Endothelium-Dependent Relaxation of Canine Basilar Arteries
Part 2: Inhibition by Hemoglobin and Cerebrospinal Fluid From Patients With Aneurysmal Subarachnoid Hemorrhage

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The effects of hemoglobin and cerebrospinal fluid from patients with subarachnoid hemorrhage (CSF-SAH) on endothelium-dependent relaxation were studied. At 10^{-6} M, hemoglobin somewhat inhibited the endothelium-dependent relaxation induced by A23187 in rings of canine basilar artery. At 3 \times 10^{-6} M, it almost completely inhibited the same response. At 3 \times 10^{-6} M, hemoglobin did not significantly inhibit smooth muscle relaxation mechanisms as papaverine-induced relaxation was not inhibited by hemoglobin. It was also demonstrated that pretreatment of arterial rings with CSF-SAH resulted in a dose-dependent inhibition of relaxation induced by A23187. The inhibitory effect of CSF-SAH was prominent in the case in which a high oxyhemoglobin concentration was measured by spectrophotometry. Normal CSF from patients without SAH did not affect endothelium-dependent relaxation. These results suggest that hemoglobin released from lysed erythrocytes inhibits endothelium-dependent relaxation of canine basilar arteries and may also play an important role in the pathogenesis of cerebral vasospasm after aneurysmal subarachnoid hemorrhage. (Stroke 1987;18:938-943)

Delayed arterial spasm demonstrated angiographically following subarachnoid hemorrhage (SAH) is still a phenomenon of unknown cause. It has generally been accepted that some vasoactive substances in the extravasated blood might be closely associated with cerebral vasospasm.\(^1\)\(^-\)\(^4\) Among the many vasoconstrictors, hemoglobin has earned attention as a possible cause of spasm.\(^5\)\(^-\)\(^7\) Furthermore, it has been reported that lysed erythrocytes have vasoconstrictive capacity in vitro and that the breakdown product of erythrocytes may be the main factor in chronic vasospasm.\(^8\)\(^-\)\(^10\)

Recent study suggests that hemoglobin inhibits acetylcholine (ACh) or A23187-induced endothelium-dependent relaxation of rabbit aortas by an action that could be related to its binding of endothelium-derived relaxing factor (EDRF).\(^11\)\(^-\)\(^12\) This observation implies that the proposed role for hemoglobin in cerebral vasospasm may be related to its inhibition of an EDRF-dependent mechanism.\(^11\) Therefore, studies of the interaction of hemoglobin and EDRF in cerebral vascular preparations seem worthwhile. Although inhibitory responses to ACh are endothelium-dependent in large feline, porcine, and rabbit cerebral arteries,\(^11\)\(^-\)\(^16\) studies of the interaction of hemoglobin and EDRF in canine basilar arteries have not yet been reported. The present investigation was undertaken to demonstrate the effects of hemoglobin and cerebrospinal fluid (CSF) obtained from 5 patients with SAH on EDRF in canine basilar arteries.

Materials and Methods
Preparation of Basilar Rings and Tension Recording
Eleven mongrel dogs of either sex, weighing about 10 kg, were anesthetized with 30 mg/kg i.v. sodium pentobarbital and killed. After the basilar arteries were isolated from the brains, the experiments were carried out as previously described.\(^17\)

The basic experimental protocol employed a control period during which the arterial rings were contracted with an approximate ED\(_{50}\) concentration of prostaglandin F\(_2\alpha\) (PGF\(_2\alpha\)) and relaxed by the cumulative addition of A23187. Values relative to papaverine-induced relaxation are presented in the text, tables, and figures. As in previous reports,\(^11\)\(^-\)\(^12\) preparations had been treated for 5–10 minutes with hemoglobin or CSF before response to A23187 was obtained.

Clinical Materials and Assessment of Cases
Five patients with ruptured intracranial aneurysms were selected for this study; a summary of the cases is shown in Table 1. Angiography was performed within 5 days after SAH in all patients. Arterial spasm detected on angiography was classified into 3 groups according to the system of Fisher et al\(^18\): 1) no vasospasm, 2) slight to moderate vasospasm, and 3) severe vasospasm. The clinical condition of the patients was graded according to the classification of Hunt and Hess.\(^19\) Four patients underwent craniotomy for clipping of their aneurysm; 1 underwent ventricular CSF drainage only and was treated conservatively. A lumbar CSF drainage system was placed in these patients at the time of aneurysm surgery, left there for 1–2 weeks,
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Table 1. Summary of Cases With Subarachnoid Hemorrhage

<table>
<thead>
<tr>
<th>Case/sex</th>
<th>Clinical</th>
<th>Site of aneurysm</th>
<th>Vasospasm</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/49/F</td>
<td>V</td>
<td>ACoA</td>
<td>N</td>
<td>Dead</td>
</tr>
<tr>
<td>2/52/M</td>
<td>IV</td>
<td>R MCA</td>
<td>S</td>
<td>Disabled</td>
</tr>
<tr>
<td>3/69/F</td>
<td>IV</td>
<td>R VA-PICA</td>
<td>M</td>
<td>Severely disabled</td>
</tr>
<tr>
<td>4/60/F</td>
<td>IV</td>
<td>R MCA</td>
<td>S</td>
<td>Dead</td>
</tr>
<tr>
<td>5/49/M</td>
<td>III</td>
<td>ACoA</td>
<td>N</td>
<td>Excellent</td>
</tr>
</tbody>
</table>

Clinical grade according to Hunt and Hess.19 ACoA, anterior communicating artery; R, right; MCA, middle cerebral artery; VA-PICA, vertebral artery-posterior inferior cerebellar artery. N, none; S, severe; M, slight to moderate.

and then either removed or replaced by internal shunts. The outcome was described as "excellent" when the patient had no residual deficit and as "disabled" when the patient was dependent on help due to major deficits. Death resulted from rebleeding or cerebral infarction.

The CSF samples were preserved at —20° C until used. Normal CSF samples were obtained at the time of lumbar anesthesia in a patient who was otherwise healthy.

Spectrophotometry of Cerebrospinal Fluid

To determine the concentration of oxyhemoglobin, spectrophotometry (Model U-3200 double-beam spectrophotometer, Hitachi Ltd., Tokyo, Japan) was performed on CSF from the 5 patients with SAH. The absorption spectrum was recorded between 350 and 600 nm. The results of spectrophotometry were also expressed according to the method of Kronholm and Lintrup,20 in which the concentrations of hemoglobin (methemoglobin and oxyhemoglobin) and bilirubin are estimated from absorption at 400, 412, and 480 nm.

Preparation of Hemoglobin

Bovine hemoglobin Type I, supplied by Sigma Chemical Co. (St. Louis, Mo.), contains a mixture of oxyhemoglobin and the oxidized derivative, metmethemoglobin. Pure hemoglobin (oxyhemoglobin) was prepared according to methods described in previous reports.11,12

Results

Effect of Hemoglobin on Endothelium-Dependent Relaxation Induced by A23187

Pretreatment of canine basilar arteries with 10^-6 to 3 x 10^-6 M hemoglobin caused a dose-dependent inhibition of relaxation induced by A23187 (Figure 1). At 3 x 10^-6 M, hemoglobin significantly inhibited maximal relaxation to 16.16 ± 11.88% but did not alter the contractile responses to 3 x 10^-6 M PGF_2 alpha (Table 2). The blockade induced with hemoglobin was not inten-

Table 2. Effect of Hemoglobin and CSF From Patients With SAH on PGF_2 alpha-Induced Contraction and A23187-Induced Relaxation in Canine Basilar Artery Rings

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>n</th>
<th>Developed tension (mg)</th>
<th>A23187-induced relaxation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Maximum (%)</td>
</tr>
<tr>
<td>Saline (control)</td>
<td>6</td>
<td>379.5 ± 88.7</td>
<td>78.51 ± 4.74</td>
</tr>
<tr>
<td>3 x 10^-6 M Hemoglobin</td>
<td>5</td>
<td>457.6 ± 90.5</td>
<td>16.16 ± 11.88*</td>
</tr>
<tr>
<td>SAH CSF (μl)</td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Case 1 (100)</td>
<td>5</td>
<td>326.5 ± 56.2</td>
<td>15.63 ± 2.96*</td>
</tr>
<tr>
<td>Case 2 (100)</td>
<td>5</td>
<td>467.3 ± 55.3</td>
<td>40.08 ± 6.42*</td>
</tr>
<tr>
<td>Case 3 (100)</td>
<td>5</td>
<td>467.4 ± 82.6</td>
<td>27.02 ± 3.59*</td>
</tr>
<tr>
<td>Case 4 (100)</td>
<td>9</td>
<td>510.4 ± 67.0*</td>
<td>29.12 ± 14.53*</td>
</tr>
<tr>
<td>Case 5 (100)</td>
<td>10</td>
<td>398.5 ± 57.3</td>
<td>57.35 ± 10.32*</td>
</tr>
<tr>
<td>Normal CSF (μl)</td>
<td>(500)</td>
<td>338.9 ± 40.7</td>
<td>72.83 ± 6.34</td>
</tr>
</tbody>
</table>

SAH, subarachnoid hemorrhage; CSF, cerebrospinal fluid. PGF_2 alpha at 3 x 10^-6 M.

*Different from control (p<0.05).
Relaxation

Relaxing Factor

Effect of Cerebrospinal Fluid From Patients With
Subarachnoid Hemorrhage on Endothelium-Derived
Relaxing Factor

Discussion

significantly enhanced the contractile response to
PGF\textsubscript{2\alpha} (Table 2). The dose–response curve for A23187
was also suppressed by this CSF pretreatment (Figure 3d).

Pretreatment with CSF from Case 5 significantly
inhibited maximal relaxation induced by A23187 with-
out affecting the contractile response to PGF\textsubscript{2\alpha} (Table
2). This inhibitory effect was apparently less than the
effect of other CSF. The dose–response curve for
A23187 was slightly suppressed by this CSF pretreat-
ment (Figure 3e).

Pretreatment with 500 µl normal CSF did not affect
the dose–response curves for A23187-induced relax-
atation (Figure 3f). There was no significant difference
between the pD\textsubscript{2} for control (100 µl saline) and those
for normal CSF (Table 2).

The amount of oxyhemoglobin in CSF estimated by
the optical densities was greater in Cases 1, 3, and 4
than in Cases 2 and 5 (Figure 4). Maximal relaxation in
the canine basilar artery rings pretreated with 100 µ1
CSF from each case is indicated in Figure 4. When
Case 5 is compared with the other cases, we suggest
that the important factor in the inhibition of EDRF may
be the amount of oxyhemoglobin in the CSF. How-
ever, the number of patients is too small to determine a
statistical significance; therefore, a correlation be-
tween the inhibitory effect of CSF from patients with
SAH on EDRF and clinical outcome of the patients
warrants further investigation.

Effect of Hemoglobin on Papaverine-Induced
Relaxation

Effect of Cerebrospinal Fluid From Patients With
Subarachnoid Hemorrhage on Endothelium-Derived
Relaxing Factor

The intense blockade of A23187-induced relaxation
produced by hemoglobin prompted an attempt to deter-
mine whether the endothelium-dependent relaxation
could be blocked by other materials, such as CSF from
patients with SAH.

Pretreatment with CSF from Cases 1, 2, or 3 signifi-
cantly inhibited maximal relaxation induced by
A23187 without affecting the contractile responses to
PGF\textsubscript{2\alpha} (Table 2). The dose–response curves for
A23187 were suppressed by pretreatment with these
samples of CSF (Figure 3, a, b, and c).

Pretreatment with CSF from Case 4 significantly
inhibited maximal relaxation induced by A23187 and
sifted noticeably with longer exposure and was com-
pletely reversed 20 minutes after the hemoprotein was
washed out of the bathing medium. The concentration
of hemoglobin tested was less than that reported by
Martin et al,\textsuperscript{11} who used rabbit aorta rings to investi-
gate the effect of hemoglobin on endothelium-depen-
dent relaxation induced by A23187. Canine basilar
arteries may be more susceptible to hemoglobin than
rabbit aortas.

Effect of Hemoglobin on Papaverine-Induced
Relaxation

To determine the selectivity of the inhibitory effect
of hemoglobin, we examined the effect of hemoglobin
on the relaxation induced by papaverine. At 3 × 10\textsuperscript{-6}
M, hemoglobin did not affect relaxation stimulated by
papaverine (Figure 2), suggesting that the inhibitory
effect of hemoglobin is specific for the endothelium-
dependent relaxation induced by A23187. Canine basilar
arteries may be more susceptible to hemoglobin than
rabbit aortas.

Reportedly, hemoglobin is a powerful inhibitor of
the endothelium-dependent relaxation induced by ACh
and A23187, as well as an inhibitor of the endoteli-
ium-independent relaxation induced by glyceryl trini-
trate in rabbit aortas.\textsuperscript{11} Hemoglobin inhibits the stimu-
lation of soluble guanylate cyclase in cell-free sys-
tems,\textsuperscript{21}\textsuperscript{23} and inhibition of this enzyme may be the
basis for its blocking action in rabbit aortas since the
increases in cyclic GMP content that normally accom-
pany relaxation induced by ACh, A23187, and gly-
ceryl trinitrate are abolished.\textsuperscript{11} The present experi-
ments confirm that hemoglobin potentially inhibits
endothelium-dependent relaxation in canine basilar ar-
tery rings. It is indeed of interest that the inhibition of
endothelium-dependent relaxation by hemoglobin may
be linked to cerebral vasospasm after SAH. In this
regard, hemoglobin also appears to inhibit the action of
a neurotransmitter of a nonadrenergic, noncholinergic
nerve that relaxes certain smooth muscles in associ-
ation with a rise in cyclic GMP levels.\textsuperscript{24}\textsuperscript{25} As a non-
adrenergic, noncholinergic nerve appears to be present
in cerebral vessels,\textsuperscript{14}\textsuperscript{26}\textsuperscript{27} blockade of neurotrans-
mission by hemoglobin\textsuperscript{28} may also contribute to cerebral
vasospasm.

Hemoglobin is also a relatively potent contractile
agent in the isolated canine cerebral artery prepara-
tion.\textsuperscript{9}\textsuperscript{10} The vasoconstrictor effect of hemoglobin
appears to depend on the oxidation state of the com-
ound\textsuperscript{5}\textsuperscript{6}\textsuperscript{10} since ferrous hemoglobin (oxyhemoglobin)
produces much greater contraction than does ferric

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Dose–response curves showing effects of pretreat-
ment with 3 × 10\textsuperscript{-6} M hemoglobin (○) on papaverine-induced
relaxation in canine basilar artery rings. ●, control.}
\end{figure}
hemoprotein (methemoglobin). Furthermore, oxyhemoglobin is a more potent inhibitor of EDRF than is methemoglobin, suggesting that the oxidation state of iron in the hemoprotein is important for inhibitory activity. Therefore, we used bovine hemoglobin after treatment with a reducing agent. It is unlikely that the blocking action of hemoglobin is due to its vasoconstrictive activity because pretreatment with $10^{-6}$ to $3 \times 10^{-6}$ M hemoglobin did not affect the contractile response to PGF$_{2\alpha}$ (Table 2) and because hemoglobin did not affect relaxation stimulated by papaverine in canine basilar arteries.

The present study demonstrates that the CSF obtained from patients with SAH significantly inhibits endothelium-dependent relaxation of canine basilar arteries. The concentration of oxyhemoglobin in the CSF was determined by spectrophotometry. Several authors have demonstrated that the maximum absorption of oxyhemoglobin is near 412 nm. The greater the concentration of oxyhemoglobin, the more inhibited the EDRF becomes (Figure 4). Although the mechanism of this inhibition is not fully understood, it is postulated that the release of oxyhemoglobin from lysed erythrocytes in the CSF might be involved in the genesis of the inhibition.

Clinically, it is well known that vasospasm is most frequently observed in the arteries near ruptured intracranial aneurysms. In addition, computed tomography strongly suggests the close relation of the incidence and location of vasospasm to the size and location of SAH.
distribution of subarachnoid clot. It has been demonstrated that oxyhemoglobin, bilirubin, and methemoglobin are the pigments that most commonly develop in colored CSF and that oxyhemoglobin appears at the onset of SAH, reaches a maximum during the first few days, and then gradually decreases. Moreover, it has been generally accepted that late vasospasm usually develops several days after SAH and persists for a few weeks or more. These findings may indicate that the progressive release of oxyhemoglobin from a blood clot during the hemolytic process in the subarachnoid space coincides with the time course of chronic vasospasm.

However, in most SAH patients, blood is found in the adventitia of the constricted vessels. This blood may be simply an indicator of extensive hemorrhage or also may be important for the pathogenesis of vasospasm. Zervas et al postulated that adventitial channels found on ultrastructural examination may act as a nutritional or waste disposal pathway in cerebral vessels. It was also demonstrated that horseradish peroxidase or tritiated leucine injected into the ventricles readily enters cerebral vessel walls via the vessel's adventitial surface and that a large molecular weight channel found on ultrastructural examination may act as a nutritional or waste disposal pathway in cerebral vessels. Horseradish peroxidase (horseradish peroxidase) penetrated as far as the endothelial cell basal lamina. Therefore, hemoglobin or other breakdown products of red blood cells may use these channels to enter the vessel wall and thus regulate EDRF.

Finally, our experiments have led us to the conclusion that hemoglobin released from lysed erythrocytes inhibits endothelium-dependent relaxation of canine basilar arteries and may be one of the causative factors for cerebral vasospasm in SAH patients.

Acknowledgment

The authors wish to thank Dr. Yukio Shimizu for his invaluable collaboration during the course of this work.

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Key Words • endothelium-derived relaxing factor • subarachnoid hemorrhage • hemoglobin • cerebrospinal fluid • cerebral vasospasm
Endothelium-dependent relaxation of canine basilar arteries. Part 2: Inhibition by hemoglobin and cerebrospinal fluid from patients with aneurysmal subarachnoid hemorrhage.

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Stroke. 1987;18:938-943
doi: 10.1161/01.STR.18.5.938

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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