Prolonged Exposure to Oxyhemoglobin Modifies the Response of Isolated Dog Middle Cerebral Arteries to Vasoactive Substances

Hisashi Onoue, MD, Norio Nakamura, MD, PhD, and Noboru Toda, MD, PhD

We exposed helical strips of dog middle cerebral arteries to oxyhemoglobin for 5 hours, rinsed them with bathing medium, and stored them overnight; we compared the responses of strips thus treated with the responses of strips without oxyhemoglobin treatment. Relaxation induced by nicotine was abolished by hexamethonium and was markedly inhibited after exposure to oxyhemoglobin. A low concentration of KCl (5 mM) elicited relaxation that was abolished by ouabain and significantly reduced by oxyhemoglobin. Endothelium-dependent relaxation caused by calcium ionophore A23187 was attenuated, and that caused by substance P was reversed to contraction after exposure to oxyhemoglobin. Contraction elicited by substance P also depended on endothelium and was abolished by indomethacin. Relaxation induced by TRK-100, a stable analogue of prostaglandin I₂, was moderately attenuated by oxyhemoglobin. On the other hand, concentration-dependent relaxation induced by papaverine and contractile responses to KCl, serotonin, and prostaglandin F₂α were not affected by oxyhemoglobin. Our results indicate that vasodilations mediated by vasodilator nerves, the electrogenic sodium pump, endothelium-derived relaxing factor, and prostaglandin I₂ were impaired in dog cerebral arteries exposed to oxyhemoglobin. After exposure to oxyhemoglobin, vascular endothelium appears to participate in cerebroarterial contraction via a release of vasoconstrictor prostaglandins. These actions of oxyhemoglobin may be involved in the genesis of cerebral vasospasm after subarachnoid hemorrhage. (Stroke 1989;20:657–663)

Thus, it seems worthwhile to systematically evaluate the effect of oxyHb on contraction and relaxation responses of cerebral arteries. Articles published so far include results relating to the acute effects of erythrocyte breakdown products.5–9 After subarachnoid hemorrhage, cerebral arteries are surrounded by blood constituents for a long time, until the spasm is evoked. Therefore, our study was undertaken to clarify the modification by prolonged exposure to oxyHb of cerebroarterial responses to vasoconstrictor and vasodilator substances with different mechanisms of action.

Materials and Methods

Sixty mongrel dogs of either sex weighing 8–15 kg were anesthetized with 50 mg/kg i.p. thiopental sodium and killed by bleeding from the common carotid arteries. The brains were rapidly removed, and the left and right middle cerebral artery trunks were isolated from the brains. The arteries were cut into helical strips approximately 20 mm long, and the strips were fixed vertically between hooks in a muscle bath containing a modified Locke-Ringer solution of the following millimolar composition: NaCl 120, KCl 5.5, CaCl₂ 2.2, MgCl₂ 1.0, NaHCO₃ 26.0, glucose 11.1, and EDTA 0.02 (pH 7.4).
FIGURE 1. Mean±SEM contractile response of dog middle cerebral artery strips to KCl at Day 1 (●) and Day 2 (○) in strips not treated with oxyhemoglobin. Contraction induced by 30 mM KCl at Day 1 was taken as 100%; mean absolute value was 1224±64 mg (n=8). *p<0.05 different from Day 1.

25.0, and dextrose 5.6. This bathing medium was maintained at 37±0.3°C and was aerated with a mixture of 95% O2 and 5% CO2. The hook anchoring the upper end of each strip was connected to the lever of a force-displacement transducer (Nihon-kohden Kogyo Co., Tokyo, Japan). The resting tension was adjusted to 1.5 g, which is optimal for inducing maximal contractions. Before the start of the experiments, all strips were allowed to equilibrate for 90–120 minutes in the bathing medium, during which time the medium was replaced every 10–15 minutes.

Isometric contractions and relaxations of the strips were displayed on an ink-writing oscillograph (Sanei Sokki Co., Tokyo, Japan). Contractions to 30 mM K+ were obtained first, and the strips were repeatedly washed in bathing medium and equilibrated again. The concentration–response relation of the strips for vasoactive agents was obtained by adding the agents directly to the bathing medium. Vasoactive agents except nicotine, substance P, and 5 mM KCl were added in a cumulative manner. Vasodilator agents were added after the strips had been partially contracted with prostaglandin (PG) F2α, and at the end 10⁻⁴ M papaverine was added to obtain maximal relaxation.

After responses to the vasoactive agents were determined to be similar in a pair of strips obtained from the same dog, one strip, the experimental strip, was exposed to a nutrient solution containing 1.6x10⁻⁴ M oxyHb for 5 hours, and the other strip, used as a control, was left untreated in the bathing medium. After 5 hours, both strips were repeatedly washed and stored in the bathing medium at 4°C overnight. On Day 2, the strips were fixed between hooks in fresh bathing medium at 37°C as described above and responses to vasoactive agents used at Day 1 were obtained. The response to 10⁻⁷ M calcium ionophore A23187 was obtained only at Day 2 because the response was markedly suppressed after the second trial.

FIGURE 2. Mean±SEM contractile response of dog middle cerebral artery strips to KCl (left), serotonin (middle), and prostaglandin F2α (right) at Day 2 in control (●) and experimental (○) strips. Contraction induced by 30 mM KCl at Day 1 in respective strips was taken as 100%; mean absolute values were 1008±64 mg (n=8) for control and 1114±124 mg (n=8) for experimental strips.
Oxyhemoglobin and Cerebroarterial Response

Results

In helical strips of dog middle cerebral arteries, 10–50 mM KCl, 10^{-5} to 2 \times 10^{-6} M serotonin and 2 \times 10^{-8} to 10^{-5} M PGF_{2\alpha} produced concentration-dependent contraction. Concentration–response curves for KCl obtained at Days 1 and 2 in the control strips are compared in Figure 1. Contractions induced by KCl concentrations of up to 30 mM did not differ between days; therefore, agonist-induced contractions at Day 2 in control and experimental strips are presented as values relative to the respective response to 30 mM KCl at Day 1. Contractions caused by KCl and serotonin tended to be attenuated by exposure to oxyHb (Figure 2, left and middle); however, the difference between control and experimental strips was not significant. PGF_{2\alpha} induced contractions were quite similar in control and experimental strips (Figure 2, right).
Control day 1 day 2
Experimental day 1 day 2

**FIGURE 5.** Mean±SEM relaxation response to 5 mM KCl in dog middle cerebral artery strips partially contracted with prostaglandin F$_{2\alpha}$ modified by exposure to oxyhemoglobin. Relaxations induced by 10$^{-4}$ M papaverine were taken as 100%; mean absolute values at Days 1 and 2 in control strips were 241±39 mg (n=14) and 204±30 mg (n=14) and those in experimental strips were 269±30 mg (n=14) and 194±19 mg (n=14), respectively.

In strips partially contracted with PGF$_{2\alpha}$, 10$^{-4}$ M nicotine produced a transient relaxation that was abolished by treatment with 10$^{-5}$ M hexamethonium (n=4). The relaxation was significantly inhibited after exposure to oxyHb (Day 2 in experimental strips) compared with the response at Day 1 (Figure 3). On the other hand, relaxations at Days 1 and 2 in the control strips did not differ significantly.

TRK-100 (10$^{-8}$ to 10$^{-6}$ M), a stable analogue of PGI$_2$, relaxed dog middle cerebral artery strips in a dose-dependent manner. Treatment with oxyHb significantly attenuated the response (Figure 4, right), despite the fact that the relaxations at Days 1 and 2 in the control strips did not differ significantly.

In strips partially contracted with PGF$_{2\alpha}$, the addition of 5 mM KCl caused a relaxation that was abolished or reversed to a contraction by treatment with 10$^{-6}$ M ouabain (n=5). The relaxation was significantly reduced, by >50%, after exposure to oxyHb (Figure 5).

Relaxations caused by 10$^{-7}$ M substance P were markedly suppressed by removal of the endothelium, and the remaining relaxations were abolished by treatment with 10$^{-6}$ M indomethacin. Relaxations were significantly reduced at Day 2 in both control and experimental strips (Figure 6), but inhibition in the experimental strips was significantly greater than that in the control strips (88.6±2.2% vs. 64.7±3.6%, p<0.001).

Relaxations caused by 10$^{-7}$ M calcium ionophore A23187 were not reproducible; therefore, the responses were compared at Day 2 in the control and experimental strips. Magnitudes of the relaxation were significantly smaller in experimental than in control strips; the mean values were 6.6±1.8% and 19.7±3.1%, respectively (n=14, p<0.05).

Papaverine (5×10$^{-8}$ to 10$^{-4}$ M) elicited concentration-dependent relaxations that did not differ at Days 1 and 2 in the control and experimental strips (n=8); EC$_{50}$ values at Day 2 were 1.76±0.25×10$^{-6}$ M and 1.56±0.35×10$^{-6}$ M, respectively.

Since the relaxation caused by substance P was considerably reduced at Day 2 compared with that at Day 1 in the control strips, reproducibility of the responses was evaluated in control and experimental strips.

Relaxations caused by 10$^{-7}$ M substance P were first obtained (C$_0$ in Figure 7, upper panel). After...
repeated washings, experimental strips were exposed to 1.6×10^{-4} \text{ M } \text{oxyHb} \text{ for 1 hour and extensively washed again, and responses to substance P were then obtained (H_1). Experimental strips were then exposed to oxyHb for 2 hours; the response to substance P is shown as H_1. Responses to substance P after exposure for 2 more hours to oxyHb are demonstrated as H_3. Exposure to oxyHb suppressed the substance P–induced relaxation at H_1 and reversed the relaxation to a contraction at H_3 and H_5, which were compared with the responses obtained in respective control strips (C_1, C_3, and C_5). Substance P–induced contractions in experimental strips were reversed to relaxations by treatment with 10^{-6} \text{ M } \text{indomethacin} (n=7). On the other hand, relaxations induced by substance P in control strips were not influenced by indomethacin (n=8). The relaxations obtained after treatment with indomethacin were significantly smaller at H_3 (15.0±3.8%) than at C_3 (41.2±6.1%). Typical recordings of the response are presented in Figure 8. Removal of endothelium by rubbing the intimal surface of experimental strips with a cotton pellet abolished the contractions caused by substance P seen in intact strips (n=5).

Changes in the relaxation caused by 5 mM KCl were also examined under the same experimental condition (Figure 7, lower panel). Relaxation at H_3 was significantly inhibited, whereas other responses (C_0–C_3 and C_0–H_3) did not differ.

Discussion
Contractions caused by KCl, serotonin, and PGF_{2α} in dog middle cerebral artery strips were not affected by exposure to oxyHb-containing solution, despite the fact that relaxations caused by a PGI_{2} analogue and agents releasing endothelium-derived relaxing factor (EDRF) were suppressed. EDRF is released

**FIGURE 7.** Mean±SEM responses to 10^{-7} \text{ M } \text{substance P} (upper panel) and 5 mM KCl (lower panel) in dog middle cerebral artery strips partially contracted with prostaglandin F_{2α} after exposure to oxyhemoglobin. Relaxations induced by 10^{-4} \text{ M } \text{papaverine} and contractions by 30 mM KCl were taken as 100% of relaxation and contraction, respectively. *p<0.001, *p<0.02 (multiple comparison test after repeated-measures analysis of variance) different from C_0 in experimental strips.
spontaneously\textsuperscript{5,15,16} and by serotonin,\textsuperscript{17,18} whereas PGI\textsubscript{2} is released by PGF\textsubscript{2α}.\textsuperscript{19} These vasodilator agents may significantly interact with the vasoconstrictor actions of serotonin and PGF\textsubscript{2α}. Therefore, the unaltered contractile response in oxyHb-treated artery strips would be explained by some reduction of arterial contractility.

The nicotine-induced relaxation was markedly inhibited in dog middle cerebral artery strips exposed to oxyHb. The chemical stimulation by nicotine of vasodilator nerves in cerebral arteries of humans, monkeys, dogs, cats, rabbits, and sheep elicits a relaxation\textsuperscript{20-24}; however, the transmitter substance has not been identified. On the other hand, nicotine-induced contractions mediated by release of norepinephrine from adrenergic nerve terminals in dog mesenteric arteries\textsuperscript{22} were not affected by treatment with oxyHb for 5 hours (unpublished data), suggesting that the inhibition of cerebroarterial relaxation by oxyHb is associated with neither an interference with nicotinic receptor function and a blockade of neuromuscular transmission nor a potentiation of contractile responses. OxyHb inhibits guanylate cyclase activity stimulated by nitrovasodilators.\textsuperscript{26-28} Relaxations caused by nicotine in dog cerebral arteries are abolished by treatment with methylene blue,\textsuperscript{8} a guanylate cyclase inhibitor.\textsuperscript{29} Therefore, inhibitions of the nicotine-induced cerebroarterial relaxation by exposure to oxyHb may result from decreased cyclic guanosine monophosphate (cGMP) production, as postulated in the bovine retractor penis muscle in response to dilator nerve stimulation.\textsuperscript{30}

Relaxations caused by 5 mM KCl were also attenuated by oxyHb. Activation of the electrogenic sodium pump is postulated to be involved in the relaxation induced by small amounts of K\textsuperscript{+} due to the fact that the relaxation is abolished by ouabain, by an increase in K\textsuperscript{+} in the bathing medium, and by substitution of Li\textsuperscript{+} for Na\textsuperscript{+}, is reduced by lowering the temperature,\textsuperscript{31,32} and is associated with hyperpolarization of smooth muscle cell membrane.\textsuperscript{33} Exposure to oxyHb-containing solution (for up to 3 hours) as well as hemolysate (containing 1.6×10\textsuperscript{-5} M hemoglobin)\textsuperscript{8} failed to inhibit K\textsuperscript{+}-induced relaxations. Therefore, prolonged exposure to oxyHb (for >5 hours) may inhibit the active extrusion of Na\textsuperscript{+} by inactivating membrane Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, by exhausting its substrate, etc.

Endothelium-dependent relaxations caused by calcium ionophore A23187 were inhibited during oxyHb exposure and after removal of oxyHb. The A23187-induced relaxation in dog cerebral arteries is supposed to be mediated by EDRF but not by cyclooxygenase products.\textsuperscript{34} It has been generally postulated that hemoglobin selectively inhibits the vasodilations associated with a rise in cGMP concentrations, including EDRF-mediated relaxations.\textsuperscript{3} Therefore, the inhibition by oxyHb of A23187-induced cerebroarterial relaxation may be related to an interference with production of cellular cGMP. However, inhibition of EDRF synthesis and release or inactivation of released EDRF cannot be excluded.

Substance P is also known to be an endothelium-dependent vasodilator.\textsuperscript{35} As shown in Figure 7 (upper panel), exposure to oxyHb reduced relaxations but potentiated contractions caused by substance P. The contractions were abolished by endothelium denudation and were reversed to relaxations by indomethacin, a cyclooxygenase inhibitor. Our previous study demonstrated that substance P elicits contractions in dog cerebral arteries, possibly by releasing vasoconstrictor prostaglandins such as PGF\textsubscript{2α}, PGE\textsubscript{2}, PGD\textsubscript{2}, and PGA\textsubscript{2} from endothelial cells\textsuperscript{14} and elicits relaxations via a mediation of EDRF and PGI\textsubscript{2} produced in smooth muscle cells. Relaxations caused by TRK-100 were also reduced by oxyHb. Therefore, it is suggested that substance P-induced relaxations in the arteries treated with
oxyHb are mainly due to the inhibition of relaxations caused by EDHF and PG1. However, possible involvement of increased production of vasoconstrictor prostaglandins in the endothelium and of decreased PG1 synthesis in the vascular wall stimulated by substance P are not disregarded. It has been reported that the synthesis of PGE2 in dog basilar artery increases, while that of PG1 decreases, after experimentally induced subarachnoid hemorrhage and that hemolysate reduces PGH2-induced relaxations in dog cerebral arteries, possibly due to inhibition of a conversion of the endoperoxide to PG1 in the vascular wall.

In summary, prolonged exposure to oxyHb interferes with the cerebral vasodilator interventions such as vasodilator nerves, electrogenic sodium pump activation, EDHF, and PG1. These antagonistic actions of oxyHb, together with its vasoconstrictor action, may be involved in the genesis of cerebral vasospasm after subarachnoid hemorrhage. Vasodilatory action of oxyHb, together with its vasoconstrictor action, may be involved in the genesis of cerebral vasospasm after subarachnoid hemorrhage.

**References**


**Key Words** • endothelium • oxyhemoglobin • vasodilation • dogs
Prolonged exposure to oxyhemoglobin modifies the response of isolated dog middle cerebral arteries to vasoactive substances.
H Onoue, N Nakamura and N Toda

Stroke. 1989;20:657-663
doi: 10.1161/01.STR.20.5.657

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/20/5/657

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/