficiency of copper-zinc superoxide dismutase (CuZnSOD; SOD1) on structure and function of cerebral arterioles.

Methods—Systemic arterial pressure (SAP) and cross-sectional area of the vessel wall (CSA) and superoxide (O$_2^-$) levels (relative fluorescence of ethidium [ETH]) were examined in maximally dilated cerebral arterioles in mice with targeted disruption of one (+/-) or both (-/-) genes encoding CuZnSOD. Wild-type littermates served as controls. Vasodilator responses were tested in separate groups of mice.

Results—CSA and ETH were significantly increased ($P<0.05$) in both CuZnSOD$^{+/−}$ and CuZnSOD$^{−/−}$ mice (CSA = 435 ± 24 and 541 ± 48 μm$^2$; ETH = 18 ± 1 and 34 ± 2%) compared with wild-type mice (CSA = 327 ± 28 μm$^2$; ETH = 6%). Furthermore, the increases in CSA and ETH relative to wild-type mice were significantly greater ($P<0.05$) in CuZnSOD$^{−/−}$ mice than in CuZnSOD$^{+/−}$ mice (CSA = 108 versus 214 μm$^2$; ETH = 12 versus 28%). In addition, dilatation of cerebral arterioles in response to acetylcholine, but not nitroprusside, was reduced by $≈25%$ in CuZnSOD$^{−/−}$ ($P<0.075$) and 50% in CuZnSOD$^{−/−}$ mice ($P<0.05$) compared with wild-type mice.

Conclusions—Cerebral arterioles in CuZnSOD$^{+/−}$ and CuZnSOD$^{−/−}$ mice undergo marked hypertrophy. These findings provide the first direct evidence in any blood vessel that CuZnSOD normally inhibits vascular hypertrophy suggesting that CuZnSOD plays a major role in regulation of cerebral vascular growth. The findings also suggest a gene dosing effect of CuZnSOD for increases in O$_2^-$, induction of cerebral vascular hypertrophy and impaired endothelium-dependent dilatation. (Stroke. 2006;37:1850-1855.)

Key Words: cerebral arteries ■ hypertrophy ■ reactive oxygen species ■ superoxide dismutase

Small resistance arteries and arterioles undergo hypertrophy in models of hypertension.$^{1,2}$ Several determinants apparently contribute to vascular hypertrophy including increases in arterial pressure$^{3,4}$ (including pulse pressure) and the renin-angiotensin system.$^{5}$ Another determinant that may influence vascular hypertrophy is nitric oxide (NO). This suggestion is based on the observations that NO donors inhibit proliferation of vascular muscle in culture,$^6$ and that cerebral arterioles undergo hypertrophy in endothelial NO synthase (eNOS)–deficient mice, even in the absence of increases in arteriolar pulse pressure.$^7$

The bioactivity of NO depends, in part, on its interaction with reactive oxygen species (ROS), particularly superoxide (O$_2^-$).$^8$ Many studies have suggested that inactivation of NO by O$_2^-$ contributes to impaired vascular function.$^9$–$^{11}$ Local levels of O$_2^-$ reflect both the rate of O$_2^-$ formation and the rate of its removal by endogenous antioxidants (primarily superoxide dismutases [SODs]). Much attention has been focused on sources of O$_2^-$ in relation to O$_2$ levels and vascular dysfunction. However, much less is known regarding the functional importance of expression or activity of SODs within the vascular wall.

SODs exist as 3 isoforms localized within specific cellular compartments: copper-zinc SOD (CuZnSOD; SOD1) located predominately within the cytosol, manganese SOD (MnSOD; SOD2) targeted to the mitochondrial matrix, and extracellular SOD (EC-SOD; SOD3) found primarily bound to heparan sulfate proteoglycan on cell surfaces. Although it is known that the 3 isoforms of SOD are expressed within the vessel wall, the predominant isoform of SOD (when expressed as percent of total SOD activity) is CuZnSOD.$^{12,13}$ The goal of this study was to examine the hypothesis that loss of CuZnSOD results in increased O$_2$ levels and altered vascular growth. To test this hypothesis, we measured O$_2$ levels and examined mechanics and structure, as well as vasodilator responses, of cerebral arterioles in wild-type and CuZnSOD-deficient mice.$^{14}$

Methods

Animals

CuZnSOD-deficient mice were produced as described previously.$^{13}$ We interbred heterozygous CuZnSOD-deficient mice to generate wild-type (WT; +/+), mice, heterozygous CuZnSOD-deficient (+/-) mice, and homozygous CuZnSOD-deficient (-/-) mice.
within the same litter. This approach allowed us to use WT littersmates as controls. Genotyping of the animals was performed by polymerase chain reaction from tail biopsies. All mice were studied at about 7 months of age (WT=7.6±0.4 months; CuZnSOD−/−=7.3±0.2 months; CuZnSOD−/−−=7.4±0.4 months). Procedures followed in this study were in accordance with institutional guidelines for care and use of experimental animals at the University of Iowa.

### In Vivo Preparation

Because anesthesia can lower arterial pressure in mice, we measured systemic arterial pressure in conscious mice before examination of cerebral arteriolar structure using a method we have described previously. Following measurement of conscious arterial pressure, animals were weighed and anesthetized with sodium pentobarbital (5 mg 100 g−1 body wt IP), intubated, and mechanically ventilated with room air and supplemental O2. Supplemental anesthetic was administered intravenously at a rate of 1.7 mg 100 g−1 body wt.

A catheter was inserted into a femoral vein for injection of drugs and fluids. A catheter was inserted into a femoral artery to withdraw blood to produce hypotension (for studies of arterial blood gases, and a catheter was inserted into the other systemic arterial pressure and obtain blood samples for measurement of systemic arterial pressure in conscious mice before examination of cerebral arteriolar structure using a method we have described previously.

### Measurement of Cerebral Arteriolar Pressure and Diameter

We measured pressure and diameter in first-order arterioles on the cerebrum through an open skull preparation that we have described previously. A catheter was inserted into a femoral vein for injection of drugs and fluids. A catheter was inserted into a femoral artery to withdraw blood to produce hypotension (for studies of vascular mechanics).

### Baseline Values in CuZnSOD-Deficient Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CuZnSOD Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (+/+)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td><strong>Before Maximal Dilatation</strong></td>
<td></td>
</tr>
<tr>
<td>Systemic Arterial Mean Pressure, mm Hg</td>
<td></td>
</tr>
<tr>
<td>Unanesthetized</td>
<td>127±7</td>
</tr>
<tr>
<td>Anesthetized</td>
<td>75±6</td>
</tr>
<tr>
<td>Cerebral Arteriolar Pressure, mm Hg</td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>38±2</td>
</tr>
<tr>
<td>Diastolic</td>
<td>28±2</td>
</tr>
<tr>
<td>Mean</td>
<td>31±2</td>
</tr>
<tr>
<td>Pulse</td>
<td>10±1</td>
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<tr>
<td>Arterial Blood Gases</td>
<td></td>
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<tr>
<td>( \text{PO}_2 )</td>
<td>39±4</td>
</tr>
<tr>
<td>pH</td>
<td>7.32±0.04</td>
</tr>
<tr>
<td>( \text{PO}_3 )</td>
<td>94±7</td>
</tr>
<tr>
<td>Internal Cerebral Arteriolar Diameter, ( \mu )m</td>
<td>35±2</td>
</tr>
<tr>
<td><strong>After Maximal Dilatation</strong></td>
<td></td>
</tr>
<tr>
<td>Cerebral Arteriolar Diameter, ( \mu )m</td>
<td></td>
</tr>
<tr>
<td>Internal</td>
<td>58±2</td>
</tr>
<tr>
<td>External</td>
<td>61±2</td>
</tr>
<tr>
<td>Wall Thickness, ( \mu )m</td>
<td>1.7±0.1</td>
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<tr>
<td>(E_t ) vs Stress</td>
<td>6.16±0.49</td>
</tr>
<tr>
<td>(N)</td>
<td>5</td>
</tr>
</tbody>
</table>

Measurements of internal diameter before maximal dilatation of cerebral arterioles were obtained at prevailing levels of arterial pressure. Measurements of internal diameter after maximal dilatation were made at an arteriolar mean pressure of 40 mm Hg. Values of external diameter after maximal dilatation were calculated from measurements of internal diameter at 40 mm Hg arteriolar pressure and histological measurements of cross-sectional area of the vessel wall. \(E_t \) vs Stress: slope of tangential elastic modulus (\(E_t \)) vs stress. Values are mean ±SEM.

*\(P<0.05 \) vs male WT mice; †\(P<0.05 \) vs female WT mice; ‡\(P<0.05 \) vs male heterozygous SOD-deficient mice; §\(P<0.05 \) vs female homozygous SOD-deficient mice.
sectional area using an approach we have described in detail previously.9,18

Measurement of Superoxide
O2 levels were evaluated in vitro in 6 to 8 μm thick frozen sections of unfixed cerebral arteries using hydroethidine-based (2 μmol/L hydroethidine) confocal microscopy as described previously.9,19 Laser settings were identical for acquisition of images, and vessels from WT, CuZnSOD+/− and CuZnSOD−/− mice were processed and imaged in parallel. Relative increases in ethidium fluorescence were determined and normalized to the cross-sectional area of the vessel wall.

Evaluation of Endothelium-Dependent Responses in Cerebral Arterioles
Endothelium-dependent and independent responses were examined in cerebral arteries using separate groups of WT, CuZnSOD+/− and CuZnSOD−/− mice and a method we have described previously.13 The diameter of 1 arteriole per animal was measured under control conditions and during topical application of acetylcholine (1 and 10 μmol/L) and nitroprusside (0.1 and 1 μmol/L). Arterial blood gases were monitored and were similar in the 3 groups: WT Pco2=33 ± 2, Po2=191 ± 24, pH=7.35 ± 0.03; CuZnSOD+/− Pco2=35 ± 2, Po2=163 ± 17, pH=7.35 ± 0.02; CuZnSOD−/− Pco2=35 ± 3, Po2=167 ± 20, pH=7.33 ± 0.01 (mean ± SE).

Statistical Analysis
Analysis of variance was used to compare systemic mean pressure and arteriolar pressures, diameters, cross-sectional area of the vessel wall, slope of tangential elastic modulus versus stress, relative density of ethidium fluorescence and dilator responses. Probability values were calculated using a Student t test. Statistics were determined using JMP statistics software (SAS Institute Inc).

Results
Blood Pressure and Baseline Characteristics
In the unanesthetized state, systemic mean arterial pressure was similar in male and female CuZnSOD+/− and WT mice (Table). In contrast, unanesthetized systemic mean arterial pressure in male and female CuZnSOD−/− mice was significantly lower than in male and female WT CuZnSOD+/− mice (Table). Anesthesia substantially reduced arterial pressure in all groups of mice (Table). During anesthesia, systemic mean arterial pressure and cerebral arteriolar mean and pulse pressure were not significantly different in any of the groups of mice (Table).

Superoxide Levels
Basal O2 levels appeared to be higher in cerebral arteries of CuZnSOD−/− mice than in WT mice (Figure 1). Quantification of ethidium signal revealed significantly higher fluorescence in cerebral arterioles of both CuZnSOD+/− and CuZnSOD−/− mice compared with WT (Figure 1). In addition, relative fluorescent intensity was significantly greater in cerebral arterioles of CuZnSOD−/− than CuZnSOD+/− mice (Figure 1). No gender differences in relative fluorescence were noted in any of the groups of mice.

Vascular Mechanics
Diameter before EDTA was similar in cerebral arterioles of male and female WT, CuZnSOD+/− and CuZnSOD−/− mice (Table). After maximal dilatation with EDTA, internal and external diameters in male and female CuZnSOD+/− and CuZnSOD−/− mice were not significantly different than in male and female WT mice (Table). Wall thickness and cross-sectional area of the vessel wall were significantly greater in cerebral arterioles in male and female CuZnSOD−/− and CuZnSOD+/− mice than in male and female WT mice (Figure 2). Furthermore, wall thickness and cross-sectional area of the vessel wall in male and female CuZnSOD+/− mice were intermediate between male and female WT and CuZnSOD−/− mice (Figure 2). Thus, cerebral arterioles in both CuZnSOD+/− and CuZnSOD−/− mice underwent hypertrophy of the vessel wall regardless of gender.

During maximal dilatation, internal diameter in cerebral arterioles in male and female CuZnSOD+/− and CuZnSOD−/− mice were similar to diameter in male and female WT mice at all levels of arteriolar pressure between 10 and 40 mm Hg (Figure 3). The stress-strain curve in cerebral arterioles in male CuZnSOD+/− mice was comparable to the curve in cerebral arterioles in male WT mice (Figure 4, left panel). In contrast, the stress-strain curves in female CuZnSOD+/− mice and male and female CuZnSOD−/− mice were shifted to the right of the curves in male and female WT mice (Figure 4). The slope of tangential elastic modulus versus stress was significantly reduced in female CuZnSOD+/− mice and male and female CuZnSOD−/− mice but not in male CuZnSOD−/− mice (Table). These findings suggest that a deficiency of CuZnSOD may result in increased distensibility of cerebral arterioles.

Vasodilator Responses
Baseline diameter of cerebral arterioles was similar (P>0.05) in WT mice (33 ± 4 μm; n=7), CuZnSOD+/− mice
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dine, were significantly increased in CuZnSOD-deficient mice. Increases in cerebral arteriolar O₂ levels occurred in both CuZnSOD<sup>+/−</sup> and CuZnSOD<sup>−/−</sup> mice, but were greater in the CuZnSOD<sup>−/−</sup> group. Second, mean and pulse pressures in cerebral arterioles of CuZnSOD<sup>+/−</sup> and CuZnSOD<sup>−/−</sup> mice were similar to cerebral arteriolar pressures in WT mice. Third, cerebral arterioles in CuZnSOD-deficient mice underwent hypertrophy of the vessel wall with the degree of hypertrophy being greater in CuZnSOD<sup>−/−</sup> than CuZnSOD<sup>+/−</sup> mice. Cerebral arteriolar hypertrophy in CuZnSOD<sup>−/−</sup> mice was associated with an increase in arteriolar distensibility. Fourth, we confirmed that endothelium-dependent dilatation of cerebral arterioles was impaired in CuZnSOD<sup>−/−</sup> mice, and we also found that this response was reduced in CuZnSOD<sup>+/−</sup> mice, though to a lesser degree. Finally, external diameter was not reduced in arterioles of CuZnSOD-deficient mice. Thus, deficiency of CuZnSOD did not result in inward or outward remodeling of cerebral arterioles. Taken together, these findings suggest that increases in basal O₂ in cerebral blood vessels attributable to deficiency of CuZnSOD produce cerebral vascular hypertrophy, even in the absence of increases in arteriolar pressure. Furthermore, the findings indicate that the loss of a single copy of the CuZnSOD gene is sufficient to induce hypertrophy and impair endothelium-dependent dilatation of cerebral arterioles.

**Hypertrophy of Cerebral Arterioles**

The finding in this study of cerebral arteriolar hypertrophy in CuZnSOD-deficient mice suggests that oxidative stress, and in particular O₂, may be another factor that contributes to vascular hypertrophy in chronic hypertension. At least 2 other lines of evidence support this possibility. First, oxidative stress is a prominent component of chronic hypertension. Second, oxidative stress may influence other factors that impact growth of vascular muscle, such as NO, epidermal growth factor–receptors, p38 mitogen-activated protein kinase, and Akt.

**Discussion**

There are several major new findings in this study. First, basal O₂ levels in cerebral arterioles, as measured using hydroethidine, were significantly increased in CuZnSOD-deficient mice. Increases in cerebral arteriolar O₂ levels occurred in both CuZnSOD<sup>+/−</sup> and CuZnSOD<sup>−/−</sup> mice, but were greater in the CuZnSOD<sup>−/−</sup> group. Second, mean and pulse pressures in cerebral arterioles of CuZnSOD<sup>+/−</sup> and CuZnSOD<sup>−/−</sup> mice were similar to cerebral arteriolar pressures in WT mice. Third, cerebral arterioles in CuZnSOD-deficient mice underwent hypertrophy of the vessel wall with the degree of hypertrophy being greater in CuZnSOD<sup>−/−</sup> than CuZnSOD<sup>+/−</sup> mice. Cerebral arteriolar hypertrophy in CuZnSOD<sup>−/−</sup> mice was associated with an increase in arteriolar distensibility. Fourth, we confirmed that endothelium-dependent dilatation of cerebral arterioles was impaired in CuZnSOD<sup>−/−</sup> mice, and we also found that this response was reduced in CuZnSOD<sup>+/−</sup> mice, though to a lesser degree. Finally, external diameter was not reduced in arterioles of CuZnSOD-deficient mice. Thus, deficiency of CuZnSOD did not result in inward or outward remodeling of cerebral arterioles. Taken together, these findings suggest that increases in basal O₂ in cerebral blood vessels attributable to deficiency of CuZnSOD produce cerebral vascular hypertrophy, even in the absence of increases in arteriolar pressure. Furthermore, the findings indicate that the loss of a single copy of the CuZnSOD gene is sufficient to induce hypertrophy and impair endothelium-dependent dilatation of cerebral arterioles.

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Potential Mechanisms of Cerebral Arteriolar Hypertrophy

Interactions with NO

One mechanism by which oxidative stress may influence growth is by reducing availability of NO to the vessel wall. Previous findings suggest that reduced availability of NO in the vessel wall may contribute to the development of cerebral vascular hypertrophy as a consequence of reduced inhibition of smooth muscle growth. The bioactivity of NO depends, in part, on its interaction with reactive O$_2$ species, particularly O$_2$–. Thus, increased levels of O$_2$– in CuZnSOD-deficient mice may lead to cerebral arteriolar hypertrophy by inactivating NO in the arteriolar wall, and thus diminishing NO’s inhibitory influence on vascular growth.

Even if interactions of O$_2$– with NO play a role, however, it is unlikely that such interactions account entirely for O$_2$–’s trophic effects on cerebral arterioles. If these interactions were entirely responsible for trophic effects, we would anticipate that deficiency of eNOS would result in levels of arteriolar hypertrophy similar to those produced by deficiency of CuZnSOD. Instead, the degree of cerebral arteriolar hypertrophy that we found previously in homozygous eNOS-deficient mice was about half that found in this study in CuZnSOD–/– mice. These findings suggest that other factors, in addition to an interaction with NO, may contribute to cerebral arteriolar hypertrophy in CuZnSOD deficiency.

Mediation by Hydrogen Peroxide

H$_2$O$_2$ is derived primarily from O$_2$– and is considered to be one of the most important ROS by virtue of its interactions with multiple signaling systems. H$_2$O$_2$ is a good candidate to play a second messenger role in signaling processes that lead to vascular growth by virtue of its being uncharged, relatively long-lasting and freely diffusible. Because levels of O$_2$– are typically increased in many conditions that stimulate vascular growth, such as hypertension and atherosclerosis, and because in most circumstances O$_2$– is quickly converted to H$_2$O$_2$ by SOD, it is possible the contribution of O$_2$– to vascular growth under conditions of oxidative stress is mediated at least in part by H$_2$O$_2$.

We think it is less likely that H$_2$O$_2$ contributed to the observation in this study of cerebral arteriolar hypertrophy in CuZnSOD-deficient mice. Because rapid conversion of O$_2$– to H$_2$O$_2$ requires an enzyme such as the SODs, one might expect in the absence of CuZnSOD a shift in the O$_2$–:H$_2$O$_2$ balance...
that favors O₂. This expectation is borne out by the finding that O₂ levels are increased and levels of H₂O₂ are significantly reduced in CuZnSOD−/− mice. Furthermore, if H₂O₂ is an important stimulus of cellular growth in response to oxidative stress, we would anticipate that overexpression of CuZnSOD would increase levels of H₂O₂ and accelerate cellular growth. At least with fibroblasts, however, overexpression of CuZnSOD suppresses growth and induces features of cell senescence, despite elevated levels of H₂O₂. Thus, our findings in this intact system support the concept that ROS influence vascular growth, but provide new evidence to suggest O₂ may be a key mediator of vascular hypertrophy in vivo.

Conclusion
We found in this study that (1) cerebral arterioles in both CuZnSOD−/− and CuZnSOD+/− mice undergo hypertrophy and (2) the degree of hypertrophy is greater in CuZnSOD−/− mice than previously observed in homozygous eNOS-deficient mice. Based on these findings we conclude that interactions of O₂ with NO cannot account entirely for the degree of cerebral arteriolar hypertrophy found in CuZnSOD−/− mice. Our study provides genetic support for the concept that ROS influence vascular growth and CuZnSOD may play an important role in the regulation of vascular growth.

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Disclosures
None.

References
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