Comparison of Intraluminal and Extraluminal Inhibitory Effects of Hemoglobin on Endothelium-Dependent Relaxation of Rabbit Basilar Artery

Kazuhiro Hongo, MD, Hisayuki Ogawa, MD, Neal F. Kassell, MD, Tadayoshi Nakagomi, MD, Tomio Sasaki, MD, Tetsuya Tsukahara, MD, and R. Michael Lehman, BA

To determine whether extraluminal or intraluminal hemoglobin inhibits endothelium-dependent relaxation, we measured the vascular responsiveness of rabbit basilar artery in an in vitro perfusion system and we performed immunohistochemical staining for hemoglobin. In the in vitro study, we applied agents from either the intraluminal or the extraluminal side of excised basilar arteries. KCl-induced contraction was the same with either application. Acetylcholine-induced maximal relaxations were 57.6±8.5% of the contraction induced by 10⁻⁵ M 5-hydroxytryptamine for control, 3.3±0.3% for intraluminal, and 34.9±8.6% for extraluminal applications. Adenosine triphosphate-induced maximal relaxations were 64.2±4.1% of the contraction induced by 10⁻⁵ M 5-hydroxytryptamine for control, 26.9±3.8% for intraluminal, and 42.2±6.0% for extraluminal applications. Hemoglobin’s inhibition of acetylcholine- and adenosine triphosphate–induced relaxation was significantly greater with intraluminal than with extraluminal application (p<0.05). The immunohistochemical study revealed hemoglobin in the outer layer of the smooth muscle and in the adventitia when 10⁻⁵ M hemoglobin was applied extraluminally for 5 minutes, whereas hemoglobin was observed on the surface of the endothelial cells after intraluminal application. Our findings suggest that hemoglobin inhibits acetylcholine- or adenosine triphosphate–induced relaxation by binding to endothelium-derived relaxing factor (EDRF) and by inhibiting production of EDRF. Hemoglobin’s inhibitory effect on endothelium-dependent relaxation may be important in the pathogenesis of vasospasm after subarachnoid hemorrhage. (Stroke 1988; 19:1550–1555)

It is well known that vascular endothelium is essential for vascular relaxation in response to several agents, such as acetylcholine (ACh), adenosine triphosphate (ATP), and bradykinin, by releasing endothelium-derived relaxing factor (EDRF).1–4 Recently, it has been reported that EDRF is released not only by vasoactive substances but also spontaneously.5–8 Furthermore, the release of EDRF, both drug-stimulated9 and spontaneous,10 was impaired after experimental subarachnoid hemorrhage (SAH). Considering these findings, it is likely that endothelium-dependent relaxation may contribute to the pathogenesis of vasospasm after SAH.

It is also accepted that hemoglobin inhibits endothelium-dependent vascular relaxation,6,11,12 although a precise mechanism for this inhibition has not been fully investigated. Hemoglobin is thought to be one mediator of the pathogenesis of vasospasm,13–15 and hemoglobin may affect the vessel from the outside. There is no confirmation of from which side of the vessel wall, that is, the intraluminal or the extraluminal side, hemoglobin inhibits EDRF; therefore, it is important to investigate this to confirm the significance of hemoglobin in the pathogenesis of vasospasm as well as to confirm the mechanism by which hemoglobin inhibits EDRF. Our experiment was conducted to investigate these issues using an in vitro perfusion system to evaluate vascular responsiveness and immunohistochemical staining.

Materials and Methods

We anesthetized 34 male New Zealand White rabbits weighing 2.7–3.2 kg by intramuscular injection of a mixture of 20 mg/kg ketamine, 5 mg/kg xylazine, and 0.25 mg/kg acepromazine. The rabbits were exsanguinated from the femoral arteries.
Each brain with the basilar artery in situ was removed and placed in a dissecting chamber filled with Krebs' solution of the following millimolar composition: NaCl 120, KCI 4.5, MgSO₄ 1.0, NaHCO₃ 27.0, KH₂PO₄ 1.0, CaCl₂ 2.5, and dextrose 10.0. Each basilar artery was dissected free under magnification. Meticulous care was taken not to pull branches from the basilar artery.

Our method was modified from that described previously. Under a dissecting microscope, approximately 15–20-mm-long whole basilar artery was cannulated from both ends with 18-gauge stainless steel needles, stretched longitudinally to the approximate in situ length, and mounted in a vessel chamber filled with Krebs' solution. All branches were tied off with 10-0 sutures; the working volume of the vessel chamber was 20 ml. The basilar arteries were intraluminally perfused with Krebs' solution by means of a peristaltic pump (Masterflex, Chicago, Illinois) at 1.5–2.5 ml/min to maintain the perfusion pressure at approximately 20 mm Hg (Figure 1). Krebs' solution of both the vessel chamber and the perfusate was continuously bubbled with 95% O₂-5% CO₂ to give a pH of 7.30–7.40. Perfusion pressure was measured with a Gould model P23ID pressure transducer (Cleveland, Ohio) placed just upstream from the basilar artery and was recorded on a Gould model 8188 polygraph. The temperature of both the intraluminal and the extraluminal perfusates was kept at 37°C by means of a heat circulator (Haake FE2, Karlsruhe, FRG). Since the intraluminal perfusion rate was constant, changes in perfusion pressure reflected changes in vasoconstriction or vasodilation. All basilar arteries were equilibrated for 60 minutes at a perfusion pressure of 20 mm Hg.

Vasoactive agents were applied intraluminally by pumping them from small (5-ml) chambers containing different concentrations of agents prepared immediately before application to the basilar arteries. Agents were applied extraluminally by adding them to the Krebs' solution in the vessel chamber (Figure 1). An equilibration period of 30 minutes was allowed between each series of experiments. For relaxation studies, submaximal tone was induced with 10⁻⁵ M 5-hydroxytryptamine (5-HT); ACh or ATP was then applied intraluminally. In the relaxation studies with ATP, the basilar arteries were treated with 10⁻⁶ M 8-phenyltheophylline (8-PT), an adenosine antagonist, 5 minutes before application of 10⁻³ M 5-HT to exclude the effect of endothelium-independent relaxation by adenosine, adenine diphosphate, and adenine monophosphate. Hemoglobin at a concentration of 10⁻⁵ M was applied 5 minutes before application of ACh or ATP either intraluminally or extraluminally. Integrity of the separation of the intraluminal and extraluminal sides was confirmed by measuring draining volume and by monitoring perfusion pressure.

5-HT, KCI, ACh, ATP, 8-PT, and human hemoglobin were obtained from Sigma Chemical Co. (St. Louis, Missouri). KCl, ACh, ATP, and 8-PT were dissolved in distilled water; 5-HT was dissolved in 0.1N HCl with 0.1% ascorbic acid to make a stock solution. Each agent was then dissolved in Krebs' solution before use such that volumes of <0.1 ml were added to each small chamber. Human hemoglobin solution was prepared according to the method of Martin. The concentration of hemoglobin was measured by the cyanomethohemoglobin method.

The vascular responsiveness data are expressed as mean±standard error of the mean (SEM). The dose–response curves were analyzed using the General Linear Models procedure of the Statistical Analysis System computer program, and Scheffé’s test was used for subgroup analysis. Multiple comparisons of the vasodilatory response to ACh or ATP at each concentration were evaluated using Scheffé’s test after analysis of variance. We considered the values to be significantly different when p<0.05.

The perfusion system we used in the vascular responsiveness study was used for the immunohisto-
FIGURE 2. Typical pattern of KCl-induced contraction of rabbit basilar artery by intraluminal (IL) and extraluminal (EL) application. Numbers with arrows indicate millimolar concentrations of KCl. W, washout.

tochemical study. After the basilar artery was treated with $10^{-5}$ M hemoglobin either intraluminally or extraluminally for 5 minutes, it was fixed in 10% neutral buffered formalin for 10 minutes. The basilar artery was then washed in Krebs' solution for 20 minutes and frozen in liquid nitrogen after filling the lumen with OCT compound (Miles Scientific, Division of Miles Laboratories Inc., Naperville, Illinois). Ten micrometer-thick cross cryosections of the basilar artery were again washed in 0.01 M phosphate-buffered saline (PBS) for 10 minutes at 4°C. The sections were incubated with sheep antihuman hemoglobin serum (New England Immunology Associates, Division of Diagnostic Assays Inc., Cambridge, Massachusetts) diluted 1:20 in normal donkey serum (NDS) (Sigma) at 4°C overnight and then washed with 0.01 M PBS. The sections were exposed to donkey anti-sheep IgG conjugated with fluorescein (Sigma) and diluted 1:20 in NDS at 37°C for 1 hour. After washing with PBS, the sections were mounted and viewed under a Zeiss fluorescence microscope (Thornwood, New York). Control sections were stained with normal sheep serum (Sigma) or with donkey anti-sheep antiserum alone.

Results

Approximately 98% of the perfusate was recovered from the draining tube.

KCl (20–100 mM) induced contraction of the rabbit basilar artery in a dose-dependent fashion by either intraluminal or extraluminal application (Figures 2 and 3). There was no significant difference in the magnitude of contraction between applications. Contraction induced by application of KCl was reversed completely after washing out the basilar artery with perfusate (Figure 2).

Intraluminal application of $10^{-4}$ to $10^{-5}$ M ACh elicited a dose-dependent relaxation of rabbit basilar arteries precontracted with $10^{-5}$ M 5-HT (Figures 4 and 5); the maximum relaxation in control arteries was 57.6±8.5%. ACh-induced relaxations after pretreatment with $10^{-5}$ M hemoglobin for 5 minutes intraluminally and extraluminally were 3.3±0.3% and 34.9±8.6%, respectively. Hemoglobin significantly inhibited the ACh-induced relaxation by either application ($p<0.001$); furthermore, intraluminal application had a greater inhibitory effect than extraluminal application ($p<0.01$).

Intraluminal application of $10^{-4}$ to $10^{-5}$ M ATP elicited a dose-dependent relaxation of rabbit basilar arteries precontracted with $10^{-5}$ M 5-HT (Figure 6); the relaxation induced in control arteries by $10^{-4}$ M ATP was 64.2±4.1%. Relaxation after pretreatment with $10^{-5}$ M hemoglobin for 5 minutes was 26.9±3.8% intraluminally and 42.2±6.0% extraluminally. Hemoglobin inhibited ATP-induced relaxation by both intraluminal and extraluminal application ($p<0.001$); moreover, intraluminal application

FIGURE 3. Effect of application site (O, intraluminal, IL; ● extraluminal) on contractile responses of rabbit basilar arteries to KCl. Data are mean±SEM, eight rabbits in each group.

FIGURE 4. Typical patterns of acetylcholine (ACh)-induced relaxation (control), hemoglobin-induced inhibition of ACh-induced relaxation by either extraluminal (EL) or intraluminal (IL) application of hemoglobin (Hb) to rabbit basilar arteries. Numbers with arrows indicate negative log-molar concentrations of ACh. W, washout.
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had a greater inhibitory effect than extraluminal application ($p<0.001$).

Hemoglobin applied extraluminally was observed by immunohistochemical staining in the adventitia and in approximately the outer third of the smooth muscle layers (Figure 7a). Figure 7b is a control section stained with normal sheep serum. When hemoglobin was applied intraluminally, it was seen on the surface of the endothelial cell layer (Figure 7c); no apparent intensity was noted inside the smooth muscle layers.

Discussion

Our experiments reveal that 1) hemoglobin inhibits ACh- and ATP-induced vascular relaxation by either intraluminal or extraluminal application and that intraluminal inhibition is more potent than extraluminal inhibition, 2) ACh-induced relaxation is inhibited more than ATP-induced relaxation by both intraluminal and extraluminal application of hemoglobin, and 3) with a 5-minute incubation hemoglobin penetrates the vascular smooth muscle layers to approximately one third the distance from the outside, whereas hemoglobin administered intraluminally is seen on the surface of the endothelial cells.

Advantages that this perfusion system offers over the conventional helical or ring preparation are the ability to isolate the intimal surface of the artery from the adventitia at the time of application of the vasoactive agent and close approximation of the physiological in situ state because the vessel is not stretched mechanically but is perfused with a certain pressure. Sercombe et al reported that the contraction of the rabbit middle cerebral artery induced by histamine was potentiated by either H$_2$-receptor blockade or endothelium denudation and that this potentiation was greater with intraluminal than with extraluminal administration. Allen and Gross showed in the canine basilar artery that intraluminal delivery of sodium nitroprusside more effectively relaxes a 5-HT–induced contraction than extraluminal application. We have not yet investigated the differences in vascular contractions to other vasoactive agents except for KCl by different application routes. As Vinall and Simeone have stated, the first advantage is particularly important when studying intracranial vessels because cerebral vessels are surrounded by two dynamically changing mediums, blood and cerebrospinal fluid, both of which offer a route for the delivery of vasoactive substances.

Because of the many perforators from the rabbit basilar artery, it is difficult to completely separate an intraluminal surface from an extraluminal one. However, by tying off those branches with 10-0 sutures under a dissecting microscope, it is possible to separate both sides sufficiently; this is confirmed by our noting that almost all the Krebs’ solution was drained from the other end of the basilar artery and that a contraction curve returned to baseline. This perfusion system, therefore, seems to be a good model with which to investigate the responsiveness of cerebral vessels.

Hemoglobin released from lysed erythrocytes may play an important role in inducing vasospasm after SAH. A pharmacologic study has shown that hemoglobin causes vasoconstriction of the cerebral smooth muscle and, moreover, that hemoglobin selectively inhibits endothelium-dependent relaxation in rabbit aorta and rabbit basilar artery. Using an isometric tension recording method, Fujisawa et al demonstrated in the rabbit basilar artery that hemoglobin inhibited the endothelium-dependent vasodilation induced by ACh and ATP. Therefore, it seems important to investigate the effect of hemoglobin on the genesis of vasospasm. Most of the previous in vitro studies, however, were performed using...
helically cut or ring segments of artery, and drugs were applied both intraluminally and extraluminally. Therefore, it was difficult to conclude how hemoglobin reached the endothelium from a subarachnoid space or whether hemoglobin worked from outside the vessel.

In our study, by using a method to separate the intraluminal and extraluminal sides in an organ bath, a more physiological condition was simulated. Our result, that extraluminal application of hemoglobin inhibits ACh- and ATP-induced vasodilation, suggests that hemoglobin in the subarachnoid space can inhibit vasodilation of the rabbit basilar artery. The mechanism for hemoglobin-induced inhibition of dilation still remains unclear; however, our finding that intraluminal inhibition was more potent than extraluminal inhibition suggests that hemoglobin inhibits EDRF by binding it or by inhibiting its production at the endothelial cells.

There is no published report demonstrating how deeply hemoglobin penetrates from the wall of the vessel. Zervas et al. have demonstrated that horse-radish peroxidase (HRP), which is smaller than hemoglobin, penetrates as far as the endothelial cell basal lamina 60 minutes after its injection into the ventricles. Our immunohistochemical staining reveals that with 5 minutes' incubation, hemoglobin penetrates the smooth muscle approximately one third of the distance from the adventitial side but did not reach the endothelial surface.

Two major possibilities for the mechanism of inhibition by hemoglobin can be postulated, inhibition of EDRF production at the endothelial cells and inhibition by binding to EDRF. Based on our finding that extraluminal applied hemoglobin, which cannot reach the endothelial cells, inhibits EDRF, it seems likely that hemoglobin inhibits EDRF by binding with it. It is interesting that hemoglobin is bound by nitric oxide because the identity of EDRF has recently been thought to be nitric oxide. This finding supports the second possibility. Moreover, hemoglobin seems to inhibit the production of EDRF in that intraluminally applied hemoglobin, which cannot reach the smooth muscle layers, inhibits EDRF.

Hemoglobin's effect on ACh-induced relaxation was different from its effect on ATP-induced relaxation; ATP-induced relaxation was not inhibited as much. Although the reason these two agents act differently is unclear at present, there is a possibility that the EDRF released by ATP is different from that released by ACh. This has been previously postulated by De Mey and Vanhoutte in the canine femoral artery. Another possibility that cannot be excluded is that hemoglobin may interact with ACh in the perfusate.
Further investigation is needed to clarify the mechanism of hemoglobin’s inhibition of endothelium-dependent relaxation. Our findings, however, lead us to propose that hemoglobin coming from a clot around the cerebral vessels can be one factor responsible for the inhibition of endothelium-dependent relaxation contributing to the development of vasospasm.

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