Impairment of Endothelium-Dependent Vasodilation Induced by Acetylcholine and Adenosine Triphosphate Following Experimental Subarachnoid Hemorrhage

Tadayoshi Nakagomi, Neal F. Kassell, Tomio Sasaki, Shigeru Fujiwara, R. Michael Lehman, and James C. Tomer

The effect of subarachnoid hemorrhage (SAH) on endothelium-dependent vasodilation of isolated rabbit basilar artery was examined using an isometric tension recording method. Thirty-five rabbits that had 2 successive blood injections were divided into 3 groups: normal animals (control), 4 days, and 3 weeks after the first SAH. Acetylcholine (ACh) (10^-6-10^-4 M) and adenosine triphosphate (ATP) (10^-5-10^-4 M) were used to evoke dose-dependent vasodilation of isolated arterial rings previously contracted by 10^-6 M serotonin. In the animals killed 4 days after the first SAH, both ACh- and ATP-induced relaxation were suppressed, and the degree of relaxation of this group was 38 ± 4.5% (mean ± SEM) and 22 ± 3.9% of the initial contractile tone in response to 10^-4 M ACh and 10^-4 M ATP, respectively. Suppression of the relaxation induced by ATP was seen even in the animals killed 3 weeks after the first SAH. Moreover, pretreatment with hemoglobin (10^-5 and 10^-4 M) inhibited endothelium-dependent vasodilation induced by ACh in the arterial rings from the animals killed 4 days after the first SAH. The present experiments suggest that impairment of the endothelium-dependent vasodilation following SAH may be involved in the pathogenesis of cerebral vasospasm.

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CEREBRAL vasospasm is the leading cause of death and disability in patients with subarachnoid hemorrhage (SAH) due to rupture of a cerebral aneurysm. Despite extensive efforts to identify measures to dilate the narrowed arteries, no reliable measure exists for preventing vasospasm from developing or for reversing it. One of the major reasons for this inadequacy is a lack in understanding the pathogenesis of vasospasm. The cause of cerebral vasospasm following SAH is likely to be multifactorial. It has been recently suggested that impairment of the vasodilatory activity of cerebral arteries following SAH may play an important role in the pathogenesis of vasospasm. Arterial wall prostacyclin, a powerful vasodilator, decreases progressively following SAH. Moreover, in normal cerebral arteries, hemoglobin selectively inhibits endothelium-dependent vasodilation as well as neurogenic vasodilation. It is well documented that endothelial damage occurs frequently after SAH. We have recently demonstrated that endothelium-dependent vasodilation induced by adenosine triphosphate (ATP) is impaired after SAH in a rabbit single hemorrhage model (T. Nakagomi, unpublished data). However, acetylcholine (ACh)-induced vasodilation was well preserved following SAH in that model. This may reflect a partial, less severe damage to the endothelium.

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The present experiments were conducted to investigate 1) the effect of SAH on endothelium-dependent vasodilation induced by ACh and ATP in a rabbit double hemorrhage model, which should result in more severe damage to the endothelium than a single injection model, and 2) to study the inhibitory effect of hemoglobin on endothelium-dependent vasodilation in basilar arteries exposed to SAH.

Materials and Methods

Animal Preparations

Thirty-five male New Zealand white rabbits weighing 2.9-3.4 kg were anesthetized with an i.m. injection of ketamine (20 mg/kg), xylazine (5 mg/kg), and acepromazine (0.25 mg/kg) in a ratio of 8:1:1. The animals were intubated and received muscular paralysis with i.v. pancuronium bromide (0.08 mg/kg). Ventilation was maintained with a Harvard dual-phase control respirator. In each animal, the left ear artery was cannulated for monitoring blood pressure and withdrawing arterial blood.

A 23-gauge butterfly needle was inserted into the cisterna magna percutaneously and connected to a pressure transducer via one outlet of a three-way stopcock. The other outlet was used for the injection of arterial blood. Intracranial pressure (ICP) was monitored immediately after the injection of arterial blood through the stopcock. Arterial blood pressure and ICP were monitored by a pressure transducer (Hewlett Packard 78342A) and recorded on a chart recorder (Hewlett Packard 78172A).

In addition to a control group of normal animals, the animals were divided into 2 groups according to the
Timing of sacrifice: 4 days or 3 weeks after the first injection of blood (Table 1). SAH was produced by 2 successive injections of fresh autologous nonheparinized arterial blood into the cisterna magna administered 48 hours apart. For the first and second SAH, 5 ml of arterial blood over 10 seconds and 3 ml over 30 seconds were injected, respectively. Then the animals were tilted with the head down for 15 minutes to facilitate settling of the blood in the basal cisterns by gravity. The first injection of 5 ml of blood transiently elevated ICP and mean arterial pressure by about 195 and 30 mm Hg, respectively. These elevations lasted for several minutes. The second SAH induced a rise of both mean arterial pressure and ICP by approximately 20 and 90 mm Hg, respectively. The animals were extubated when they were fully awake. The mortality rate of this procedure was <5%.

### Artery Preparation and Tension Recording

According to the fixed time schedule noted above, the animals were reanesthetized with ketamine (60 mg/kg i.m.) and exsanguinated from the femoral arteries. The brain with the basilar artery in situ was removed and placed in a dissection chamber filled with Krebs solution (millimolar composition: NaCl, 120; KCl, 4.5; MgSO4, 1.0; NaHCO3, 27.0; KH2PO4, 1.0; CaCl2, 2.5; and dextrose, 10.0). The basilar arteries were dissected free under magnification, and 3-mm long arterial rings were prepared. Each specimen was pretreated with 10^-6 M papaverine for 3 minutes to facilitate the insertion of the L-shaped stainless steel rod into the lumen, as well as to reverse mechanical constriction during the procedure. The specimen was then suspended between two L-shaped rods in an organ bath with a 10 ml working volume, gassed with 95% O2 and 5% CO2. The pH of the solution ranged from 7.40 to 7.50. The resting tension was adjusted to 400 mg since preliminary experiments demonstrated that the best relaxation by ACh could be obtained in arterial rings precontracted by 10^-6 M serotonin, 5-hydroxytryptamine (5-HT). The preparation was allowed to equilibrate at 37°C for 90 minutes before use. Isometric tension was recorded using a Grass FT.03 force-displacement transducer and was displayed on a Soltec 3418 chart recorder.

The contractile response to 40 mM KCl was first obtained on each arterial ring. After confirming a consistent contractile response to KCl by repeated washings, experiments with 5-HT were begun. For relaxation studies, submaximal tone was induced with 10^-6 M 5-HT, then ACh or ATP was added in a cumulative fashion. The relaxation induced by ACh or ATP was expressed as percent of the tonic phase of contraction induced by 10^-6 M 5-HT. In the relaxation studies with ATP, 10^-5 M 8-phenyltheophylline, an adenosine antagonist, was used to pretreat the arterial rings 5 minutes before application of 10^-6 M 5-HT to exclude the effect of endothelium-independent relaxation by adenosine.

To investigate the effect of hemoglobin on ACh-induced vasodilation in basilar arteries exposed to SAH, each specimen was pretreated with 10^-6 or 10^-5 M hemoglobin after control relaxation by ACh was obtained. Hemoglobin was added to the organ bath 2–3 minutes before application of 10^-6 M 5-HT.

### Morphologic Examination

Transmission (TEM) and scanning electron microscopy (SEM) were performed to assess the morphologic changes of the arterial walls after SAH. In each group, 3 animals apart from the in vitro experiments were perfusion-fixed by transthoracic cannulation of the left ventricle with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) under a pressure of 120 cm H2O. The basilar arteries were removed from the brain and immersed in a cacodylate buffered fixative (pH 7.4) for 5 hours at 4°C and then kept overnight at 4°C in 0.1 M sodium cacodylate buffer (pH 7.3). For TEM, samples were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.4, dehydrated in graded alcohols, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and examined in an Hitachi HU-12A electron microscope. For SEM, the dehydrated specimens were immersed in isooamyl acetate, critical-point dried, and coated with palladium gold. The specimens were viewed and photographed on a JEOL JSM-35C scanning electron microscope.

Immediately after the in vitro experiment the arterial rings were also examined in the scanning electron microscope to check whether endothelial cells were injured during the in vitro experiments. After the in vitro studies the arterial rings were immersed in the same fixative mentioned before and processed for SEM in the same manner.

### Drugs

5-HT, ACh, papaverine, ATP, and 8-phenyltheophylline were obtained from Sigma Chemical Co. (St. Louis, Mo.). To make stock solutions, all drugs except 5-HT were dissolved in distilled water. Then, these drugs were diluted in Krebs solution before use such that volumes of <0.1 ml were added to the organ bath. 5-HT was dissolved in 0.1N HCl with 0.1% ascorbic acid.

Human hemoglobin solution was isolated by a modification of the method of Williams and Tsay described elsewhere. The concentration of hemoglobin was measured by the cyanomethohemoglobin method. Preliminary experiments demonstrated that 10^-6 and 10^-5 M hemoglobin induced contractions in the rabbit basilar artery of 10.1 ± 1.5% (mean ± SEM) and 22.1 ± 2.7%, respectively, of the maximum con-

### Table 1. Experimental Groups and Number of Animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Relaxation study</th>
<th>Morphologic study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>4 days after the first SAH</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>3 weeks after the first SAH</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>

SAH, subarachnoid hemorrhage.

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traction induced by a standard dose of 40 mM KCl (n = 12).

Statistical Analysis
The data were expressed as means ± SEM. Statistical analysis of the dose–response curves of ACh- or ATP-induced relaxation was done using the general linear models procedure of SAS (Statistical Analysis System computer program), and Scheffe’s test was used for subgroup analysis. Multiple comparisons of the contractile response to 40 mM KCl or 10^-6 M 5-HT and of the vasodilatory response to ACh or ATP at each specific concentration were evaluated by Scheffe’s test after analysis of variance (ANOVA). The values were considered to be significantly different when p < 0.05.

Results
Effect of SAH on Relaxation Induced by ACh and ATP
There was no significant difference between contractions to 40 mM KCl among the 3 groups. The initial contractile tone induced by 10^-6 M 5-HT was greater for the arterial rings from the animals killed 4 days after the first SAH than for those of the other groups, but this was not significant (Table 2).

5-HT at 10^-6 M induced a phasic and tonic contraction complex. ACh (10^-6–10^-4 M) and ATP (10^-6–10^-4 M) resulted in dose-dependent vasodilation of the basilar arteries in each group of animals (Figure 1). In the control animals, relaxation of approximately 87% and 86% of the initial contractile tone induced by 10^-6 M 5-HT occurred with 10^-4 M ACh and 10^-4 M ATP, respectively (Figures 2 and 3). In the animals killed 4 days after the first SAH, both ACh- and ATP-induced relaxation were significantly suppressed. Relaxation of 38 ± 4.5% and 22 ± 3.9% of the initial contractile tone occurred with 10^-4 M ACh and 10^-4 M ATP, respectively. ACh-induced relaxation in the animals killed 3 weeks after the first SAH was not different from that of the controls. Relaxation induced by ATP, however, was still significantly suppressed. Papaverine at 10^-4 M completely relaxed the arterial rings to the basal tension in each experiment.

Effect of Hemoglobin on ACh-Induced Relaxation
There was no significant difference in the initial contractile tone induced by 10^-6 M 5-HT between the groups pretreated with hemoglobin and the group that was not. Pretreatment of arterial rings from the animals killed 4 days after the first SAH with hemoglobin for 2–3 minutes reduced at 10^-6 M, and abolished at 10^-3 M, the relaxation induced by ACh (Figure 4). The blockade of hemoglobin was completely reversed 45 minutes after washing out the hemoglobin.

Morphologic Observation of the Basilar Arteries
In the animals killed 4 days after the first SAH, thick subarachnoid clot was observed over the basal surface of the brain around the major cerebral arteries. All of

Table 2. Effect of Subarachnoid Hemorrhage on Isometric Contractions Evoked by KCI and Serotonin

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>40 mM KCI</th>
<th>10^-6 M 5-HT</th>
<th>40 mM KCI</th>
<th>10^-6 M 5-HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>1.43 ± 0.112</td>
<td>0.71 ± 0.096</td>
<td>1.36 ± 0.091</td>
<td>0.79 ± 0.106</td>
</tr>
<tr>
<td>4 days after the first SAH</td>
<td>14</td>
<td>1.38 ± 0.098</td>
<td>0.97 ± 0.119</td>
<td>1.33 ± 0.093</td>
<td>0.96 ± 0.129</td>
</tr>
<tr>
<td>3 weeks after the first SAH</td>
<td>14</td>
<td>1.47 ± 0.114</td>
<td>0.82 ± 0.136</td>
<td>1.50 ± 0.115</td>
<td>0.81 ± 0.162</td>
</tr>
</tbody>
</table>

Data are means ± SEM, increase in tension (grams) above baseline for 14 arterial rings.
ACh, acetylcholine; ATP, adenosine triphosphate; 5-HT, serotonin; SAH, subarachnoid hemorrhage.
FIGURE 2. Effect of acetylcholine (ACh) on serotonin (5-HT)-induced contraction of rabbit basilar arteries following subarachnoid hemorrhage (SAH). ACh induced a dose-dependent vasodilation. Vasodilatory response to ACh (10^{-6}-10^{-4} M) was impaired in the animals killed 4 days after the first SAH at each concentration. There was no significant difference in the dose-response curve between the control animals and those killed 3 weeks after the first SAH. Data are expressed as percent of the contraction induced by 10^{-6} M 5-HT. Vertical bars, ± SEM; n, number of specimens checked; **p<0.01 vs. control.

the basilar arteries from this group showed slight to moderate constriction under the dissecting microscope at the time of the preparation for the in vitro study. A small amount of fibrous tissue was seen around the basilar arteries in the animals killed 3 weeks after the first SAH, and constriction of the basilar arteries was no longer seen. In 3 animals that were perfusion-fixed 4 days after the first SAH, degenerative changes of the endothelial cells were noted on TEM although denudation of the endothelium was not seen. The most frequent finding was vacuolation adjacent to the interendothelial space (Figure 5B). This vacuole formation was also seen in the animals killed 3 weeks after the first SAH. In addition to this finding, the following were occasionally observed: vacuolations in the cytoplasm, openings of the interendothelial spaces, and "sick endothelia," which appear more dense than normal endothelia by TEM. No morphologic change of the smooth muscle cells could be seen in any of the 3 groups. Under SEM, the basilar arteries from control animals had normal interendothelial borders, endothelial cell shape, and orientation (Figure 6A). In the basilar arteries from the animals killed 4 days or 3 weeks after the first SAH, endothelial cell shape was almost normal, but orientation of the endothelial cells was irregular in places. Crater-like formation, which corresponded to vacuolation in the endothelium, was frequently seen near the endothelial border. No denudation was seen over the entire surface of the vascular lumen. Examination of the arterial rings after the in vitro experiments did not demonstrate damage to the endothelial cells (Figure 6B).

Discussion

The present study demonstrated that SAH inhibits relaxation of rabbit basilar arteries induced by ACh and ATP. It has been recently shown by Furchgott and Zawadzki that an intact endothelium is required for the vasodilatory effects of several endogenous vasoactive agents such as ACh and ATP. The endothelium acts by releasing a diffusible unstable factor, "endothelium-derived relaxing factor" (EDRF), the chemical nature of which remains to be elucidated.

In the present experiments, contractile responses to 40 mM KCl and 10^{-6} M 5-HT were not significantly different among the 3 groups. Therefore, it seems reasonable to compare the effect of SAH on the endothelium-dependent vasodilation among the different groups.

Inhibition of the relaxation induced by ACh or ATP is considered to be due to the impairment of endothelium-dependent relaxation since constrictor action of

FIGURE 3. Effect of adenosine triphosphate (ATP) on serotonin (5-HT)-induced contraction of rabbit basilar arteries following subarachnoid hemorrhage (SAH). ATP evoked a dose-dependent vasodilation. Vasodilatory response to ATP (10^{-6}-10^{-4} M) was severely impaired in the animals killed 4 days after the first SAH at each concentration and remained impaired even 3 weeks after the first SAH. Data are expressed as percent of the contraction induced by 10^{-6} M 5-HT. Vertical bars, ± SEM; n, number of specimens checked; *p<0.05; **p<0.01 vs. control.
ACh or ATP seems minimal at the concentrations used in these experiments.\textsuperscript{14}

Three major causes for the impairment of the endothelium-dependent vasodilation following SAH can be postulated: 1) denudation of the endothelium; 2) inhibition of the production or release mechanism of EDRF in the endothelium, including damage to a specific receptor for ACh or ATP; and 3) decreased or absent responsiveness of the smooth muscle cells to EDRF. Endothelial damage accompanied by morphologic change in the endothelium may explain impairment of the endothelium-dependent vasodilation after SAH. By both TEM and SEM we observed degenerative changes of the endothelium, including vacuolations adjacent to the interendothelial space or in the cytoplasm, opening of the interendothelial space, and "sick endothelium," despite the absence of denudation. Two successive hemorrhagic insults, therefore, may have resulted in severe functional damage to the endothelium, which could result in attenuation of the production or release mechanism of EDRF in the endothelium.

Decreased or absent responsiveness of the smooth muscle cells to EDRF is another possible cause for the inhibition of the relaxation. However, it is unlikely that damage to the smooth muscle cells impairs the endothelium-dependent relaxation since degenerative changes in the smooth muscle cells could not be seen on TEM. Moreover, arterial rings from the animals with SAH responded well to $10^{-4}$ M papaverine with good relaxation.

In the present experiments, ATP-induced relaxation was more profoundly impaired in the animals killed 4 days after the first SAH than that induced by ACh. Moreover, relaxation induced by ATP was still suppressed even 3 weeks after the first SAH, while ACh-induced relaxation was not significantly different from that of the control value. These results suggest not only that ATP-induced relaxation is more sensitive to endothelial damage following SAH than that induced by ACh, but also raise the possibility that EDRF released by ATP is different from that released by ACh. This has also been postulated by De Mey and Vanhoutte working with canine femoral artery.\textsuperscript{15}

Hemoglobin released from lysed erythrocytes probably plays an important role in the development of cerebral vasospasm after SAH. It has been demonstrated that hemoglobin has a preferential vasoconstrictive

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Effect of hemoglobin on acetylcholine (ACh)-induced vasodilation. Top, a, b, c: Typical pattern of the effect of hemoglobin ($10^{-6}$ M and $10^{-5}$ M) on ACh-induced relaxation. Bottom, d: Dose-response relation of the basilar arteries to hemoglobin. Hemoglobin attenuated ACh-induced vasodilation at $10^{-6}$ M and completely abolished the relaxation at $10^{-5}$ M in the animals killed 4 days after the first subarachnoid hemorrhage (SAH). The numbers with arrows indicate the log-molar concentration of ACh. Pap, $10^{-4}$ M papaverine. Data expressed as percent of the contraction induced by $10^{-6}$ M serotonin (5-HT). Vertical bars, ± SEM; *p<0.05; **p<0.01 vs. 4 days after SAH.}
\end{figure}
FIGURE 5. Transmission electron micrographs of the basilar arteries from a control animal (A) and an animal killed 4 days after the first subarachnoid hemorrhage (SAH) (B). Vacuolations can be seen near the interendothelial space (arrows) of the basilar artery from the animal killed 4 days after the first SAH. Sick endothelium (SE) appears more dense than normal endothelium and has many small vacuoles (arrowheads) at the luminal front. Smooth muscle cells look normal. E, endothelium; el, elastic lamina; SE, sick endothelium; *, interendothelial space; sm, smooth muscle; VL, vascular lumen. Bars = 2 μm.
activity on cerebral arterial smooth muscle. Moreover, hemolysates have been reported to inhibit neurogenic vasodilation and to potentiate neurogenic vasoconstriction. In the present experiments, ACh-induced relaxation of the arterial rings from animals killed 4 days after the first SAH was inhibited by pretreatment with $10^{-6}$ M hemoglobin and completely abolished by $10^{-3}$ M hemoglobin. The present result is consistent with that seen in normal rabbit basilar arteries and suggests that hemoglobin has basically the same inhibitory action on the ACh-induced relaxation in normal arteries and those exposed to SAH.

Endothelial damage, including swelling or vacuolation of the endothelium, denudation, and loss of endothelial tight junctions, occurs frequently after SAH. Damage to the endothelium after SAH results in decreased production of prostacyclin, a potent vasodilator, in the arterial wall, which brings about impairment of vasodilation. The present experiments demonstrated that endothelium-dependent vasodilation is impaired by SAH itself. Moreover, hemoglobin derived from lysed erythrocytes in bloody cerebrospinal fluid completely inhibits endothelium-dependent relaxation of the cerebral arteries, which may contribute to the arterial wall narrowing. Based on all of these results, impairment of the vasodilatory activity of the cerebral arteries after SAH is likely to be associated with the pathogenesis of cerebral vasospasm.

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References

Key Words • acetylcholine • adenosine triphosphate • cerebral vasospasm • endothelium-dependent vasodilation • subarachnoid hemorrhage
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