Selective hemoglobin inhibition of endothelium-dependent vasodilation of rabbit basilar artery


Department of Neurological Surgery, University of Virginia School of Medicine, Charlottesville, Virginia

The effect of hemoglobin on endothelium-dependent vasodilation of the isolated rabbit basilar artery was examined using an isometric tension recording method. Acetylcholine (ACh) (10^{-7}-10^{-4} M) evoked a dose-dependent vasodilation of isolated rabbit basilar artery previously contracted by 10^{-6} M serotonin. This vasodilating action disappeared after removal of the endothelium. The ACh-induced vasodilation of rabbit basilar artery is thought to be strictly endothelium-dependent. Hemoglobin (10^{-7}-10^{-5} M) inhibited this ACh-induced endothelium-dependent vasodilation conditional upon the dose. Adenosine triphosphate (ATP, 10^{-7}-10^{-4} M) also relaxed isolated rabbit basilar artery already contracted by 10^{-6} M serotonin. This vasodilating action was slightly inhibited by adenosine antagonist, 8-phenyltheophylline (8-PT), and markedly attenuated by removal of the endothelium. This ATP-induced vasodilation is thought to be composed of ATP itself (endothelium-dependent) and ATP degradation products (endothelium-independent) such as adenosine monophosphate or adenosine. Hemoglobin markedly inhibited ATP-induced vasodilation, but there still remained a small vasodilation, which was blocked by 8-PT. Papaverine-induced vasodilation was not affected by removal of the endothelium, and hemoglobin did not inhibit the papaverine-induced vasodilation. These results suggest that rabbit basilar artery has endothelium-dependent vasodilating mechanisms induced by ACh and ATP, and that hemoglobin selectively blocks the endothelium-dependent vasodilation. This finding may relate to the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage: there is a possibility that the presence of hemoglobin released from lysed erythrocytes inhibits the endothelium-dependent vasodilation of cerebral arteries; furthermore, the endothelial degeneration following subarachnoid hemorrhage may impair the vasodilating mechanisms of cerebral artery smooth-muscle cells.

KEY WORDS • hemoglobin • endothelium-dependent vasodilation • basilar artery • rabbit

Cerebral vasospasm has a grave influence on the prognosis of patients with subarachnoid hemorrhage (SAH) due to ruptured aneurysms. While a variety of hypotheses as to the pathogenesis of cerebral vasospasm have been developed from extensive clinical and experimental studies, the subject is still controversial. Recently, it has been considered that vasospasm is related to an impairment of the vasodilating activity of cerebral arteries following aneurysm rupture, since endothelial degeneration and diminished production of vasodilatory substances in cerebral arteries have been reported following SAH.

Largely from the work of Furchgott, et al., it has been demonstrated that an intact vascular endothelium is required for the vasodilatory effects of several pharmacological agents, including acetylcholine (ACh), bradykinin, and adenosine nucleotides, to occur. Furthermore, recent work from the same group has shown that hemoglobin and methylene blue exert a selective blockade of endothelium-dependent relaxation in rabbit aorta. Accordingly, we hypothesized that cerebral vasospasm may be caused by impairment of hemoglobin in the endothelium-dependent relaxation mechanism.

The purpose of this study was to define the presence of the endothelium-dependent vasodilator mechanism in the rabbit basilar artery, and to examine the effect of hemoglobin on endothelium-dependent vasodilation.

Materials and Methods

Artery Preparation and Tension Recording

Male albino rabbits (each weighing 2.7 to 3.2 kg) were anesthetized with ketamine (60 mg/kg intramus-
cularly), and then exsanguinated from the femoral arteries. The brain with the basilar artery in situ was removed and placed in a dissecting chamber filled with Krebs solution (millimolar composition: NaCl 120; KCl 4.5; MgSO4 1.0; NaHCO3 7.0; KH2PO4 1.0; CaCl2 2.5; and dextrose 10.0). The basilar artery (0.3 to 0.4 mm in diameter) was dissected free under magnification, and 3-mm long rings were prepared. Each specimen was suspended between L-shaped stainless steel rods in an organ bath with a 10-ml working volume, which was gassed with 95% O2 and 5% CO2. The pH of the solution ranged from 7.40 to 7.50. Isometric tension was recorded using a Grass FT.03 force-displacement transducer and was displayed on a Grass Model 7 or Soltec 3418 polygraph.*

For relaxation studies, submaximal tone was first induced with 10^-6 M of serotonin (5-HT), then drugs were added in a cumulative fashion. The relaxation induced by ACh, adenosine triphosphate (ATP), or papaverine was expressed as a percentage of the tonic phase of contraction induced by 10^-6 M of 5-HT. Each specimen was pretreated with each concentration of hemoglobin, 8-phenyltheophylline (8-PT), or atropine 5 to 10 minutes before application of 10^-6 M of 5-HT. The preparations were washed at least three times with 20 ml of Krebs solution and allowed to equilibrate for 45 minutes after each exposure to a vasoactive substance.

**Removal of Endothelial Cells**

In some rings, the endothelium was removed by the following procedure. The specimen was placed in a chamber filled with Krebs solution. A No. 30 needle, which was connected to the gas supply (95% O2 and 5% CO2), was introduced into the top of the basilar artery. A gentle stream of gas was passed along the lumen of the vessel for 10 minutes to produce a drying injury of the endothelium. The specimen was then refilled with Krebs solution containing 10^-5 M papaverine to reverse the contraction induced by the above procedure, and the endothelial cells were removed by gentle insertion of a PE 20 polyethylene tube. After the in vitro experiments, some of the rings, including those with and without removal of endothelium, were sectioned transversely and examined with light and transmission electron microscopy.

**Preparation of Oxyhemoglobin**

Hemoglobin solution was isolated by a modification of the method of Williams and Tsay. Blood was obtained from normal adult humans (laboratory personnel) by venipuncture and was put into tubes containing citrate. 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 cold 0.9% NaCl, 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 suspension of lysed cells was removed from the tube and subjected to centrifugation (10,000 G for 30 minutes). Solution was removed from the center two-thirds of the tube and was diluted with an equal volume of 0.05 M Tris, pH 8.0. The diluted solution was subjected to a second centrifugation at 10,000 G for 30 minutes. From the center layer of the tube, hemoglobin containing solution was removed and then applied to a diethylaminoethyl (DEAE)-Sephadex A-50 column (4 x 60 cm) equilibrated with 0.05 M Tris, pH 7.60. Elution of the column was carried out with 0.05 M Tris, pH 7.60. The hemoglobin fraction thus obtained was concentrated by absorption on a DEAE-Sephadex A-50 collection column (2 x 20 cm) equilibrated with 0.05 M Tris, pH 8.0. Then hemoglobin was slowly eluted from the collecting column with 0.2 M NaCl in 0.1 M Tris buffer, pH 7.4. Concentration of hemoglobin was measured by the cyanmethemoglobin method. Vasoactivity of the hemoglobin was confirmed by application to canine and rabbit basilar artery ring preparations (Fig. 1). The contractile responses of rabbit basilar artery evoked by hemoglobin were smaller than those of canine basilar artery.

**Drug Preparation**

All the drugs except 5-HT, ACh, papaverine, atropine sulfate, ATP, and 8-PT‡ were dissolved in distilled water such that volumes of less than 0.1 ml were added to the organ baths. The 5-HT was dissolved in 0.1 N HCl with 0.1% ascorbic acid.

* Grass Model 7 polygraph manufactured by Grass Instrument Co., Quincy, Massachusetts.

† Diethylaminoethyl (DEAE)-Sephadex A-50 column manufactured by Biorad Laboratories, Richmond, California.
‡ Drugs obtained from Sigma Chemical Co., St. Louis, Missouri.
Hb inhibition of endothelium-dependent vasodilation

Fig. 2. Electron microscopic views of the rabbit basilar artery. A: Section after 6 to 7 hours in the organ bath, which has been superfused with hemoglobin for 20 minutes two or three times. B: Section from which endothelial cells were intentionally removed. Bars = 2 μ.

Statistical Analysis

The data were expressed as means ± standard deviation. Statistical comparisons between responses of rings with and without endothelium, or in the presence and absence of drugs, were made using Student’s t-test. Multiple comparisons of specimens pretreated with multiple drugs were evaluated by Scheffe’s test after analysis of variance (ANOVA). A probability of 0.05 or less was considered significant.

Results

Integrity of Endothelium

After 6 to 7 hours in the organ bath, vessels that were not subjected to the procedure to remove the endothelium maintained an intact endothelium over nearly all of the intimal surface with the exception of the areas that had been in continual contact with the mounting rods. Even the specimens that had been superfused with hemoglobin maintained an intact endothelium (Fig. 2A). All eight arterial segments with the endothelium intentionally removed were completely devoid of endothelial cells, and there was no notable injury to the elastic lamina or smooth-muscle cell layers (Fig. 2B).

Vasodilating Effect of ACh on 5-HT-Induced Contraction

Phasic and tonic contraction complexes were induced by 10⁻⁶ M 5-HT in rabbit basilar artery (Figs. 3, 5, and 9). Recordings of the typical pattern of dilator responses induced by ACh in basilar artery with and without endothelium are shown in Fig. 3A and B, respectively. In eight preparations, ACh (10⁻⁷ to 10⁻⁴ M) inhibited the tonic responses of basilar artery with endothelium in a dose-dependent manner.

Removal of the endothelium did not significantly affect the response of basilar artery to 5-HT. However, the inhibitory effect of ACh was abolished by the removal of the endothelium (Fig. 3C, eight preparations). At higher concentrations of ACh, relaxation changed to contraction (Fig. 3B and C). The inhibitory effect of ACh was also abolished by pretreatment with 10⁻⁶ M atropine (Fig. 4, nine preparations).

Effect of Hemoglobin on ACh-Induced Vasodilation

When hemoglobin (10⁻⁷ to 10⁻³ M) was added to basilar artery rings relaxed by ACh, a rapid reversal of the relaxation was observed and the tone of the ring returned to the level of tension before the addition of
ACh (Fig. 5A). Treatment of basilar artery rings with hemoglobin for 5 minutes reduced, at concentrations of $10^{-7}$ to $10^{-6}$ M, and abolished, at $10^{-5}$ M, the relaxation induced by ACh (Figs. 5B and 6, nine to 14 preparations). The blockade induced by hemoglobin ($10^{-7}$ to $10^{-5}$ M) was completely reversed 45 minutes after washout of the hemoglobin.

**Effect of Hemoglobin on Papaverine-Induced Vasodilation**

To determine the selectivity of the inhibitory action of hemoglobin, we examined the effect of hemoglobin against papaverine-induced vasodilation, which occurs independently of an action on endothelial cells. Papaverine ($10^{-7}$ to $10^{-4}$ M) produced vasodilation of basilar artery rings previously contracted by $10^{-6}$ M of 5-HT. This vasodilating action was not influenced by the removal of the endothelium. Treatment of basilar artery rings with $10^{-5}$ M hemoglobin for 10 minutes did not block the papaverine-induced vasodilation (Fig. 7, eight to 12 preparations).

**Effect of Vasodilation by ATP on 5-HT-Induced Contraction**

The intense blockade of ACh-induced relaxation produced by hemoglobin prompted us to determine whether the endothelium-dependent vasodilation induced by another agent, such as ATP, could also be blocked. Adenosine triphosphate ($10^{-7}$ to $10^{-4}$ M) produced a dose-dependent vasodilation of basilar arteries with intact endothelium (Figs. 8 and 9, eight preparations). High concentrations of ATP ($10^{-4}$ M) induced an initial contraction and slow relaxation complex (Fig. 9A). This vasodilating action of ATP was markedly

---

**Fig. 3.** Effect of acetylcholine (ACh) on serotonin (5-HT)-induced contraction of rabbit basilar artery with or without endothelium. A: Typical pattern of dilator response induced by ACh in basilar artery with intact endothelium. B: Typical pattern of effect of ACh on basilar artery after removal of endothelium. The number with the arrow indicates the log molar concentrations of ACh or papaverine in the bath. C: Dose-response relationships to ACh of basilar arteries with endothelium (eight preparations) and without endothelium (eight preparations). Data are expressed as percentage of the contraction induced by $10^{-6}$ M of 5-HT. Vertical bars indicate two standard deviations. * = p < 0.05; ** = p < 0.01.

**Fig. 4.** Acetylcholine (ACh) dose-response relationships of nine control preparations and after $10^{-6}$ M atropine pretreatment (nine preparations). Data are expressed as percentage of contractions induced by $10^{-6}$ M serotonin (5-HT). Vertical bars indicate two standard deviations. * = p < 0.05; ** = p < 0.01.
Hb inhibition of endothelium-dependent vasodilation

FIG. 5. Typical patterns of blockade of the acetylcholine (ACh)-induced vasodilation of rabbit basilar artery previously contracted by 10^{-6} M serotonin (5-HT). A: Hemoglobin, 10^{-6} M, was applied during an ACh-induced relaxation. B: The specimen was pretreated with 10^{-6} M hemoglobin (Hb) 5 minutes before application of 10^{-6} M 5-HT. The numbers with the arrow indicate the log molar concentration of ACh or papaverine in the bath.

attenuated by removal of the endothelium (Fig. 8, six preparations). Furthermore, pretreatment with 10^{-6} M of 8-PT of basilar artery rings without endothelium abolished the ATP-induced vasodilating actions (Fig. 8, six preparations).

FIG. 6. Acetylcholine (ACh) dose-response relationships of control and hemoglobin (Hb)-pretreated basilar arteries. Data are expressed as percentage of the 10^{-6} M serotonin (5-HT)-induced contraction of each condition (nine to 14 preparations). *= p < 0.05; ** = p < 0.01.

Effect of Hemoglobin on ATP-Induced Vasodilation

Recordings of the typical pattern of the effect of hemoglobin on ATP-induced vasodilation of basilar artery rings that were or were not pretreated with 8-PT are shown in Fig. 9A and B. When hemoglobin (10^{-7} to 10^{-5} M) was added to basilar artery rings that had not been pretreated with 8-PT during ATP-induced relaxation, a rapid reversal of the relaxation was observed. However, the tone of the rings did not return to the level of tension before the addition of ATP (Fig. 9A and C, eight to 10 preparations). After treatment of basilar artery rings with 10^{-6} M of 8-PT, the ATP-induced vasodilating effect was slightly inhibited and, furthermore, this ATP-induced vasodilating effect was completely blocked by hemoglobin (Fig. 9B and C, eight preparations).

FIG. 7. Papaverine dose-response relationships of control and 10^{-5} M hemoglobin (Hb)-pretreated basilar arteries. Data are expressed as a percentage of 10^{-6} M serotonin (5-HT)-induced contraction of each condition (eight to 12 preparations). Vertical bars indicate standard deviation.

FIG. 8. Adenosine triphosphate (ATP) dose-response relationships of basilar arteries with and without endothelium, and without endothelium after pretreatment with 10^{-6} M 8-phenyltheophylline (8-PT). Data are expressed as percentage of 10^{-6} M serotonin (5-HT)-induced contraction of each condition (eight preparations). Vertical bars indicate two standard deviations. *= p < 0.05, and ** = p < 0.01, control vs. absent endothelium; † = p < 0.01 (absent endothelium vs. absent endothelium with 8-phenyltheophylline (6-PT)).
Discussion

Recently, it has been shown that the vasodilating action of acetylcholine (ACh) on several types of vascular smooth muscle is mediated indirectly by release of a relaxant substance from the endothelial cells. This substance was later termed the "endothelium-derived relaxing factor" (EDRF). Subsequent studies have also shown that other vasodilators, such as ATP, cause relaxation by an endothelium-dependent mechanism. In this experiment, ACh-induced vasodilation was shown to be generated through muscarinic receptors, and to be strictly endothelium-dependent (that is, pretreatment of the specimen with atropine and removal of the endothelium abolished the relaxation); at higher concentrations of ACh, vasoconstriction occurred. Vasodilation induced by ATP was also attenuated by removal of endothelium. However, a small degree of vasodilation remained, which was completely blocked by the adenosine antagonist, 8-phenyltheophylline (8-PT). This means that the ATP-induced vasodilation was due both to ATP itself (endothelium-dependent) and to ATP degradation products such as adenosine or adenosine monophosphate (endothelium-independent). On the other hand, papaverine-induced vasodilation was not affected by removal of the endothelium.

Hemoglobin released from lysed erythrocytes may play an important role in causing the prolonged cerebral vasospasm that occurs after SAH. Pharmacological studies of hemolysate or hemoglobin have shown that hemoglobin has a preferential vasoconstrictive activity on cerebral arterial smooth muscle, and inhibits the neurogenic vasodilation of cerebral arteries. Recently, a selective blockade by hemoglobin of endothelium-dependent relaxation in rabbit aorta was reported. Therefore, it seemed worthwhile to examine the effect of hemoglobin on endothelium-dependent relaxation in cerebral arteries, in order to clarify the contribution of hemoglobin to the genesis of cerebral vasospasm.

In this experiment, hemoglobin at concentrations of 10⁻⁷ to 10⁻⁵ M selectively inhibited the endothelium-dependent vasodilation produced by ACh and ATP.

**Fig. 9.** Effect of adenosine triphosphate (ATP) on serotonin (5-HT)-induced contraction of rabbit basilar artery with or without pretreatment with 10⁻⁶ M 8-phenyltheophylline (8-PT) and effect of 10⁻⁵ M hemoglobin (Hb) on these ATP-induced vasodilations. A and B: Typical patterns of ATP-induced vasodilation of rabbit basilar artery without (A) pretreatment with 10⁻⁶ M 8-PT or with (B) pretreatment, and inhibition by 10⁻⁵ M Hb of the ATP-induced vasodilation. C: The ATP dose-response relationships of basilar artery with (open triangles) pretreatment with 10⁻⁶ M 8-PT or without (open circles) pretreatment, and Hb dose-dependent blockade of the 10⁻⁷ M ATP-induced vasodilation with (closed triangle) pretreatment with 10⁻⁶ M 8-PT or without (closed circles) pretreatment (eight to 10 preparations). *= p < 0.05; **= p < 0.01 (control vs. Hb treatment); †= p < 0.01 (without 8-PT vs. with 8-PT).
Hb inhibition of endothelium-dependent vasodilation

but did not inhibit the endothelium-independent vasodilation produced by papaverine or ATP degradation products such as adenosine. The blocking action of hemoglobin was not due to its vasoconstrictive activity, because 1) the vasoconstrictive activity of hemoglobin was weak in rabbit basilar artery, as shown in Fig. 1; and 2) this blocking action was observed both when hemoglobin was applied during an ACh-induced vasodilation or when it was applied before the application of ACh. The blocking action of hemoglobin was also not due to the destruction of endothelium, because the endothelium was intact even after exposure to hemoglobin, as shown in Fig. 2A; this blockade was completely reversed after washout of the hemoglobin.

The nature of the EDRF has not yet been established, and the mechanisms by which hemoglobin inhibits relaxation induced by EDRF are also unclear at the present time. However, there is general agreement that endothelium-dependent relaxation is associated with a rise in the cyclic guanine monophosphate (GMP) content of the smooth-muscle cells. Hemoglobin is a large protein, which is undoubtedly confined to the extracellular space. Therefore, hemoglobin may inhibit endothelium-dependent relaxation by binding EDRF in the extracellular space before it is able to diffuse to the smooth-muscle cells and stimulate an increase of the cyclic GMP level. This would explain the rapid onset and recovery from the blockade induced by hemoglobin.

When hemoglobin is added to rabbit aortic rings with intact endothelium during relaxation induced by ACh or the calcium ionophore A23187, there is sometimes not only a reversal of relaxation, but also an overshoot in tone to a level higher than that present before application of these relaxing agents. This overshoot in tone may be due to inhibition of the effect of spontaneously released EDRF in addition to blockade of the effects of induced EDRF.

In our experiment, such an overshoot was rarely seen. If the above hypothesis was applicable to our results, the activity of spontaneously released EDRF in basilar artery should be quite minimal. Another possibility is that the overshoot may be due in part to the direct contractile effect of ACh on the smooth-muscle cells, which is normally not observed because the relaxing effect of ACh predominates. This notion is supported by the observation that the direct contractile activity of ACh on the basilar artery smooth-muscle cells was not as strong as that seen in rabbit aorta. Our experiments do not elucidate these mechanisms, and further investigation is required.

Conclusions

Rabbit basilar artery has an endothelium-dependent vasodilatory mechanism induced by ACh and ATP, which is selectively blocked by hemoglobin. This may play a role in the pathogenesis of cerebral vasospasm after SAH: there is a possibility that the presence of hemoglobin released from lysed erythrocytes inhibits the endothelium-dependent vasodilation of cerebral arteries and contributes to the arterial narrowing. Furthermore, the endothelial degeneration that occurs following SAH may impair the vasodilating mechanism of cerebral arterial smooth-muscle cells.

Acknowledgment

We are grateful to Lucille Staiger for typing the manuscript.

References


Manuscript received July 10, 1985.
Address reprint requests to: Neal F. Kassell, M.D., Department of Neurosurgery, Box 212, Medical Center, University of Virginia, Charlottesville, Virginia 22908.