Cerebral vasospasm: contractile activity of hemoglobin in isolated canine basilar arteries

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Recent studies suggest the possible role of the red blood cell (RBC) in causing chronic cerebral vasospasm. However, the basic action of hemoglobin (Hb), the major component of the RBC, on cerebral arteries remains unknown. The present study was undertaken to analyze the contractile effects of human Hb (purified by ion-exchange chromatography) on canine arteries in vitro. The contractile activity of lysed RBC was shown to be derived from Hb. Hemoglobin in oxygenated form (oxyHb) caused a maximum contraction equal to about 70% of that induced by serotonin in the basilar artery. Ferrous Hb's (oxyHb and carboxyHb) produced much greater contraction than ferric Hb's (methemoglobin and cyanmethemoglobin), suggesting that superoxide radicals, an active species of oxygen, may be related to the contractile activity of Hb. Neither methysergide, phentolamine, mepyramine, nor aspirin inhibited the vasoconstrictive activity of oxyHb. This finding indicates that the activation of serotonergic, alpha-adrenergic, or histaminergic H1 receptors, or prostaglandin synthesis may not be involved in the mechanism of action of oxyHb. The constituents of Hb caused little or no contraction as compared with Hb as a whole. The basilar artery was more highly sensitive to Hb than arteries from other anatomical locations. Cyclic adenosine monophosphate caused a very slight decrease in the Hb-induced contraction. It is concluded that oxyHb can contract cerebral arteries in vitro. These results, coupled with recent reports on the participation of the RBC in producing chronic vasospasm, strongly suggest that oxyHb released from RBC's plays an important role in the pathogenesis of chronic cerebral vasospasm.

KEY WORDS - vasospasm - subarachnoid hemorrhage - hemoglobin - oxyhemoglobin - red blood cell - superoxide radical

Cerebral vasospasm is a major cause of morbidity and mortality in patients with subarachnoid hemorrhage (SAH). Despite many intensive investigations in the past, the pathogenesis of this vasospasm has not yet been fully elucidated. It has been generally believed that some vasoactive substance in the extravasated blood might be closely associated with cerebral vasospasm. Among many vasoconstrictors, serotonin released from platelets has been most strongly suspected as a causative factor because of its potent contractile effect on the cerebral artery. However, serotonin may be subject to rapid degradation in cerebrospinal fluid (CSF) and lose its vasoconstrictive activity in the chronic phase of cerebral vasospasm. Serotonin is thus thought to contribute only to acute cerebral vasospasm.

Recent investigations have been focused on the red blood cell (RBC), especially in relation to prolonged or chronic cerebral vasospasm. Barrows, et al., found that the concentration of oxyhemoglobin (oxyHb) in CSF increased progressively during hemolysis and reached peak levels a few days after SAH. Saito, et al., reported that the onset of vasospasm was delayed for at least 4 days after the hemorrhage. These findings suggest that there may be a close time relationship between the increase of oxyHb in CSF and the development of cerebral vasospasm. On the other hand, no such substance showing a similar time course has been demonstrated. Furthermore, Osaka, and Ozaki and Mullan have recently reported that lysed RBC's have vasoconstrictive capacity, and that the breakdown product of the RBC may be the main factor of the chronic vasospasm. The RBC is known to be composed largely of hemoglobin (Hb). It is therefore reasonable to assume that Hb...
which is released from the RBC may manifest vasocostrictive activity in the chronic phase of cerebral vasospasm. Yet very little is known about the contractile activity of Hb.

The present study was designed: 1) to demonstrate the contractile activity of Hb; 2) to elucidate the basic pharmacological properties of Hb; and 3) to assess the role of Hb in the etiology of chronic vasospasm.

Materials and Methods

Preparation of Hemoglobin Solutions

Blood was obtained from normal adults by venipuncture and was put into tubes containing citrate (4 ml of 3.2% sodium citrate per 20 ml of blood). The following procedures were carried out at 4°C. The blood was centrifuged at 1500 G for 10 minutes, and the plasma was discarded. The cells were washed five times with three to four volumes of ice-cold 0.9% sodium chloride, and the buffy coat was removed. An equal volume of distilled water was added to the packed cells, and the mixture was agitated for 5 minutes. The lysed red cells were then centrifuged at 15,000 G for 20 minutes to remove stroma. The destomatized hemolysate was chromatographed on a Sephadex G-100 column (2.0 x 80 cm)* with 0.05 M Tris- HCl buffer (pH 8.5) to remove low-molecular substances.

Hemoglobin A0 (Hb-A0), the major component of Hb in normal adults, was isolated by a modification of the method of Winterhalter and Huehns.6 The Hb-containing fraction collected by the above procedures was applied to a DEAE-Sephadex A-50 column (1 x 65 cm) equilibrated with 0.05 M Tris- HCl buffer (pH 8.5). The chromatogram was developed with a linear pH gradient (pH 8.5-7.0). Hemoglobin-A0 was eluted between pH 7.8 and 7.7. The Hb-A0 fraction thus obtained was concentrated by adsorbing on a DEAE-Sephadex A-50 column (1 x 20 cm) and eluting with 0.05 M Tris- HCl buffer (pH 6.9) containing 0.5 M sodium chloride. Finally, the concentrated Hb-A0 solution was passed through a Sephadex G-25 column. Carboxyhemoglobin (CO-Hb) was prepared by bubbling CO gas through the Hb-A0 solution.

The concentrations of these Hb solutions were calculated with the equations of Benesch, et al.8 The total Hb concentration was determined by conversion to cyanmethHb using a molar extinction coefficient of 11.5 x 103 (per heme) at 540 nm. Measurements were made with a spectrophotometer.†

Starch gel electrophoresis was used to check the purity of the Hb-A0 solution. The K+ concentration in the Hb solution was determined using a flame photometer.‡ Globin was prepared from Hb by the method of Winterhalter and Huehns.58

Study of Contraction

Mongrel dogs of both sexes, weighing 10 to 15 kg, were anesthetized with intravenous sodium pentobarbital (30 mg/kg) and sacrificed by exsanguination from the femoral artery. The brain and heart were rapidly removed. The basilar artery and the ventral interventricular branch of the left coronary artery were dissected out. Distal portions of the mesenteric and femoral arteries were also removed. Ring segments, 4 mm in length, of the arteries were mounted between L-formed stainless steel holders8,18 in a 20-ml bath containing a nutrient solution that was maintained at 37 ± 0.5°C and saturated with 95% O2 and 5% CO2. The composition of the nutrient solution was (mM): NaCl, 120; KCl, 4.5; CaCl2, 2.5; MgSO4, 1.0; NaHCO3, 27.0; KH2PO4, 1.0; and glucose, 10.0. The pH of the solution ranged from 7.4 to 7.5.

Isometric tension was measured using a force-displacement transducer, an amplifier, and an ink-writing recorder.§ The resting tension was adjusted to 1.5 gm. A period of 60 to 90 minutes was allowed for stabilization of the arterial segments. Before each experiment, the response of the artery to 40 mM K+ was determined, and all agonist responses were expressed as a percentage of 40 mM K+ contraction in the respective artery. The total volume of each test agent added to the bath never exceeded 2 ml per test.

The following drugs were used: serotonin, protoporphyrin, hematin, methysergide hydrogen maleinate, phenolamine methanesulfonate, mephramine maleate, aspirin, and dibutylcyclic AMP. Preparations were exposed for 15 minutes to all of these blocking agents. In the present study, the concentration of each agent was expressed as its final molar concentration in the bath. Median effective concentrations (ED50) were determined by a graphical method. Results shown in the text and figures represent mean values ± standard error of the mean. The data were statistically evaluated by Student's t-test.

†Spectrophotometer, model 624, manufactured by Hitachi, Tokyo, Japan.
‡Flame photometer, model FLM 3, manufactured by Radiometer, Copenhagen, Denmark.

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FIG. 1. Representative recording of contraction induced by purified oxyhemoglobin (that is, hemoglobin Ao in fully oxygenated form) in the canine basilar artery.

Results

The elution curve obtained in the present study was essentially similar to that described by Huisman and Dozy. The HB-Ao fraction was electrophoretically pure. The Hbs, except for CO-Hb, metHb, and cyanmetHb, were proved to be in fully oxygenated form, namely, oxyHb, when added to the bath media. A flame photometric examination revealed that the destromatized hemolysate contained 20 to 30 mEq/liter of potassium, which was not detectable after passage through a Sephadex G-100 column. The K+ concentration increased in the media after the application of the destromatized hemolysate was less than 2 mEq/liter.

The cumulative addition of Hb-Ao at concentrations ranging from 10⁻⁸ M to 10⁻⁴ M caused a dose-related contraction in the isolated canine basilar artery (Fig. 1). The dose-response curve of Hb-Ao was attained at 10⁻⁴ M. The mean absolute value was 2556 ± 110 mg for 12 samples. It was clearly demonstrated that purified Hb, or Hb-Ao, caused contraction in the basilar artery.

Contractile activity was compared between the following three Hb solutions differing in purity: A) the destromatized hemolysate, which contained potassium, adenosine, vitamins, a variety of enzymes, and other substances as well as Hb; B) the destromatized hemolysate, which was obtained by removing low-molecular substances from the solution A through a Sephadex G-100 column; and C) the Hb-Ao fraction, which was regarded as pure Hb solution (Fig. 2). The maximum contractions induced by these three Hb solutions (A, B, and C) at 10⁻⁴ M Hb were 59.1 ± 2.8 (12 samples), and 66.1 ± 2.7% (12 samples), respectively. These values were not significantly different. The contractile activity of the destromatized hemolysate (solution A) was shown to be identical with that of pure Hb (solution C). This result indicates that the contractile activity of the destromatized hemolysate was due to Hb.

The contractile activity of Hb on the basilar artery was compared with that of serotonin (Fig. 3). The maximum contractions produced by 10⁻⁵ M serotonin and 10⁻⁴ M Hb were 94.2 ± 13.7 (10 samples) and 66.1 ± 2.7% (12 samples), respectively. Hemoglobin produced a maximum contraction equal to about 70% of that induced by serotonin. The median effective concentrations (ED₅₀) were (2.9 ± 0.5) × 10⁻⁷ M for serotonin and (3.8 ± 0.6) × 10⁻⁴ M for Hb.

Figure 4 illustrates the contractile responses of different arteries to Hb. Solution B was used as Hb in this experiment because it was identical with pure Hb in contractile activity, as shown in Fig. 2. The maximum contractions induced by 10⁻⁴ M Hb in the basilar, coronary, femoral, and mesenteric arteries were 59.1 ± 2.8 (12 samples), 17.3 ± 1.6 (nine samples), 2.9 ± 1.0 (12 samples), and 2.4 ± 0.4% (12 samples), respectively. The basilar artery was much more strongly contracted by Hb than arteries from other anatomical locations.
Figure 3. Dose-response curves of serotonin and purified hemoglobin (or Hb-Ao in oxy-form) in the canine basilar arteries. The ED$_{50}$ values were $(2.9 \pm 0.5) \times 10^{-7}$ M for serotonin and $(3.8 \pm 0.6) \times 10^{-6}$ M for hemoglobin.

Figure 4. Dose-response curves of hemoglobin in the canine basilar, coronary, femoral, and mesenteric arteries. Mean absolute values induced by 40 mM K$^+$ in the basilar, coronary, femoral, and mesenteric arteries were 3435 ± 139 (12 samples), 2467 ± 182 (nine samples), 4898 ± 202 (12 samples), and 4600 ± 212 mg (12 samples), respectively. Note that the basilar artery was more highly sensitive to hemoglobin than the other arteries.

Figure 5 presents the dose-response curves of different Hb derivatives. The maximum contractions produced by oxyHb, CO-Hb, metHb, and cyanmethHb at $10^{-4}$ M were 66.1 ± 2.7 (12 samples), 64.4 ± 3.6 (10 samples), 22.7 ± 2.6 (10 samples), and 20.7 ± 1.8% (12 samples), respectively. It was found that ferrous Hb's produced greater contractions than ferric Hb's.

The contractile activity of each constituent of Hb (that is, hematin, protoporphyrin, globin, ferrous chloride, and ferric chloride) was examined to elucidate which factors primarily contribute to the contractile activity of Hb as a whole. However, these constituents of Hb were proved to cause little or no contraction of the basilar artery (Fig. 6). Ferrous heme was not examined because it was impossible to prepare this substance by available techniques.

The average contractions produced by $10^{-4}$ M Hb after the application of $10^{-6}$ M methysergide (a serotonergic blocker), $10^{-6}$ M phentolamine (an adrenergic blocker), $10^{-6}$ M mepyramine (a histaminergic blocker), and $10^{-6}$ M aspirin (an inhibitor of prostaglandin synthesis) were 76.7 ± 7.6, 66.9 ± 4.6, 62.2 ± 10.9, and 58.5 ± 6.2%, respectively, for six samples each (Fig. 7). Compared with the control value, none of these blocking agents significantly inhibited the contractile activity of Hb. On the contrary, methysergide significantly potentiated the Hb-induced contraction (p < 0.05).
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Figure 6. Responses of the canine basilar arteries to globin, protoporphyrin, hematin, ferrous chloride, and ferric chloride. Concentrations indicated by the numbers 8 to 4 are $10^{-8}$ M to $10^{-4}$ M. These constituents caused little or no contraction as compared with hemoglobin as a whole.

Figure 8 shows the effect of cyclic adenosine monophosphate (AMP) on the Hb-induced contraction of the basilar artery. The contraction induced by $10^{-4}$ M Hb after treatment with cyclic AMP ($5 \times 10^{-5}$ gm/ml) was $47.9 \pm 3.6\%$ in seven samples. This value significantly differed from the control ($p < 0.05$). However, as can be seen from Fig. 8, the inhibitory effect of cyclic AMP was slight.

Discussion

The vasoconstrictive activity of lysed RBC's on the cerebral artery was confirmed in the present study. This property of lysed RBC's was attributed to Hb (specifically oxyHb) since no significant difference in contractile activity was found between hemolysate and purified Hb. Although the vasoconstrictive activity of purified Hb has not yet been described, the present study demonstrated that purified Hb produced a maximum contraction equal to about 70% of that induced by serotonin, which is known to contract the cerebral artery strongly. The technical limitations of currently available column chromatography precluded the preparation of Hb solutions at concentrations higher than $10^{-3}$ M (or $10^{-8}$ M in the bath). More sufficient evidence in favor of the vasoconstrictive activity of Hb can be obtained if this technical problem is settled.

Interestingly, Hb caused a more marked contraction in the cerebral artery than in the peripheral arteries, probably because of histological differences among these arteries. This finding supports the author's hypothesis that Hb may be a true factor in causing cerebral vasospasm.

![Image](https://example.com/image.png)
during the autoxidation of oxyHb to metHb, may participate in the contractile activity of oxyHb. Second, configurational changes may occur, probably depending upon different activities of various Hb's, during the autoxidation of oxyHb to metHb in such a manner as observed during the conversion of deoxyHb to oxyHb. The mechanism of action of oxyHb awaits further elucidation.

Fox reported that ferrous chloride caused marked vasodilatation, while ferric chloride induced mild but definite vasoconstriction. On the other hand, the author obtained the different finding that ferrous chloride, ferric chloride, and three other constituents of Hb exercised little or no influence on the tonus of the cerebral artery. This suggests that the contractile activity of Hb (specifically oxyHb) may be attributed to ferrous heme, which could not be examined in the present study.

Neither methysergide, phentolamine, mepyramine, nor aspirin inhibited the vasoconstrictive activity of Hb. These findings indicate that the activation of serotoninergic, alpha-adrenergic, or histaminergic H receptors, or prostaglandin synthesis may not be inhibited by aspirin. Based on this ability to inhibit Hb-induced contraction (p < 0.05). This finding may support the suggestion that methysergide also has vasocontractile activity.

It is known that the smooth muscle-relaxing effects of beta-adrenergic agents are related to increases in the level of cyclic AMP. Based on this ability to increase cyclic AMP in vascular smooth muscle, iso-proterenol, a beta-adrenergic agonist, has been used in the treatment of clinical vasospasm. On the other hand, some investigators have demonstrated that the cerebral artery was less sensitive to beta stimulators than peripheral arteries, suggesting that cyclic AMP may not be involved in the mechanism of relaxation of cerebral vascular smooth muscle. The present study also demonstrated that cyclic AMP very slightly inhibited Hb-induced contraction of the basilar artery, although a significant difference from the control sample was found. The therapeutic effect of cyclic AMP on cerebral vasospasm may therefore not be expected.

It has been reported that the oxyHb level in CSF from patients with SAH is gradually elevated and becomes maximal a few days after the hemorrhage. This finding may indicate that the progressive release of oxyHb from blood clot during the hemolytic process in the subarachnoid space coincides with the time course of chronic vasospasm, the onset of which is delayed for at least 4 days. Moreover, in recent studies, the RBC, which is composed largely of Hb, has been demonstrated to contribute to chronic vasospasm.

From these previous works, it seems reasonable to assume that oxyHb released from RBC's during hemolysis might be responsible for chronic vasospasm. Based on the above consideration, the author has analyzed the basic pharmacological action of purified human Hb on isolated canine arteries. It is concluded that oxyHb has a vasocontractile capacity in cerebral arteries. These data suggest that oxyHb may play an important role in the causation of chronic cerebral vasospasm. Further studies, especially in vivo experiments, are necessary to confirm the present findings.

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References


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