Effect of Hemorrhagic Hypotension on Cerebrovascular Reactivity and Ultrastructure in the Cat

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Background and Purpose: The goal of this study was to determine the alterations in contractile and dilatory responses and ultrastructure of the feline middle cerebral artery after hemorrhagic hypotension.

Methods: In the sodium pentobarbital anesthetized cats, a steady 50 mm Hg level of hypotension was reached by bleeding into a reservoir and maintained at this level by further bleeding or autotransfusion for 2 hours. Rings of the arteries, from control animals and from animals after hypotension, were suspended for isometric tension recording in organ chambers filled with modified Krebs-Henseleit solution, aerated with 95% O₂-5% CO₂ at 37°C, and their reactions to contractile and relaxant agents were tested. Vascular ultrastructure was studied by electron microscope.

Results: Endothelium-dependent relaxations induced by 10⁻⁴ M acetylcholine were enhanced, whereas there was a marked inhibition of the relaxation at 10⁻⁶ M. Relaxations induced by adenosine triphosphate and adenosine showed an impairment. Contractions induced by norepinephrine and prostaglandin F₂α remained unchanged, whereas 5-hydroxytryptamine caused a more pronounced contraction after hypotension. No alterations in the morphology of endothelium or smooth muscle were found after hemorrhage. There was, however, a marked decrease in the number of transmitter vesicles in the perivascular nerve terminals.

Conclusions: The present results show marked alterations in cerebrovascular reactivity and ultrastructure of the adventitia after hypotension. These alterations might play an important role in the development of cerebral vasoconstriction during and after this hemorrhagic state. (Stroke 1991;22:1541-1547)

Endothelium-dependent relaxation, a key mechanism regulating vascular function plays an important role in the maintenance of the cerebrovascular resistance under resting conditions.¹⁻⁴ Endothelium-derived relaxing factor (EDRF) released from the vascular endothelium has been identified as nitric oxide also in the cerebral vessels.³⁻⁴

Cerebrovascular reactivity is profoundly altered in several pathophysiological states, including hypertension and ischemia-reperfusion.²⁻⁵ One of the most important alterations is the dysfunction of the vascular endothelium, which has been suggested to play a role in the impairment of the organ perfusion.

There is a general ischemia of the tissues during hemorrhagic hypotension and shock. In later phases of shock, retransfusion of the blood does not restore the depressed organ blood flow.⁷ Such phenomena, as well as marked metabolic and circulatory alterations, have also been observed in the brain during hypoxia and shock, in spite of the well-developed cerebral autoregulatory mechanisms.⁷⁻⁹ The exact processes leading to the development of such no-reflow phenomenon are not yet clarified. The possible alterations in the cerebrovascular, in particular, endothelium-dependent responsiveness in hemorrhagic hypotension, have not been investigated previously. In the present work we examined the changes in the contractile and dilatory responsiveness of the middle cerebral artery of the cat after hemorrhagic hypotension. Contractions were induced by norepinephrine, prostaglandin F₂α, and 5-hydroxytryptamine, whereas relaxations...
were induced by acetylcholine, adenosine triphosphate (ATP), and adenosine. Possible alterations in the vascular responsiveness to these agents might have a pronounced influence on the cerebral blood flow, since most of the vasoactive agents tested in the present study are involved in the regulation of the cerebral circulation.\(^1\) We also investigated whether the changes in reactivity were accompanied by changes of the ultrastructure of the intima, media, and adventitia of the vessels.

**Materials and Methods**

Eighteen cats of both sexes, weighing 1.9–2.8 kg, were anesthetized with sodium pentobarbital (Nembutal, 30 mg/kg i.p.) and ventilated with a Harvard respirator. Tidal volume was controlled, and blood gases were kept within physiological ranges. Arterial blood pressure was measured through a femoral artery with a Grass pressure transducer and polygraph. A steady 50 mm Hg level of hypotension was reached by bleeding into a reservoir through the contralateral femoral artery, and it was maintained at this level by further bleeding or autotransfusion during 2 hours (nine cats). Rectal temperature was measured and maintained at 37°C during the experiments. The anesthetized control animals (nine cats) and the hemorrhaged animals were killed by exsanguination via a femoral artery catheter.

After quickly removing the brain from the skull, we carefully removed the middle cerebral artery using microsurgical methods and a Zeiss operation microscope. We studied several segments from each cat. One- to 3-mm-long vessel segments were placed on two L-shaped stainless steel specimen holders (0.1 mm in diameter), one of which was attached to a Grass FT03 force transducer. The position of the other holder could be adjusted by a micromanipulator. The preparations were immersed into a tissue chamber containing Krebs-Henseleit solution of the following millimolar composition: NaCl 119, KCl 4.6, CaCl\(_2\) 1.5, MgCl\(_2\) 1.2, NaHCO\(_3\) 15, NaH\(_2\)PO\(_4\) 1.2, and glucose 6. The solution was bubbled with a gas mixture containing 95% O\(_2\) and 5% CO\(_2\), the temperature was kept at 37°C, and the pH was 7.4.\(^{11}\) The vessels were incubated for 60 minutes at a tension of 4–500 mg. Norepinephrine, prostaglandin F\(_{2\alpha}\), and 5-hydroxytryptamine were applied cumulatively. Acetylcholine, ATP, and adenosine were applied cumulatively after a stable precontractile tone was induced by 5×10\(^{-6}\) M prostaglandin F\(_{2\alpha}\). The effect of N\(^\text{6}\) -nitro-L-arginine (10\(^{-4}\) M) was also studied on the relaxant responses of control arteries. All measurements were carried out in the presence of 5×10\(^{-6}\) M indomethacin and 5×10\(^{-7}\) M propranolol to block the cyclooxygenase enzyme and \(\beta\)-receptor activation, respectively. Contractile responses are presented in milligrams, and dilatatory responses are expressed in the percent of the preconstricted tension.

For the morphological studies, segments 1 mm long were cut from the vessels and placed into a 4°C fixative solution containing 2.5% paraformaldehyde and 2.5% glutaraldehyde in 1.0 M phosphate buffer. The materials were then postfixed in Epon. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined with a Tesla BS 500 electron microscope.

All drugs were obtained from Sigma Chemical Co., St. Louis, Mo., and dissolved in saline, except indomethacin and prostaglandin F\(_{2\alpha}\), which were dissolved in 50% ethanol.

Student’s t test for unpaired samples was used for comparison of the control responses and the responses of the vessels from the animals after hemorrhage, and a difference was considered as significant if \(p<0.05\). Data are presented as mean±SEM.

**Results**

Contractions were studied in the concentration range of 10\(^{-8}\) to 10\(^{-7}\) M. The contractions induced by norepinephrine and prostaglandin F\(_{2\alpha}\) showed no significant alterations in the vessels from the hemorrhage-subjected cats when compared with the responses of the control animals (Figure 1, left and middle parts). In contrast, 5-hydroxytryptamine–induced contractions showed statistically significant enhancement at lower concentrations (10\(^{-8}\) and 10\(^{-7}\) M) after this hypotensive state (Figure 1, right part). Acetylcholine-, ATP-, and adenosine-evoked relaxations were studied in the concentration range of 10\(^{-8}\) to 10\(^{-6}\) M (acetylcholine), 10\(^{-8}\) to 10\(^{-6}\) M (ATP), and 10\(^{-8}\) to 10\(^{-5}\) M (adenosine). After hemorrhage, acetylcholine caused a more pronounced relaxation than in the control vessels at 10\(^{-8}\) M. Elevation of the concentration of acetylcholine did not result in further relaxations, and the dilatory action was significantly inhibited at 10\(^{-6}\) M (Figure 2, left panel).

In the vessels, prepared from the animals after hemorrhage, there was a statistically significant inhibition of the ATP-induced relaxations at 10\(^{-6}\) M. Furthermore, at concentrations higher than 10\(^{-6}\) M a pronounced attenuation of the adenosine-induced relaxations was found. (Figure 2, middle and right panels).

The finding that also adenosine-induced relaxation showed an impairment after hemorrhagic shock can be explained either by a nonspecific attenuation of the relaxant ability of the vessel after shock or by a possible endothelium- or nitric oxide–mediated component of the adenosine-induced relaxations in the present vascular bed (see “Discussion”). To clarify which of these possibilities is true, we tested the effect of N\(^\text{6}\) -nitro-L-arginine, a specific blocker of nitric oxide synthesis on acetylcholine-, ATP-, and adenosine-induced relaxations. As expected, N\(^\text{6}\) -nitro-L-arginine (10\(^{-4}\) M) inhibited acetylcholine-induced relaxations (relaxations of 98.4±1% and 7.4±6.4% of the response to 10\(^{-6}\) M acetylcholine for vessels before and after N\(^\text{6}\) -nitro-L-arginine treatment, respectively, \(n=5\), \(p<0.01\)), and ATP-induced relaxations (relaxations of 92.8±3.9% and 41.2±13.7% of the response to 10\(^{-6}\) M ATP for vessels before and after N\(^\text{6}\) -nitro-L-arginine treat-
ment, respectively, \( n=5, p<0.05 \). Inhibition was also found when the effect of \( N^G \)-nitro-L-arginine on adenosine-induced relaxations was tested (relaxations of 81.6±6.4\% and 22.6±6.1\% of the response to \( 10^{-6} \) M adenosine for vessels before and after \( N^G \)-nitro-L-arginine treatment, respectively, \( n=5, p<0.01 \)).

Electron microscopic studies showed no alterations in the endothelial or smooth muscle layer of the blood vessels after hemorrhage (not shown). In the adventitial layer, however, marked changes were found. In the control animals, a number of transmitter vesicles—small clear, small granulated, and large granulated vesicles—were seen in the nerve terminals (Figure 3). After hypotension, however, the number of vesicles decreased markedly; only a few granulated vesicles of 80–100 nm diameter remained in the nerve fibers. Only neurofilaments, microtubules, and mitochondria could be observed in the nerve terminals (Figure 4).

**Discussion**

Although the effect of ischemia, hemorrhagic hypotension, and shock on cerebral circulation and metabolism has been extensively studied in various models,\(^7-9\) there were no available data examining the effect of hemorrhagic hypotension on the vaso-motor responses of the isolated cerebral artery. There are several new findings of the present study. First, there is a profound impairment of the endothelium-dependent relaxation of the feline middle cerebral artery in response to \( 10^{-6} \) M acetylcholine

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**Figure 1.** Contractile responses of isolated middle cerebral artery rings to norepinephrine (NE), prostaglandin \( F_2\alpha \) (PGF\( _2\alpha \)), and 5-hydroxytryptamine (5-HT). •, Control vessels; ○, vessels from animals after hemorrhagic hypotension. Asterisks indicate significant differences between the responses of the vessels from the control and hemorrhage-subjected groups. Values are mean±SEM; \( n \), number of vessel segments.

**Figure 2.** Dilatory responses of isolated middle cerebral artery rings to acetylcholine (ACh), adenosine triphosphate (ATP), and adenosine (ADO). •, Control vessels; ○, vessels from animals after hemorrhagic hypotension. Data are expressed as a percentage of the initial vascular tone induced by prostaglandin \( F_2\alpha \) (5\( \times \)10\(^{-6} \) M). Asterisks indicate significant differences between the responses of the vessels from the control and hemorrhage-subjected groups. Values are mean±SEM; \( n \), number of vessel segments.
and ATP after hemorrhage. In addition, relaxations caused by adenosine are also inhibited after this hypotensive state. Second, relaxations to acetylcholine, but not ATP or adenosine, are markedly enhanced at $10^{-8}$ M. Third, contractile responses to 5-hydroxytryptamine, but not norepinephrine or prostaglandin $F_2\alpha$, are enhanced at lower concentrations. Fourth, the alterations of the vascular response are not accompanied by any ultrastructural change of the endothelium or smooth muscle. In contrast, a marked degranulation of the transmitter vesicles in the nerve terminals was found in the adventitia. Acetylcholine and ATP have been shown to dilate arteries in an endothelium-dependent fashion.\textsuperscript{1,2,12} The nature of the adenosine-induced relaxations, however, seems to vary between species and vascular beds.\textsuperscript{13-15} In some vessels, such as the rat\textsuperscript{14} or pig\textsuperscript{15} aorta, the relaxant action of adenosine has two components: first, release of EDRF from the endothelium, and second, a direct relaxant effect on the smooth muscle. We found, that $N^O$-nitro-L-arginine, a potent inhibitor of the nitric oxide/EDRF producing enzyme,\textsuperscript{16} markedly inhibits the relaxation elicited by adenosine in the present vascular preparation, suggesting a nitric oxide-depen-
dent component in its relaxant action. This finding is in contrast to previous results, where the relaxant effect of adenosine was still present after endothelium removal in the same vascular preparation. An explanation for this difference might be that adenosine releases nitric oxide directly from the smooth muscle, causing an endothelium-independent but nitric oxide-dependent relaxation. The existence of smooth muscle–derived nitric oxide has recently been shown by Wood et al.

We found a marked impairment of the relaxations induced by acetylcholine ($10^{-6}$ M), ATP ($10^{-6}$ M), and adenosine ($10^{-9}$–$10^{-3}$ M) after hemorrhagic hypotension. These changes suggest that the nitric oxide–mediated relaxing capacity of the vessel is impaired probably because of a decreased production or enhanced degradation of nitric oxide after hemorrhagic shock. Similar impairment of the relaxant responses to acetylcholine ($5 \times 10^{-6}$ M) as well as to adenosine ($10^{-4}$ M) were found after cerebral ischemia followed by reperfusion in cat pial arteries as measured in vivo.

In addition, we found an enhanced relaxant response at low dose ($10^{-8}$ M) in the case of acetylcholine. It should be pointed out that acetylcholine-
induced endothelium-dependent relaxations or EDRF release might be biphasic or might have two thresholds in some vascular beds, and that these different phases are not necessarily mediated by the same relaxing substance.\textsuperscript{19–21} Thus, one possible explanation for the present finding might be that hemorrhagic hypotension differentially affects these different endothelial pathways leading to the release of EDRFs.

The results showed that 5-hydroxytryptamine-induced contractile responses are enhanced after hemorrhagic shock, whereas prostaglandin $F_{2\alpha}$- and norepinephrine-induced contractions remained unchanged. 5-Hydroxytryptamine, acting on 5-hydroxytryptamine-$\textsubscript{1}$ receptors of the endothelium, causes endothelium-dependent relaxations. Contractile responses to 5-hydroxytryptamine are enhanced in endothelium-denuded preparations.\textsuperscript{22} Such a mechanism might explain this enhancement of this contractile response after hemorrhage. As expected, the responses to prostaglandin $F_{2\alpha}$, an agonist which does not interfere with endothelium-dependent processes, remained unchanged after shock. Interestingly, however, the contractile response to norepinephrine, an agonist, which also has an EDRF-mediated dilatory component\textsuperscript{22} did not show significant enhancement.

No alterations in the ultrastructure of the endothelium or smooth muscle layer of the cerebral artery were found presently after this hemorrhagic state. Similarly, interleukin-2 infusion or coronary ischemia and reperfusion have been shown to depress endothelium-dependent relaxation with only occasional changes of the endothelium.\textsuperscript{23,24}

We found, however, marked alterations in the ultrastructure of the perivascular nerve terminals: a nonselective degranulation of the transmitter vesicles. Cerebral vessels have a dense perivascular innervation. On the basis of histochemical techniques as many as 12 transmitter candidates have been demonstrated in nerve terminals associated with the cerebrovascular bed.\textsuperscript{25} Among others, cerebral vessels have a dense sympathetic innervation.\textsuperscript{25–27} Norepinephrine is mainly stored in the large and small granular vesicles of the postganglionic nerve fibers. The presence of small granular vesicles is diagnostic for norepinephrine containing neurons.\textsuperscript{25–27} The present finding, showing a depletion of these vesicles in the nerve terminals, is consistent with the well-known sympathetic activation during hemorrhagic hypotension,\textsuperscript{7–9} as well as with the experimental data showing a marked depletion of norepinephrine from the nerve terminals in hemorrhagic shock.\textsuperscript{7,28} The present data also suggest that similarly to norepinephrine, all other perivascular transmitters like neuropeptide Y, substance P, or calcitonin gene–related peptide can be released during this hemorrhagic state. Further studies are needed to elucidate whether there is a correlation between the endothelial dysfunction after hemorrhagic hypotension and the changes in the perivascular morphology. In this regard it is worthwhile to notice that perivascular depletion of substance P and calcitonin gene–related peptide by capsaicin depresses acetylcholine-induced arterial relaxations.\textsuperscript{39}

Cytokine interactions are activated and play an important role in various shock states, including hemorrhagic shock. They cause the adherence of platelets and neutrophils to the endothelial surface followed by their activation. The concomitant cytokine feedback processes and free radical release might lead to microcirculatory collapse and endothelial cell impairment.\textsuperscript{30} Some cytokines and also free radicals have been shown to inhibit endothelium-dependent relaxations.\textsuperscript{31–33} Free radicals as final mediators causing endothelial cell dysfunction have been also implicated in several other pathophysiological conditions, including hypertension and ischemia-reperfusion,\textsuperscript{23,6,24,30} and might also be involved in the present changes. Another mechanism responsible for the endothelial dysfunction might be the exhaustion of the endothelium-dependent relaxant capacity due to an excessive release of EDRF during shock. Many humoral factors that are known to be released during hemorrhage have endothelium-dependent relaxant actions and can contribute to such exhaustion. These agents include catecholamines, arginine vasopressin, histamine, 5-hydroxytryptamine, bradykinin, angiotensin, and purine derivatives.\textsuperscript{7,34} There are also other possibilities, such as changes at receptorial level. Such changes (in opiate receptors) have been shown even after shorter ischemic periods in the brain.\textsuperscript{35} An enhanced production of endothelium-derived contractile factors\textsuperscript{36} is another possible explanation, although, considering the presence of indomethacin in our studies, if an endothelium-derived contractile factor is involved, it is not a cyclooxygenase product. All these possible mechanisms, however, do not answer the question why acetylcholine-induced relaxations are enhanced at low dose in the present experiments.

In conclusion, the present results demonstrate that cerebrovascular relaxations are impaired at higher agonist concentrations after hemorrhagic hypotension, which is similar to the abolishment of the endothelium-dependent relaxations in the cerebral circulation after ischemia-reperfusion.\textsuperscript{10} Contractile responsiveness, however, is maintained or even facilitated. These changes in vascular reactivity might elevate arterial tone leading to the impairment of the cerebral perfusion during and after hemorrhagic shock.

\section*{Acknowledgments}

The authors express their gratitude to G. Kiss and G. Csubá for the technical assistance and to E. Molnár for making the drawings.

\section*{References}


Key Words • endothelium, vascular • hypotension • cats
Effect of hemorrhagic hypotension on cerebrovascular reactivity and ultrastructure in the cat.

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*Stroke*. 1991;22:1541-1547
doi: 10.1161/01.STR.22.12.1541

*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

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/22/12/1541

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