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

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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.

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Prolonged cerebral vasospasm frequently threatens the life of patients with subarachnoid hemorrhage following ruptured cerebral aneurysms. Despite extensive, long efforts, the pathogenesis of cerebral vasospasm is still controversial. It has been generally accepted that constituents of erythrocytes and substances produced during hemolysis of subarachnoid blood clots are related to the subsequent arterial narrowing. Among the erythrocyte breakdown products, oxyhemoglobin (oxyHb) is considered to be a key substance in the genesis of cerebral vasospasm because of its vasoconstrictor activity in cerebral arteries and its inhibitory effect on endothelium-dependent vasodilations.

Since the vascular tone is controlled by vasoconstrictor and vasodilator interventions, cerebroarterial spasm is attributed possibly to an increased contractility and a decreased relaxation potential.

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. 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...
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. Vasoreactive 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) F₂₀ 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 1.6×10⁻⁴ 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.

Mean±SEM contractile response of dog middle cerebral artery strips to KCl (left), serotonin (middle), and prostaglandin F₂₀ (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.
Louis, Missouri) containing a mixture of oxyHb and methemoglobin was reduced by Na₂S₂O₄. After removal of the reducing agent by extensive dialysis against distilled water, the purity of the solutions of oxyHb was determined spectrophotometrically. Chemicals used were PGF₂α, tris-(hydroxymethyl)aminomethane salt (Nippon Upjohn Ltd., Tokyo, Japan), dog hemoglobin, indomethacin (Sigma), serotonin creatinine sulfate, ouabain octahydrate (E. Merck, Darmstadt, FRG), substance P (Protein Research Foundation, Osaka, Japan), ionophore A23187 (C.H. Boehringer Ingelheim Ltd., Elmsford, New York), TRK-100 (sodium (+)-4-[(1R,2R,3aS,8bS)-1,2,3a,8b-tetrahydro-2-hydroxy-1-[(3S,4RS)-3-hydroxy-4-methyl-oct-6-yne-(E)-1-enyl]-5-cyclopenta[b]benzofuranyl] butyrate, Toray-Kaken Pharmaceutical Co., Tokyo, Japan), papaverine hydrochloride (Dainippon Pharmaceutical Co., Osaka, Japan), and others (Nakarai Chemicals, Ltd., Kyoto, Japan).

Results
In helical strips of dog middle cerebral arteries, 10–50 mM KCl, 10⁻⁵ to 2×10⁻⁶ M serotonin and 2×10⁻⁸ to 10⁻⁵ M PGF₂α 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. PGF₂α-induced contractions were quite similar in control and experimental strips (Figure 2, right).

Figure 3. Mean±SEM relaxation response to 10⁻⁴ M nicotine in dog middle cerebral artery strips partially contracted with prostaglandin F₂α 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 274±25 mg (n=8) and 402±69 mg (n=8) and those in experimental strips were 316±35 mg (n=8) and 370±42 mg (n=8), respectively.

Results are expressed as mean±SEM and were analyzed using Student’s paired and unpaired t tests and the multiple comparison test after repeated-measures analysis of variance. OxyHb was prepared as described previously. Briefly, dog hemoglobin (Sigma Chemical Co., St.

Figure 4. Mean±SEM relaxation response to TRK-100 in dog middle cerebral artery strips partially contracted with prostaglandin F₂α modified by exposure to oxyhemoglobin. Relaxations induced by 10⁻⁴ M papaverine were taken as 100%; mean absolute values at Day 1 (●) and Day 2 (○) in control strips (left) were 285±27 mg (n=10) and 304±29 mg (n=10) and those in experimental strips (right) were 278±25 mg (n=10) and 343±32 mg (n=10), respectively. *p<0.001, #p<0.01 different from Day 1.
In strips partially contracted with PGF_2alpha, 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 (Figure 4, left).

In strips partially contracted with PGF_2alpha, 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} M oxyHb for 1 hour and extensively washed again, and responses to substance P were then obtained (H1). Experimental strips were then exposed to oxyHb for 2 hours; the response to substance P is shown as H2. Responses to substance P after exposure for 2 more hours to oxyHb are demonstrated as H3. Exposure to oxyHb suppressed the substance P-induced relaxation at H1 and reversed the relaxation to a contraction at H2 and H3, which were compared with the responses obtained in respective control strips (C1, C3, and C5). Substance P-induced contractions in experimental strips were reversed to relaxations by treatment with 10^{-6} M 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 H3 (15.0±3.8%) than at C3 (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 H3 was significantly inhibited, whereas other responses (C0–C3 and C0–H3) did not differ.

**Discussion**

Contractions caused by KCl, serotonin, and PGF_{2a} in dog middle cerebral artery strips were not affected by exposure to oxyHb-containing solution, despite the fact that relaxations caused by a PGI2 analogue and agents releasing endothelium-derived relaxing factor (EDRF) were suppressed. EDRF is released

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**FIGURE 7.** Mean±SEM responses to 10^{-7} M substance P (upper panel) and 5 mM KCl (lower panel) in dog middle cerebral artery strips partially contracted with prostaglandin F_{2a} after exposure to oxyhemoglobin. Relaxations induced by 10^{-4} M 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 C0 in experimental strips.
unaffected 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.26-28 Relaxations caused by nicotine in dog cerebral arteries are abolished by treatment with methylene blue,8 a guanylate cyclase inhibitor.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.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⁺ due to the fact that the relaxation is abolished by ouabain, by an increase in K⁺ in the bathing medium, and by substitution of Li⁺ for Na⁺, is reduced by lowering the temperature,31,32 and is associated with hyperpolarization of smooth muscle cell membrane.33 Exposure to oxyHb-containing solution (for up to 3 hours) as well as hemolysate (containing 1.6x10⁻⁵ M hemoglobin)4 failed to inhibit K⁺-induced relaxations. Therefore, prolonged exposure to oxyHb (for >5 hours) may inhibit the active extrusion of Na⁺ by inactivating membrane Na⁺,K⁺-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.34 It has been generally postulated that hemoglobin selectively inhibits the vasodilations associated with a rise in cGMP concentrations, including EDRF-mediated relaxations.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.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 PGF₂α, PGE₂, PGD₂, and PGA₂ from endothelial cells14 and elicits relaxations via a mediation of EDRF and PGI₂ produced in smooth muscle cells. Relaxations caused by TRK-100 were also reduced by oxyHb. Therefore, it is suggested that substance P-induced contractions in the arteries treated with...
oxyHb are mainly due to the inhibition of relaxations caused by EDRF and PGI2. However, possible involvement of increased production of vasconstricor prostaglandins in the endothelium and of decreased PGI2 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 PGI2 decreases, after experimentally induced subarachnoid hemorrhage and that hemolyse reduces PGH2-induced relaxations in dog cerebral arteries, possibly due to inhibition of a conversion of the endoperoxide to PGI2 in the vascular wall.

In summary, prolonged exposure to oxyHb interferes with the cerebral vasodilator interventions such as vasodilator nerves, electrogenic sodium pump activation, EDRF, and PGI2. These antagonistic actions of oxyHb, together with its vasconstrictor action, may be involved in the genesis of cerebral vasospasm after subarachnoid hemorrhage. Vascular endothelium appears to contribute to cerebroarterial contractions via a release of intrinsic vasoconstrictors.

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