The Effect of Superoxide Anion on Autoregulation of Cerebral Blood Flow

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Background and Purpose—Recent studies have suggested that autoregulation of cerebral blood flow (CBF) is impaired after traumatic and ischemic brain injury. Given that the levels of superoxide anion (O$_2^-$) are increased in these conditions, we postulate that O$_2^-$ contributes to the impairment of CBF autoregulation.

Methods—CBF was monitored with laser Doppler flowmetry during increases in blood pressure.

Results—During the control period, CBF was well autoregulated after the increase in mean arterial pressure (MAP) from 98±3 to 140±6 mm Hg. The autoregulation index (AI; ΔCBF/ΔMAP) averaged 0.25±0.02 (n=6). O$_2^-$ in the brain was then increased by subdural perfusion of xanthine/xanthine oxidase (different concentrations) and catalase. Low concentrations of O$_2^-$ decreased basal CBF by 10±1.6% but had no effect on autoregulation (AI, 0.19±0.02; n=6). Higher concentrations of O$_2^-$ (0.2 mmol/L xanthine and either 3 or 20 mU xanthine oxidase) increased basal CBF by 30±2% and 42±4%, respectively, and impaired autoregulation of CBF (AI, 0.55±0.03 and 0.76±0.02; n=6). Inclusion of superoxide dismutase in the O$_2^-$-generating system restored autoregulation (AI, 0.28±0.05; n=6). Neither inhibition of NO synthase nor the addition of defer spinexine had any effect on the ability of higher concentrations of O$_2^-$ to impair autoregulation of CBF (AI, 0.65±0.07 and 0.72±0.05 respectively; n=6). O$_2^-$ also increased the activity of K$_{Ca}$ channels in cerebral smooth muscle cells (VSMCs; n=8).

Conclusion—These results suggest that O$_2^-$ increases basal CBF and impairs autoregulation of CBF, likely through the activation of K$_{Ca}$ channels in cerebral VSMCs. (Stroke. 2005;36:2589-2594.)

Key Words: free radicals • cerebral blood flow

Considerable evidence suggests that superoxide anion (O$_2^-$) has direct and indirect effects on vascular tone. O$_2^-$ increases the basal tone of vessels by reducing the availability of NO in a variety of tissues.1–3 However, recent studies suggest that O$_2^-$ can also dilate vessels by directly activating potassium (K$_{Ca}$) channels in vascular smooth muscle cells (VSMCs).4–6 As in other vascular beds, cerebral blood flow (CBF) is well autoregulated and remains constant despite fluctuations in arterial blood pressure. The importance of autoregulation of blood flow in cerebral circulation is greater than that in the peripheral vasculature because neuronal activity and viability is highly dependent on constant delivery of oxygen and glucose. In addition, increases in vascular resistance after elevations in blood pressure are required to protect the cerebral microcirculation from elevations of capillary hydrostatic pressure and overperfusion. Impaired autoregulation of CBF significantly contributes to disturbances of Frank-Starling forces and vasogenic brain edema, suggesting the importance of this mechanism in physiological and pathological conditions.7 The exact mechanism of CBF autoregulation remains elusive.8 Two major mechanisms, myogenic and metabolic, have been proposed, but it is unclear which plays a dominant role. Abnormalities in autoregulation of CBF have been reported after cerebral ischemia/reperfusion or traumatic brain injury, resulting in a shift of the autoregulatory curve toward higher pressures.9,10 Pretreatment of animals with antioxidants and scavengers of reactive oxygen species (ROS) have been reported to prevent the impairment in autoregulation of CBF and suggest that ROS may be involved.11,12 However, the influence of O$_2^-$ on autoregulation of CBF in the intact animal has not been studied directly. Thus, the present study examines the effect of various concentration of O$_2^-$ on autoregulation of CBF produced by superfusion of the brain of rats with artificial cerebrospinal fluid (aCSF) containing an O$_2^-$-generating system.

Materials and Methods

Experiments were performed on 8- to 10-week-old male Sprague-Dawley rats purchased from Harlan Laboratories (Indianapolis, Ind). The rats were housed in the animal facility at the Medical College of Wisconsin, which has been approved by the American Association for Laboratory Animal Care. The institutional animal care committee approved all protocols involving animals.
Surgical Preparation

Anesthesia was induced by using 4.5% isoflurane and maintained at 2.0% using a gas anesthesia mask for small rodents (model 51610; Stoelting). The femoral vessels were cannulated with polyurethane (PE, 50) tubing (Intramedic; Clay Adams) for intravenous infusion and measurement of arterial pressure. Body temperature was maintained at 37°C using a thermostatically regulated heated surgical table. The head was fixed in a stereotaxic apparatus (model 900; David Kopf), and the closed cranial window was made in the right parietal bone using a low-speed drill as described previously. Two small holes were drilled on the opposite sides of the cranial window, and PE-10 tubing (pulled to a diameter of $\approx$100 to 200 $\mu$m) was inserted under the dura and advanced to the border of the cranial window for subdural perfusion. The subdural space was perfused continuously with aCSF at a rate of 3 $\mu$L/min and inflow pressure averaged between 5 and 8 mm Hg. Composition of the aCSF was (in mmol/L): 2.9 KCl, 38 MgCl$_2$, 1.99 CaCl$_2$, 131.9 NaCl, 19 NaHCO$_3$, 6.63 urea, and 3.69 glucose, with pH adjusted daily to 7.4. After surgery, the isoflurane anesthesia was withdrawn, and anesthesia was maintained by continuous intravenous infusion of $\alpha$-chloralose (40 mg/kg; Sigma). Cortical microvascular blood flow was monitored with a laser Doppler flowmeter (PF3; Perimed) and a flow probe (PF316; Perimed).

Experimental Protocol

After surgery and a 30-minute equilibration period, CBF was monitored as arterial pressure was increased by increasing the rate of intravenous infusion of phenylephrine from 10 $\mu$g/mL per minute to 30 $\mu$g/mL per minute. Steady-state cortical blood flow was recorded over a 3-minute period after reaching a stable level of mean arterial pressure (MAP). After a control autoregulation curve was obtained, the infusion of phenylephrine was terminated and the subdural perfusion was switched to aCSF containing the $O_2^-$-generating system. After a 15-minute equilibration period, the experimental relationship between CBF and MAP was re-evaluated.

Autoregulation of CBF was also tested under the condition in which basal CBF was increased. In these experiments, rats were intubated, ventilated, and normobaric normocapnic hypoxia was used to increase CBF. Hypoxic conditions were achieved by decreasing oxygen concentration in the inspired mixture from 21% to 10% and confirmed by blood gas analysis in the arterial blood samples (0.07 mL; Stat Profile; Nova Biomedical). After 15 minutes of hypoxia, during which the increase of basal CBF stabilized, autoregulation of CBF was determined by increasing blood pressure with phenylephrine infusion.

Additional experiments were performed to determine the role of NO in mediating the effects of $O_2^-$, in these experiments, animals were pretreated by subdural perfusion of aCSF containing 50 $\mu$L/L of N (omega)-nitro-L-arginine methyl ester (l-NAME) at a rate 3 $\mu$L/min for 45 minutes before perfusion with an $O_2^-$-generating system.

Generation of $O_2^-$

$O_2^-$ was produced using a xanthine (0.2 mmol/L)/xanthine oxidase (XO; 0.2, 2, 3, and 20 mM/L) reaction. Components of generating system were dissolved in aCSF, and catalase (250 U/L) was added to prevent accumulation of hydrogen peroxide. The steady-state concentration of $O_2^-$ generated by this system was measured, in vitro, by the reduction of ferricytochrome C (25 $\mu$mol/L horse heart type III; Sigma) in the presence and absence of superoxide dismutase (SOD; 150 U/mL; Sigma). Reduction of ferricytochrome C was determined spectrophotometrically at 550 nm (Beckman DU-640), and the concentration of $O_2^-$ was calculated using a molar extinction coefficient ($\varepsilon$) of 21 000.$^{13}$

Dihydroethidine Staining

Dihydroethidine (DHE) was used to determine the distribution of $O_2^-$ in the brain after superfusion with the generating system. In response to $O_2^-$, DHE is oxidized intracellularly to the fluorescent product ethidium bromide (EB$^+$), which is membrane impermeable and trapped within the cell by intercalation with DNA.$^{14,15}$ In these experiments, the brains of rats were superfused with aCSF containing the $O_2^-$-generating system for 15 minutes followed by superfusion of the brain with aCSF containing DHE (3 $\mu$L/L) at a rate of 3 $\mu$L/min for 10 minutes. The brain was then quickly removed and frozen in liquid nitrogen. Frozen brains were sectioned on a cryostat, mounted, cover slipped, and examined using a Nikon E-600 microscope equipped with epifluorescence.

Measurement of NO Synthesis

NO synthase (NOS) activity in the brain was determined by measuring the conversion of L-[15$^3$H]-arginine to L-citrulline using reverse-phase high-performance liquid chromatography equipped with a radioactive flow detector as described previously.$^{16,17}$ Comparisons of NOS activity were made between rats treated with l-NAME (50 $\mu$L/L) and vehicle (aCSF).

Patch-Clamp Experiments

Vascular muscle cells were freshly isolated from middle cerebral arteries of rats as described previously.$^{18}$ Membrane currents were recorded at room temperature using a GeneClamp 500 amplifier (Axon Instruments) and a Digidata 1200A analog-to-digital converter (Axon Instruments). Currents were recorded at 10-kHz bandwidth with a low-pass Bessel filter at 1 kHz. Data were analyzed using pCLAMP 6.0 software (Axon Instruments). Patch pipettes were pulled from borosilicate glass (No. 7052; Garner Glass) by use of a vertical puller (Narashige) and gently heat polished using a microforge (model MF-83, Narashige). The indifferent electrode was an Ag-AgCl plug connected to a bath chamber via a 140-mmole/L agar bridge. The effect of $O_2^-$ on K$_{Ca}$ channel current activity was examined using the cell-attached configuration and symmetrical K$^+$ solutions (145 mmole/L). K$_{Ca}$ channel current activity was compared in the presence and absence of $O_2^-$ generated by adding xanthine and XO to the bath solution (final concentration 0.2 mmole/L xanthine and 20 mU XO).

Statistics

Mean values±SE are presented. Changes in baseline CBF are expressed as the percent change from control values. The autoregulatory capacity of cerebral vessels was expressed as an autoregulation index (AI, ΔCBF/ΔBP). Differences between groups were analyzed using ANOVA with Holm–Sidak post hoc test. A P value <0.05 was considered to be statistically significant.

Results

During the control period, CBF was well autoregulated and increased by 11±4% when MAP was increased from 98±3 to 140±6 mm Hg. The AI averaged 0.25±0.02 (Figure 1A and 1B). Subdural perfusion of the brain with the lowest concentration of $O_2^-$ (0.2 mmole/L xanthine and 0.2 mU XO) studied had no significant effect on basal CBF (Figure 2) or autoregulation of CBF (data not shown). A slightly higher concentration of $O_2^-$ decreased basal CBF by 10±1.6% (Figure 2) but had no significant effect on autoregulation of CBF (AI averaged 0.19±0.02). Concentration of $O_2^-$, produced by superfusion of the brain with 0.2 mmole/L xanthine and 3.0 or 20 mU XO, increased basal CBF by 30±2 and 42±4%, respectively (Figure 2; n=6), and significantly impaired autoregulation of CBF. The AI averaged 0.55±0.03 and 0.76±0.02, respectively (Figure 1B). Addition of SOD (150 U) to the superfusate prevented the effect of $O_2^-$ on baseline flow and autoregulation of CBF (AI, 0.28±0.05; n=6; Figure 1B).

Because $O_2^-$ significantly increased basal CBF, we determined autoregulation of CBF, in which a similar increase in basal CBF was induced by hypoxia. Hypoxic conditions were
confirmed by blood gas analysis (control pH 7.41 ± 0.01, Po2 98.5 ± 4.4, Pco2 33 ± 0.03; hypoxia pH 7.44 ± 0.01, Po2 40.4 ± 4, Pco2 29 ± 0.02). Hypoxia increased basal CBF by 38 ± 7%; however, increasing blood pressure under hypoxic conditions resulted in no further increase in CBF (control AI, 0.11 ± 0.01; hypoxia AI, 0.08 ± 0.03; n = 6; Figure 3A and 3B).

Other experiments were performed to determine whether blocking the synthesis of NO affects the ability of O2•− to impair autoregulation of CBF. NOS activity in the brain was inhibited by perfusion of the subdural space with aCSF containing the nonselective inhibitor L-NAME (50 μmol/L). The ability of L-NAME to inhibit NOS activity was confirmed by an 88 ± 7% reduction of the conversion of L-[14C]-arginine to L-citrulline in the homogenized brain tissue (data not shown). Superfusion of the brain with L-NAME decreased basal CBF by 18 ± 2% (n = 6; data not shown) and blocked the effect of the low concentration of O2•− (0.2 mmol/L xanthine and 0.2 μU XO) on basal CBF. In addition, L-NAME significantly enhanced autoregulation of CBF compared with control conditions (AI, 0.13 ± 0.01; Figure 4B). In contrast, L-NAME did not alter the ability of a higher concentration of O2•− to increase basal CBF and impair autoregulation. In animals pretreated with L-NAME, higher concentrations of O2•− (0.2 mmol/L xanthine and 20 μU XO) still raised basal

Figure 1. A, Relationship between CBF and blood pressure. MAP was increased by graded intravenous infusion of phenylephrine. CBF is expressed as percentage change from control. Mean values ± SE from 6 rats per group are presented; *P < 0.05 vs control (aCSF). ▲ indicates aCSF; △, aCSF containing 0.2 mmol/L xanthine (X), 3.0 μU XO; *, aCSF containing 0.2 mmol/L X, 20 μU XO; ◇, aCSF containing 0.2 mmol/L X, 20 μU XO, and 150 U/L SOD. B, Effects of subdural perfusion of the brain with aCSF containing 0.2 mmol/L X, different concentrations of XO on AI. Values are mean ± SE obtained from 6 rats per group; *P < 0.05 vs control (aCSF).

Figure 2. Effects of subdural perfusion of aCSF containing 0.2 mmol/L xanthine (X) or 0.2 mmol/L X+XO (0.2, 2.0, 3.0, and 20 μU) on basal CBF. CBF is expressed as a percentage change from control flow. Values are mean ± SE from 6 rats; *P < 0.05 vs control (aCSF).
CBF by 63±6% and impaired autoregulation of CBF (AI, 0.65±0.07; n=6; Figure 4A and 4B).

To determine the role of hydroxy (OH') radical in mediating the effects of O₂' on basal CBF and autoregulation, we examined the effect of adding the iron chelator deferoxamine (DFX) to the O₂'-generating system. The effects of O₂' on basal CBF and autoregulation were not altered by addition of 1 mmol/L of DFX to perfusate solution. Under these conditions, basal CBF increased by 39±9%, and the AI averaged 0.72±0.05 (n=4; data not shown).

Measurement of O₂' Production
The steady-state concentration of O₂' produced by the generating system was measured in vitro by the reduction of cytochrome C. The results indicate that the production of O₂' from the generating system was well correlated with the concentration of XO (data not shown).

DHE Staining
The results of these experiments are presented in Figure 5. Under control conditions (aCSF perfusion), there is some EB' fluorescence, which most likely represents endogenously produced O₂' (Figure 5A). Superfusion of the brain with low concentration of O₂' had no effect on the intensity of EB' staining (Figure 5B), suggesting that at this concentration, O₂' most likely remains in the extracellular space. In contrast, superfusion of the brain with a higher concentration of O₂' markedly increased the fluorescence intensity (Figure 5C). This response was abolished by the addition of cell-impermeable SOD to the perfusate solution (Figure 5D), suggesting that the observed changes were attributable to diffusion of extracellularly generated O₂'. Figure 5E and 5F depict the cerebral arterioles stained with DHE after exposure to low (Figure 5E) and high (Figure 5F) concentration of O₂'.

Electrophysiology
The effect of O₂' on KCa channel current activity in cerebral smooth muscle cells was examined using a cell-attached configuration of the patch-clamp technique. Comparisons of the current activity were made before and after addition of the O₂'-generating system to the bath. The addition of the O₂'-generating system to the bath solution significantly increased KCa channel current activity (Figure 6A). The mean open state probability of the channel increased up to 5-fold (Figure 6B). The opening frequency of the channel was not affected by an inactive generating system (boiled XO).

Discussion
Considerable evidence supports a vasoactive role for O₂'. Previous studies suggest that elevated levels of O₂' dilate cerebral arterioles. In the present study, we confirmed that superfusion of the rat brain with an O₂'-generating system that raised intracellular levels of O₂' increased baseline cortical blood flow and impaired autoregulation of CBF. In contrast, lower concentrations of O₂' that did not change
intracellular levels of $O_2^{\cdot -}$ decreased baseline CBF and had no significant effect on autoregulation of CBF. The ability of low concentrations of $O_2^{\cdot -}$ to reduce CBF appears to be related to the scavenging of extracellular NO because this response was completely blocked by the L-NAME pretreatment. The ability of higher $O_2^{\cdot -}$ concentrations to increase baseline CBF and impair autoregulation of CBF is independent of NO because these responses were not affected by the inhibition of NOS activity. The increase in baseline CBF and impairment of autoregulation of CBF were prevented by the addition of SOD, indicating that $O_2^{\cdot -}$ likely mediates these effects. However, these effects may be mediated by secondary reactive species such as hydrogen peroxide or hydroxyl radicals. The inclusion of catalase in the perfusion solution prevented the formation of hydrogen peroxide in the present experiment and therefore excludes hydrogen peroxide as the

Figure 5. Coronal section of rat cortex stained with 3 $\mu$mol/L DHE after exposure to the $O_2^{\cdot -}$-generating system (details in the Methods section). Pictures were taken with a digital camera connected to a Nikon E-600 microscope equipped with epifluorescence. A, aCSF. B, aCSF containing 0.2 mmol/L xanthine (X), 2.0 mU XO, and 250 U/L catalase. C, aCSF containing 0.2 mmol/L X, 20 mU XO, and 250 U/L catalase. D, aCSF containing 0.2 mmol/L X, 20 mU/L XO, 250 U/L catalase, and 150 U/L SOD. E and F, Cerebral vessel exposed (in situ) to low and high concentration of $O_2^{\cdot -}$.

Figure 6. Effect of $O_2^{\cdot -}$ on single $K_{Ca}$ channel current activity from freshly isolated cerebral VSMCs in the presence and absence of $O_2^{\cdot -}$. A, Representative single-channel tracing in cell attached configuration with symmetrical K$^+$ (145 mmol/L KCl) solution (n=8). B, Mean open state probability of $K_{Ca}$ channels from cerebral VSMCs (n=8) in the presence and absence of $O_2^{\cdot -}$; Student $t$ test; *P<0.05 from control. PP indicates patch potential; O, open state of channel; C, closed state of channel.
mediator of the effects. In addition, responses of baseline CBF and autoregulation to higher concentration of O$_2$$^{-}$ were also not altered by the addition of DFX to the perfusate solution, suggesting that these effects are not likely to be mediated by OH$^\cdot$ radicals.

The opposite effects of different concentrations of O$_2$$^{-}$ on CBF in our experiments are very likely attributable to the presence of O$_2$$^{-}$ in different tissue compartments. By crossing the cell membrane, O$_2$$^{-}$ is capable of reacting and modifying a number of transmembrane and intracellular proteins that affect cellular responses. In this regard, recent studies have indicated that KCa channels play an important role in the regulation of basal CBF and autoregulation of CBF. Furthermore, the $\beta$-regulatory subunit of this channel is highly redox sensitive, making it an attractive target for modulation by ROS. This led us to examine the effects of O$_2$$^{-}$ on the activity of KCa channels in cerebral VSMCs. Our results indicate that O$_2$$^{-}$ markedly increases the open probability of KCa channels. This would be expected to hyperpolarize VSMCs and reduce the activity of voltage-gated Ca$^{2+}$ channels, which are crucial for influx of Ca$^{2+}$ and the maintenance of vascular tone. The opposite effects of different concentrations of O$_2$$^{-}$ on KCa channels activity in vitro are also consistent with our observation that superfusion of the brain with the higher concentration of O$_2$$^{-}$ increases basal CBF and impaired autoregulation of CBF.

In conclusion, the results of the present study indicate that the effects of O$_2$$^{-}$ on basal CBF and autoregulation are concentration dependent. Increased amounts of O$_2$$^{-}$ in the extracellular space decreased basal CBF, likely by scavenging NO. In contrast, superfusion of the brain with higher concentrations of O$_2$$^{-}$ that also increased intracellular levels of O$_2$$^{-}$ in the brain dilated cerebral arteries and impaired autoregulation of CBF by the mechanism that might involve activation of KCa channels in VSMCs.

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