Endothelium-Dependent and -Independent Responses to Vasodilators of Isolated Dog Cerebral Arteries

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We compared responses to calcium ionophore A23187, vasopressin, and substance P in helical strips of dog middle cerebral, basilar, and posterior communicating arteries to obtain a better understanding of humoral control of cerebrovascular tone in different brain regions and its potential impact on mechanisms of cerebral vasospasm. A23187 relaxed these different arterial strips partially precontracted with prostaglandin F$_2$ to a similar extent. Vasopressin produced concentration-dependent relaxation in basilar and posterior communicating arterial strips, whereas middle cerebral arterial strips either contracted or relaxed slightly. Relaxations induced by A23187 and vasopressin were either abolished or converted to contractions by removal of the endothelium. In contrast, the relaxation of cerebral arterial strips to substance P was markedly attenuated but not abolished by endothelium denudation; the remaining relaxation was suppressed by indomethacin. In some cerebral arterial strips with intact endothelium, substance P caused a transient contraction that was reversed to relaxation by indomethacin or ONO-3708, a prostaglandin antagonist. In arterial strips denuded of endothelium from the same dogs, substance P always produced relaxations. Relaxations of cerebral arterial strips to A23187 and vasopressin appear to be mediated by endothelium-derived relaxing factor. The function of vasopressin receptors in endothelial cells differs markedly in basilar and posterior communicating arteries versus middle cerebral arteries. Substance P-induced relaxations appear to be primarily associated with endothelium-derived relaxing factor and with prostaglandin I$_2$, whereas contractions appear to be mediated by endothelium-derived prostaglandins. (Stroke 1988;19:1388–1394)

Vascular endothelium plays an important role in vasodilation induced by various chemical substances, including acetylcholine, bradykinin, angiotensin II, histamine, ATP, substance P, and calcium ionophore.\textsuperscript{1,2} Vasodilator substances such as endothelium-derived relaxing factor (EDRF)\textsuperscript{1} and cyclooxygenase products, mainly prostaglandin (PG) I$_2$,\textsuperscript{3,4} are liberated by chemical stimulation of endothelial cells. Removal or damage of the endothelium, the source of the vasodilators, is expected to abolish or reverse vasodilation to vasoconstriction or to potentiate vasoconstriction. Since hemoglobin markedly inhibits endothelium-dependent relaxation,\textsuperscript{5} impaired endothelial cell function has been proposed as a mechanism of cerebral vasospasm following subarachnoid hemorrhage.\textsuperscript{6–8} Cerebral arteries from different brain regions differ in their responsiveness to chemical stimuli.\textsuperscript{9} These differences in response, which may be mediated by EDRF and PGs, have not been defined although such heterogeneity in responsiveness could influence the genesis of cerebral vasospasm.

Vasopressin and calcium ionophore A23187 reportedly produce endothelium-dependent relaxation in dog basilar arteries.\textsuperscript{10,11} Substance P is proposed to be a neurotransmitter or a neuromodulator in cerebral perivascular nerve fibers\textsuperscript{12–14} and induces endothelium-dependent relaxation in peripheral arteries from various species.\textsuperscript{15} A recent study suggested a possible involvement of depressed immunoreactivity of neuronal substance P after subarachnoid hemorrhage in the genesis of cerebral vasospasm.\textsuperscript{16} However, little is known concerning the mechanism of action of substance P in cerebral arteries. Therefore, our study was undertaken to determine the mechanism of action of substance P in dog cerebral arteries and to compare responses to substance P, vasopressin, and A23187 in dog middle cerebral, basilar, and posterior communicating arteries.
Materials and Methods

Forty-five mongrel dogs of either sex weighing 8–15 kg were anesthetized with 50 mg/kg i.p. thiopental sodium and were killed by bleeding from the common carotid arteries; the brains were rapidly removed. Middle cerebral and basilar arteries and posterior portions of the circle of Willis (posterior communicating arteries) were isolated from the brains and were helically cut into strips approximately 20 mm long. The arterial strips were fixed vertically between hooks in a muscle bath containing a modified Locke-Ringer solution of the following millimolar composition: 120 NaCl, 5.5 KCl, 2.2 CaCl$_2$, 1.0 MgCl$_2$, 25.0 NaHCO$_3$, and 5.6 dextrose, maintained at 37 ± 0.3° C and aerated with 95% O$_2$ and 5% CO$_2$. The hook anchoring the upper end of the arterial strips was connected to the lever of a force-displacement transducer (Nihon-kohden Kogyo Co., Tokyo, Japan). Resting tension was adjusted to 1.5 g, which is optimal for inducing maximal contraction. Before the start of the experiments, all arterial strips were allowed to equilibrate for 90–120 minutes in the solution, which was replaced every 10–15 minutes.

Isometric contractions and relaxations were displayed on an ink-writing oscillograph (Sanei Sokki Co., Tokyo). Contraction to 30 mM K$^+$ was measured, then the arterial strips were repeatedly washed and equilibrated for 30 minutes. Arterial strips were partially contracted with 3 × 10$^{-7}$ to 3 × 10$^{-4}$ M PGF$_2$- before the addition of vasodilator agents; contractions ranged between 25% and 35% of that induced by 30 mM K$^+$. The concentration–response relations for vasopressin and A23187 were obtained by adding the agents directly to the solution in cumulative concentrations. However, substance P in single experiments because of tachyphylaxis. At the end of each experiment, 10$^{-4}$ M papaverine was added to obtain maximal relaxation. The agonist-induced relaxation relative to that induced by papaverine and the contraction relative to that induced by 30 mM K$^+$ are presented in the text and figures.

The endothelium was removed by gently rubbing the intimal surface of the arterial strips with a cotton pellet. Unrubbed arterial strips from the same dogs were compared. Preservation or removal of the endothelium was verified by the AgNO$_3$ staining method.

Results are expressed as mean ± SEM and were compared using Student’s paired and unpaired $t$ tests and Tukey’s method after one-way analysis of variance. The agents used were substance P, [Arg$_8$]-vasopressin (Protein Research Foundation, Osaka, Japan), calcium ionophore A23187 (C.H. Boehringer Ingelheim Ltd., Elmsford, New York), indomethacin, methylene blue trihydrate (Nakarai Chemicals, Ltd., Kyoto, Japan), ONO-3708 (9,11),(11,12)-dideoxa-9a,11a-dimethylmethano-11,12-methano-13,14-dihydro-13-azo-14-oxo-15-cyclopentyl-16,17, 18,19,20-pentanor-15-epithrromboxane A$_2$ (Ono Pharmaceutical Co., Osaka), PGF$_2$ tris(hydroxy-
methyl)aminomethane salt (Nippon Upjohn Ltd., Tokyo), serotonin creatinine sulfate (Merck, Darmstadt, FDR), and papaverine hydrochloride (Dainippon Pharmaceutical Co., Osaka).

The addition of 10$^{-10}$ to 10$^{-8}$ M substance P produced a concentration-dependent relaxation in two middle cerebral, two basilar, and one posterior communicating arterial strips partially contracted with 3 × 10$^{-7}$ to 3 × 10$^{-6}$ M prostaglandin F$_2$-.

Significantly different from values obtained in the presence of endothelium, p < 0.001.
the second or third trial, $10^{-7}$ M substance P produced a phasic contraction followed by relaxation. The remaining 17 (six middle cerebral, six basilar, and five posterior communicating) arterial strips responded only with relaxation of similar magnitudes during repeated trials. Maximal relaxation was obtained $0.65 \pm 0.08$ minutes ($n=14$) after the addition of $10^{-7}$ M substance P, and the initial level of tension was slowly restored in 5–12 minutes. The relaxation in endothelium-denuded arterial strips developed more slowly than in intact arterial strips; the peak response was obtained $1.71 \pm 0.18$ minutes ($n=14$, $p<0.001$) after the addition of $10^{-7}$ M substance P.

In the 17 arterial strips responding only with relaxation, effects of indomethacin and methylene blue on substance P–induced relaxation were investigated. When endothelium was present, treatment with $10^{-6}$ M indomethacin failed to significantly reduce the relaxation (Figure 2) but tended to slow the recovery of tension from the maximally relaxed level; recovery half times before and after indomethacin treatment were $2.76 \pm 0.40$ and $3.83 \pm 0.50$ minutes, respectively ($57.0 \pm 19.5\%$ prolongation, $n=14$, $p<0.02$). Relaxation was markedly attenuated by additional treatment with $10^{-5}$ M methylene blue (Figure 2, left). In contrast, relaxation obtained in endothelium-denuded arterial strips was significantly suppressed by treatment with $10^{-5}$ M indomethacin alone (Figure 2, right).

In six (one middle cerebral, three basilar, and two posterior communicating) arterial strips with intact endothelium from six separate dogs in which phasic contractions became dominant during repeated applications of $10^{-7}$ M substance P, the response was reversed to relaxation by treatment with $10^{-6}$ M indomethacin and $10^{-7}$ M ONO-3708, an antagonist to vasoconstrictor PGs (Figure 3). In the endothelium-denuded arterial strips obtained from these same dogs, $10^{-7}$ M substance P always produced relaxations during repeated applications. The addition of $10^{-8}$ and $10^{-7}$ M A23187 caused a concentration-dependent relaxation in arterial strips partially contracted with PGF$_{2\alpha}$. There was no significant difference in the magnitudes of relaxation in the middle cerebral, basilar, and posterior communicating arteries (Figure 4). Relaxation was abolished almost completely by removal of the endothelium.
In basilar and posterior communicating arterial strips partially contracted with PGF$_{2a}$, the addition of $10^{-10}$ to $10^{-8}$M vasopressin caused concentration-dependent relaxations (Figure 5, middle and right). On the other hand, in seven of 12 middle cerebral arterial strips, the same doses of vasopressin produced concentration-dependent contractions despite the presence of endothelium. In the remaining five middle cerebral arterial strips, up to $10^{-8}$M vasopressin produced only relaxation, which, however, was significantly less than that in the basilar and posterior communicating arterial strips (Figure 5, left). Increasing the concentration of vasopressin to $10^{-7}$M produced a contraction from the maximally relaxed level. Relaxation induced in the posterior communicating arterial strips by vasopressin was abolished by removal of the endothelium, and relaxations in the middle cerebral and basilar arterial strips were reversed to slight contractions (Figure 5). The concentration-dependent contraction seen in seven middle cerebral arterial strips with intact endothelium was not influenced by endothelium denudation (Figure 5, left).

To confirm the different responses of middle cerebral arterial strips, the effects of substance P, A23187, and vasopressin were compared in the

**Figure 4.** Graph of relaxation of dog middle cerebral (MCA), basilar (BA), and posterior communicating (PCA) arterial strips with (●) and without (○) endothelium in response to application of A23187. Arterial strips were partially contracted with $3 \times 10^{-7}$ to $3 \times 10^{-6}$M prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$). Relaxation induced by $10^{-4}$M papaverine was taken as 100%; mean±SEM absolute values in intact and endothelium-denuded strips were $328\pm39$ (n=12) and $215\pm30$ (n=12) mg for MCA, $265\pm34$ (n=12) and $221\pm29$ (n=12) mg for BA, $201\pm33$ (n=10) and $210\pm17$ (n=10) mg for PCA, respectively. Significantly different from control at a) *p*<0.001, b) *p*<0.01, or c) *p*<0.05.

**Figure 5.** Response of dog middle cerebral (MCA), basilar (BA), and posterior communicating (PCA) arterial strips with (●, △) and without (○, ▽) endothelium to application of [Arg$^8$]-vasopressin. Triangles, data from seven pairs of MCA strips that responded only with contraction. Arterial strips were partially contracted with $3 \times 10^{-7}$ to $3 \times 10^{-6}$M prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$). Relaxation induced by $10^{-4}$M papaverine was taken as 100%; mean±SEM absolute values in intact strips of MCA (●), BA, and PCA and endothelium-denuded strips of PCA were $203\pm31$ (n=5), $198\pm29$ (n=12), $217\pm17$ (n=10), and $235\pm32$ (n=10) mg, respectively. Contraction induced by 30 mM K$^+$ was taken as 100%; mean±SEM absolute values in intact strips of MCA (●), BA, and PCA and endothelium-denuded strips of PCA were $698\pm158$ (n=7), $596\pm142$ (n=7), $912\pm244$ (n=5), and $686\pm98$ (n=12) mg, respectively. a) Significantly different from control at *p*<0.001.
same preparations from 16 middle cerebral, 16 basilar, and 14 posterior communicating arterial strips. Relaxations caused by $10^{-7}$ M substance P and $10^{-8}$ and $10^{-7}$ M A23187 in these arterial strips did not differ significantly. However, $10^{-10}$ to $10^{-8}$ M vasopressin relaxed only six of 16 middle cerebral arterial strips but relaxed all 16 basilar and all 14 posterior communicating arterial strips in a dose-dependent manner. Maximal relaxation in these middle cerebral arterial strips (21.5 ± 6.3% at $10^{-8}$ M vasopressin) was <50% of that in the basilar and posterior communicating arterial strips (60.3 ± 4.2% and 55.5 ± 4.4% at $10^{-8}$ M vasopressin, respectively). In the other 10 middle cerebral arterial strips, $10^{-10}$ to $10^{-8}$ M vasopressin caused a concentration-dependent contraction (42.3 ± 7.4% contraction at $10^{-8}$ M vasopressin) that was not influenced by treatment with $10^{-6}$ M indomethacin ($n = 5$) or $10^{-7}$ M ONO-3708 ($n = 4$).

**Discussion**

Our study demonstrates that substance P produced a concentration-dependent relaxation in helical strips of dog cerebral arteries partially contracted with PGF$_2$α that was markedly inhibited by endothelium denudation and treatment with methylene blue, a guanylate cyclase inhibitor. Endothelium-dependent relaxation caused by substance P has also been reported in various arteries (renal, celiac, and mesenteric) from different species (rabbit, dog, and cat). Removal of the endothelium markedly attenuated but did not abolish substance P–induced relaxation. The remaining relaxation after endothelium denudation was abolished by application of indomethacin, a cyclooxygenase inhibitor. Our findings suggest that the relaxation of dog cerebral arteries in response to substance P is mainly mediated by EDRF and vasodilator PGs, such as PG$_1$, produced in smooth muscle cells.

During repeated applications, $10^{-7}$ M substance P caused contraction in some basilar arterial strips. The contractile response depended on the endothelium and was reversed to relaxation by treatment with indomethacin or ONO-3708. Contractions of dog cerebral arteries caused by arachidonic acid and PGH$_2$ depend on the endothelium, suggesting that vasoconstrictor PGs are synthesized mainly in the endothelium in cerebral arteries. Involvement of thromboxane A$_2$ in the contractile response is excluded because of the inability of OKY-046, a thromboxane synthetase inhibitor, to attenuate the response. Therefore, substance P produces basilar artery contractions, possibly by releasing vasoconstrictor PGs from endothelial cells. The findings obtained so far suggest the involvement of three mechanisms underlying the response of cerebral arteries to substance P (Figure 6); substance P releases EDRF to produce a rapid relaxation (A), releases vasoconstrictor PGs such as PGF$_2$, PGE$_2$, PGD$_2$, and PGA$_2$ to produce a transient contraction (B), and releases vasodilator PGs, possibly PG$_1$, to produce a slow and small relaxation (C). Substance P does not always act as a cerebral vasodilator, and its effect appears to be modulated by functions of the endothelium, which are impaired by oxyhemoglobin.

A23187 relaxed dog middle cerebral, basilar, and posterior communicating arterial strips in a similar pattern and to similar magnitudes. Since the relaxation was abolished almost completely by removal of the endothelium, A23187 does not seem to act directly on smooth muscle cells. A23187 also produces relaxations that depend on the endothelium in rabbit aortas and in guinea pig pulmonary and human coronary arteries. The relaxation induced by A23187 in isolated dog basilar arteries is not affected by indomethacin. Therefore, the A23187-induced relaxation appears to be mediated exclusively by EDRF.

Concentrations of vasopressin in plasma and cerebrospinal fluid obtained from patients with subarachnoid hemorrhage or from cats with experimental subarachnoid hemorrhage induced by injection of blood into the subarachnoid space are increased. In our study, vasopressin relaxed basilar and posterior communicating arterial strips with intact endothelium, whereas vasopressin produced a contraction in 10 of 16 middle cerebral arterial strips and a slight relaxation in the remaining six. The concentration-dependent contraction caused by vasopressin in middle cerebral arterial strips was endothelium-independent and was not influenced by indomethacin or ONO-3708, indicating that vasopres-
sin acts directly on smooth muscle cells. Vasoactive substances such as EDRF and PGs in concentrations sufficient to significantly alter the arterial tone are not released from endothelial cells in response to vasopressin. The fact that vasopressin elicited endothelium-independent contraction in approximately two thirds of our middle cerebral arterial strips and endothelium-dependent relaxations plus endothelium-independent contractions in one third may be explained by an inconsistent, weak function of vasopressin receptors located in the endothelium of dog middle cerebral arteries. In the middle cerebral arterial strips that responded to vasopressin only with relaxations, the response was significantly smaller than that in basilar arterial strips, despite the fact that magnitudes of vasopressin-induced contraction were quite similar in endothelium-denuded strips from both arteries. Therefore, the lesser magnitude of relaxation of middle cerebral arterial strips would be associated not with greater contraction but with lesser sensitivity or quantity of vasopressin receptors in endothelial cell membranes. In basilar and posterior communicating arterial strips, vasopressin produced relaxations that were abolished or were reversed to contractions by endothelial denudation. Vasopressin-induced relaxation is postulated to be mediated by $V_1$-vasopressin receptors in endothelial cells of dog basilar arteries. $V_1$-vasopressin receptors in endothelial cells of dog femoral arteries are also considered to be mediated by $V_1$ receptors. Whether vasopressin-induced relaxation and contraction in cerebral arteries are associated with activation of the same subtype of vasopres- sin receptors remains to be clarified.

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**KEY WORDS** cerebral arteries • endothelium, vascular • vasodilation • dogs
Endothelium-dependent and -independent responses to vasodilators of isolated dog cerebral arteries.
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doi: 10.1161/01.STR.19.11.1388

*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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