Superoxide levels and function of cerebral blood vessels after inhibition of CuZn-SOD

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Didion, Sean P., Christopher A. Hathaway, and Frank M. Faraci. Superoxide levels and function of cerebral blood vessels after inhibition of CuZn-SOD. Am J Physiol Heart Circ Physiol 281: H1697–H1703, 2001.—The goal of this study was to examine the role of endogenous copper/zinc (CuZn)-superoxide dismutase (SOD) on superoxide levels and on responses of cerebral blood vessels to stimuli that are mediated by nitric oxide (acetylcholine) and cyclooxygenase-dependent mechanisms (bradykinin and arachidonic acid). Levels of superoxide in the rabbit basilar artery were measured using lucigenin-enhanced chemiluminescence (5 μM lucigenin). Diethyldithiocarbamate (DDC; 10 mM), an inhibitor of CuZn-SOD, increased superoxide levels by ~2.4-fold (P < 0.05) from a baseline value of 1.0 ± 0.2 relative light units·min⁻¹·mm⁻² (means ± SE). The diameter of cerebral arterioles (baseline diameter, 99 ± 3 μm) was also measured using a closed cranial window in anesthetized rabbits. Topical application of DDC attenuated responses to acetylcholine, bradykinin, and arachidonate, but not nitroprusside. For example, 10 μM arachidonic acid diluted cerebral arterioles by 40 ± 5 and 2 ± 2 μm under control conditions and after DDC, respectively (P < 0.05). These inhibitory effects of DDC were reversed by the superoxide scavenger 4,5-dihydroxy-1,3-benzenedisulfonic acid (10 mM). Arachidonate increased superoxide levels in the basilar artery moderately under normal conditions and this increase was greatly augmented in the presence of DDC. These findings suggest that endogenous CuZn-SOD limits superoxide levels under basal conditions and that EC-SOD may also play an important role in normal activity of CuZn-SOD.

cerebral arterioles; basilar artery; acetylcholine; nitric oxide; arachidonic acid; bradykinin

THE BIOACTIVITY OF NITRIC OXIDE (NO) depends, in part, on its interaction with reactive oxygen species, particularly superoxide. Although there has been considerable effort to define the role of NO in regulation of cerebral vascular tone (11), the role of vascular superoxide in the brain is poorly understood. Substances that spontaneously generate superoxide impair relaxation of cerebral blood vessels in response to endothelium-depen-
dent stimuli (12, 38, 59). Wei et al. (62) provided the first evidence that superoxide inactivates NO (endothelium-derived relaxing factor) in vivo. Since that initial finding, other studies (13, 18, 21, 30, 37) have suggested that inactivation of NO by superoxide contributes to impaired NO-mediated dilatation of carotid and cerebral blood vessels under several pathophysiological conditions.

Local steady-state levels of superoxide are dependent on both the rate of production of superoxide as well as the activity of endogenous superoxide dismutase (SOD). There are three main isoforms of SOD: manganese (Mn)-SOD, copper/zinc (CuZn)-SOD, and extracellular (EC) CuZn-SOD (EC-SOD) (8, 15). Within blood vessels, the predominant isoforms of SOD are CuZn-SOD and EC-SOD (42, 56). It has been suggested that CuZn-SOD is required for release of NO from endothelium (35) and that EC-SOD is needed to protect NO as it diffuses through the vascular wall (42). Relatively little is known regarding the role of endogenous SOD in protecting NO-mediated responses in the cerebral circulation. Thus, the first goal of the present study was to examine the effects of the CuZn-SOD inhibitor diethyldithiocarbamate (DDC) (16) on responses of cerebral arterioles to acetylcholine, an endothelium-dependent agonist that produces NO-dependent dilatation of the cerebral microcirculation (4, 8–11, 48, 52).

In addition to protecting NO-mediated responses, endogenous SOD may also play an important role in modulating vascular responses to stimuli that result in formation of reactive oxygen species. For example, one major source of superoxide in the brain is the cyclooxygenase (COX) pathway (22). Dilatation of cerebral arterioles and increases in cerebral blood flow in response to arachidonic acid and bradykinin are dependent on activity of COX (COX-1 specifically) and mediated by reactive oxygen species (3, 7, 22, 27, 39, 53, 54, 61). Thus the second goal of this study was to examine the hypothesis that endogenous SOD has an important influence on COX-dependent vascular responses. For these studies, we considered two possible outcomes. The first was that inhibition of SOD activity would...
enhance vascular responses mediated by COX and reactive oxygen species (arachidonic acid and bradykinin). Conversely, dilator responses of cerebral arterioles to bradykinin and arachidonic acid are mediated (at least in part) by hydrogen peroxide (22, 53, 54), the formation of which is dependent on the activity of SOD. Thus we also considered the possibility that vascular responses of cerebral arterioles to arachidonate and bradykinin would be impaired after inhibition of endogenous SOD.

**METHODS**

**Animal preparation.** Experiments were performed on 66 New Zealand White rabbits (2.5–3.5 kg) anesthetized with pentobarbital sodium (40 mg/kg iv). Pentobarbital was supplemented regularly at ~10 mg·kg⁻¹·h⁻¹. The trachea was cannulated and the animals were ventilated mechanically with air and supplemental oxygen. Arterial blood gases were monitored and were stable throughout the experiment (see RESULTS). A femoral artery was cannulated for measurement of systemic pressure and to sample arterial blood. A femoral vein was cannulated for infusion of anesthetic.

Rabbits were placed in a head holder and a closed cranial window was placed over the parietal cortex as described previously (10). The cranial window was filled with artificial cerebrospinal fluid warmed to 37°C. Diameters of pial arterioles were measured using a microscope equipped with a television camera coupled to a video monitor. Images were recorded on videotape and vessel diameters were measured later with an image analyzer. One cerebral arteriole was studied in each animal. In all animals, responses of cerebral arterioles to acetylcholine were measured initially to establish reactivity of the vessels. Flushing the window with artificial cerebrospinal fluid did not alter baseline diameter of arterioles.

**Experimental protocol.** In different groups of animals (the n for each group is outlined in RESULTS), we examined responses of cerebral arterioles to acetylcholine (1 and 10 μM), nitroprusside (1 and 10 μM), arachidonic acid (1 and 10 μM), and bradykinin (1–100 nM) under control conditions and then in the presence of DDC (10 mM). We have shown in previous experiments and in preliminary studies that responses of cerebral arterioles to these agonists are reproducible in this model. For DCC experiments, the cranial window was treated with the inhibitor for 30 min and DCC was present in the window during the second application of an agonist. DCC has been used widely for this purpose in non-vessel-free preparations, was subtracted from the readings obtained with vessels. Superoxide levels were measured after preincubation with vehicle or DCC (10 mM) for 30 min. The surface area of the vessel lumen was imaged with a video camera and calculated with the use of NIH Image software to normalize superoxide levels. This approach has been used extensively for measurement of superoxide in blood vessels (2, 5, 14, 17, 28, 31). The results obtained using this adaptation of the lucigenin assay are similar to those obtained using the ferricytochrome c reduction assay and with electron spin resonance measurements of superoxide in intact vessels as well as by ferricytochrome c reduction in vascular homogenates (5, 14, 55).

The purpose of these experiments was to verify that inhibition of SOD with DDC increased levels of superoxide in cerebral blood vessels. Measurements of superoxide were made under basal conditions and during treatment of vessel segments with arachidonic acid (in the absence and presence of DDC). In one group of vessels, we also examined the effect of indomethacin (10 μM) on superoxide levels after treatment with DDC. These biochemical studies were performed using the basilar artery because cerebral arterioles are too small to be used for reliable detection of superoxide using lucigenin-enhanced chemiluminescence.

**Statistical analysis.** For comparison of vessel diameter or superoxide levels under control conditions and during administration of an inhibitor, statistical analysis was performed using paired Student’s t-tests. All values are expressed as means ± SE. A P value <0.05 was considered significant.

**RESULTS**

**Control conditions.** Baseline diameter of cerebral arterioles for all groups (n = 48) was similar and averaged 99 ± 3 μm (means ± SE). Mean arterial pressure averaged 77 ± 1 mmHg and was not affected by treatment with agonists or inhibitors in the cranial window. Arterial blood gases were monitored and were stable throughout the experiment (PCO₂, 36 ± 1 mmHg; PO₂, 110 ± 2 mmHg; pH, 7.43 ± 0.01).

**Effect of DDC on vascular responses.** Acetylcholine (1 and 10 μM) produced dilatation of cerebral arterioles. Baseline diameter of cerebral arterioles in this group was 104 ± 8 μm under control conditions and 96 ± 8 μm in the presence of DCC. The response to the two concentrations of acetylcholine was inhibited by ~50 and 30% in the presence of DCC, respectively (Fig. 1A). For example, 1 μM acetylcholine increased diameter of cerebral arteries by 30 ± 6 μm in the absence of and 15 ± 6 μm in the presence of DCC. This inhibitory effect of DCC was completely reversed by Tiron, a scavenger of superoxide (Fig. 1B). In contrast to the effects on responses to acetylcholine, vasodilatation in response to nitroprusside (1 and 10 μM) was similar in the absence and presence of DCC (Fig. 2). In addition, treatment of the cranial window with DCC and Tiron did not alter vasodilator responses to nitroprusside (data not shown).

To determine if inhibition of SOD altered responses to other stimuli, we also examined the effects of DCC on vasodilator responses to arachidonic acid and bradykinin. Dilatation of cerebral arterioles in response to both arachidonic acid and bradykinin was markedly...
The inhibitory effects of DDC were reversed by the use of Tiron. In separate experiments, we found that treatment with Tiron alone did not inhibit dilatation of cerebral arterioles in response to bradykinin or the high concentration of arachidonic acid (data not shown).

**Effects DDC on vascular levels of superoxide.** Under baseline conditions, levels of superoxide in the basilar artery (as detected using lucigenin-enhanced chemiluminescence) were relatively low (Fig. 5). Treatment with DDC increased superoxide levels in the basilar artery by ~2.4-fold (Fig. 5) (n = 8), suggesting that endogenous CuZn-SOD limits superoxide levels in cerebral blood vessels under control conditions.

Under baseline conditions (in the absence of DDC), arachidonic acid increased levels of superoxide in the basilar artery. The levels of superoxide after treatment with 1 and 10 \( \mu M \) arachidonic acid were ~1.4- and 2.3-fold above control, respectively (P < 0.05, Fig. 6). In the presence of DDC, the increases in superoxide in
response to arachidonic acid were enormously augmented (by ~18- and 170-fold, respectively) (Fig. 7).

The marked increase in superoxide levels in response to arachidonic acid after treatment with DDC was greatly reduced in vessels treated with Tiron or indomethacin (Fig. 7).

**DISCUSSION**

There are several major new findings in the present study. First, treatment of normal cerebral vessels with DDC, an inhibitor of CuZn-SOD, increased levels of superoxide. These findings suggest that endogenous CuZn-SOD(s) limit increases in superoxide within the cerebral vascular wall under basal conditions. Second, DDC inhibited responses of cerebral arterioles to acetylcholine, an endothelium-dependent agonist that produces NO-mediated dilatation of cerebral arterioles. Third, arachidonic acid produced increased levels of superoxide in cerebral vessels. This increase in superoxide was markedly augmented in vessels treated with DDC. The COX pathway appears to be the major source of superoxide generated in response to arachidonic acid in vessels treated with DDC. Fourth, DDC produced marked impairment of vasodilator responses to arachidonic acid and bradykinin, agonists that are known to produce COX-mediated dilatation of cerebral arterioles. These findings suggest that endogenous CuZn-SOD limits levels of superoxide under basal conditions and have a marked influence on increases in superoxide levels in vessels treated with arachidonic acid. To our knowledge, this is the first study to examine the functional importance of endogenous SOD in influencing responses in the cerebral microcirculation.

**Inhibition of CuZn-SOD increases superoxide and impairs endothelium-dependent, NO-mediated responses.** Endothelium is a major regulator of vascular tone. Under normal conditions, NO produced by endothelial NO synthase (eNOS) influences basal tone and is the primary mediator of relaxation of carotid and cerebral vessels in response to the endothelium-dependent agonist acetylcholine (11). For example, relaxation of rat, mouse, rabbit, and human cerebral arterioles in response to acetylcholine is essentially completely abolished by inhibitors of NOS (4, 6, 9, 10, 48, 52).

The bioactivity of NO within the vessel wall depends, in part, on its interaction with superoxide. Although there has been considerable effort to define the role of NO in regulation of cerebral vascular tone (11), the role of vascular superoxide and the interaction of NO with superoxide in the brain and within the vessel wall are poorly understood. Substances that result in generation of superoxide (i.e., tetrahydrobiopterin, amyloid-β peptide, or pyrogallol) impair relaxation of cerebral blood vessels in response to endothelium-dependent stimuli that are mediated by release of NO by eNOS (12, 38, 59).

In the present study, DDC (a well-known and selective inhibitor of CuZn-SOD) (16, 19) was used to examine the role of endogenous CuZn-SOD in limiting basal levels of superoxide within cerebral vessels and protecting endothelium-dependent responses under normal conditions. This approach has been used by others to produce this form of oxidative stress in studies of extracranial vessels (17, 29, 39, 43, 44, 49). For example, DDC increases superoxide and impairs endothelium-dependent relaxation in aorta and carotid artery (17, 29, 39, 43, 44, 49).

The role of the various SODs within the cerebral circulation has been largely unstudied. Wambi-Kiesse and Katusic (60) recently reported that DDC impaired relaxation of the basilar artery in vitro to bradykinin,
an agonist that produces eNOS-mediated relaxation in the basilar artery (11, 41). Levels of superoxide were not measured in that previous study. Our finding that DDC increased superoxide levels by ~2.5-fold in the basilar artery is consistent with previous functional data in cerebral arteries (60) and very consistent with previous work in extracranial vessels, in which superoxide levels were measured biochemically (17, 43, 44, 49). The results of the present study also extend the findings in the basilar artery (60) by suggesting that CuZn-SOD protects eNOS-related function within the microcirculation of the brain in vivo.

The effects of DDC in this study and others (43, 44, 60) were reversed by Tiron or other scavengers of superoxide, suggesting that effects were selective and mediated by superoxide. In addition, DDC had little effect on responses to nitroprusside (present study) or another donor of NO (i.e., diethylamine-NONOate) (60). Because DDC impairs vascular responses that are mediated by NO produced by eNOS, it is somewhat surprising that DDC did not similarly affect responses to pharmacological donors of NO in this or in previous studies (60). It is not completely clear why superoxide may affect endogenously produced NO to a greater extent than exogenously applied NO. However, such a relationship has been observed in other models of oxidative stress (59) and under pathophysiological conditions with increased oxidative stress in blood vessels (35), including the carotid and cerebral circulation (13, 30). One possibility is that the subcellular distribution of superoxide is such that NO released by eNOS is more susceptible to inactivation by superoxide as it diffuses through the vessel wall, compared with NO released by NO donors, which may occur intracellularly in vascular muscle (25). Another possibility may be because nitroprusside (as opposed to eNOS, which produces NO) releases NO\(^-\), which does not react with superoxide (44).

**Inhibition of CuZn-SOD greatly increases arachidonate-induced increases in superoxide and impairs COX-mediated vasodilatation.** Under baseline conditions (in the absence of DDC), arachidonic acid increased levels of superoxide in the basilar artery (2–3 fold). It has been known that arachidonic acid increases superoxide in brain tissue when applied in vivo into a cranial window (22). However, the present data are the first to our knowledge to use biochemical measurements to demonstrate that arachidonate increases superoxide within the cerebral vascular wall. In the presence of DDC, the increases in superoxide in cerebral arteries in response to arachidonate were enormously augmented. These findings suggest that endogenous CuZn-SOD plays a major role in limiting increases in superoxide that occur within the vessel wall in response to arachidonic acid. More importantly, we found that indomethacin greatly reduced increases in superoxide in response to arachidonic acid after inhibition of CuZn-SOD. This finding suggests that COX is the main source of superoxide produced by the vessel wall in response to arachidonic acid.

To determine if inhibition of SOD affected vasodilator responses to stimuli other than those mediated by NO, we also examined effects of DDC on responses to bradykinin and arachidonic acid. Dilatation of cerebral arterioles in response to bradykinin is known to be endothelium dependent and dependent on activity of COX (11, 22, 39, 53). Similarly, dilatation of cerebral arterioles in response to arachidonate is mediated by a COX-dependent mechanism (3, 7, 27, 39, 54, 61). Recent findings by Niwa et al. (39), which were obtained with the use of gene-targeted mice, indicate that COX-1 is the isoform that mediates increases in cerebral blood flow in response to bradykinin and arachidonic acid. In the present study, dilatation of cerebral arterioles in response to both these agonists was inhibited markedly by DDC. The combination of these findings suggests that DDC produces inhibition of endothelium-dependent responses of cerebral arterioles as well as responses that are mediated by COX. Our findings do not exclude the possibility that superoxide and/or other reactive oxygen species might directly inhibit activity of COX or eNOS. This possibility is supported by studies (21, 33, 45, 51) in which reactive oxygen species have been shown to inhibit activity of COX and eNOS (in some system).

The restoration of dilator responses to arachidonic acid and bradykinin by Tiron in DDC-treated arterioles merits comment. Dilatation of cerebral arterioles in response to arachidonic acid and bradykinin are mediated to a large extent by hydrogen peroxide (22, 53, 54). For example, catalase, which converts hydrogen peroxide to water and oxygen, alone inhibits dilator responses to both these agonists (22, 53, 54). In addition to scavenging superoxide, Tiron is known to promote the formation of hydrogen peroxide (26, 32). Thus DDC may reduce vasodilatation in response to arachidonic acid and bradykinin by inhibiting endogenous production of hydrogen peroxide. Tiron, which promotes formation of hydrogen peroxide as it scavenges superoxide, may restore vasodilatation in the presence of DDC by increasing levels of hydrogen peroxide. Because reactive oxygen species are interrelated and difficult to measure, it would be extremely challenging to demonstrate that the amount of hydrogen peroxide generated by Tiron is proportional to the degree of inhibition of CuZn-SOD by DDC. However, the finding that DDC, an inhibitor of SOD activity (i.e., hydrogen peroxide formation), greatly impairs dilator responses of cerebral arterioles in response to bradykinin and arachidonic acid provides additional evidence that this vasodilator response may be mediated by hydrogen peroxide.

**Implications in relation to vascular disease.** The concept that interaction of NO with superoxide could have important vascular effects was first supported by Wei et al. (62), who provided evidence that superoxide inactivates NO after acute hypertension. Impaired endothelium-dependent relaxation that is normally mediated by NO can be improved with SOD (or the combination of SOD and catalase) in cerebral blood vessels in animal models of traumatic brain injury,
ischemia, diabetes, inflammation, and Alzheimer’s disease (13, 18, 21, 30, 37). Thus inactivation of NO by superoxide is thought to contribute to endothelial dysfunction in several disease states.

An implication of the present study is that in addition to changes in production of superoxide, reductions in the expression or activity of endogenous SODs within the vessel wall can have major effects on local levels of superoxide and vascular responses. At this time, little is known regarding changes in expression or activity of SOD in cerebral vessels in disease states. One study (57) has suggested that activity of SOD in the basilar artery is markedly reduced after subarachnoid hemorrhage. Some evidence (20, 34) suggests that superoxide is increased in cerebral vessels in subarachnoid hemorrhage. Also, both endothelium-dependent relaxation (11, 41, 58) and relaxation of cerebral arteries in response to arachidonic acid are impaired after subarachnoid hemorrhage (50).

In summary, these results suggest that CuZn-SOD protects responses of the cerebral microcirculation to NO-mediated stimuli. The study also supports the concept that endogenous CuZn-SOD plays an important role in limiting levels of superoxide in cerebral vessels, both under normal conditions and particularly after exposure to arachidonic acid. This latter finding may have major implications as metabolism of arachidonic acid by COX is thought to be a key source of superoxide during disease states and in response to brain injury (including acute hypertension, ischemia with reperfusion, traumatic brain injury, meningitis, and asphyxia/reventilation) (1, 22, 46, 47).

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ROLE OF SOD IN CEREBRAL CIRCULATION