Flow-Induced Relaxation of the Rabbit Middle Cerebral Artery Is Composed of Both Endothelium-Dependent and -Independent Components

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Background and Purpose: The flow-induced relaxation of a branch of the rabbit middle cerebral artery was examined to determine if an endothelial-independent as well as -dependent component occurs in pial as well as systemic small arteries and the possible role of products of the cyclooxygenase and the L-arginine nitric oxide synthase pathways.

Methods: Intraluminal flow was achieved by the infusion of a tissue bath solution into isometrically mounted rabbit pial arteries in a resistance artery myograph through a small pipette.

Results: Intraluminal flow caused relaxation of the artery segment precontracted with 10 μM histamine. Treatment of endothelium-intact vessels with the nitric oxide synthase inhibitors Nω-nitro-L-arginine (L-NNA) (100 μM) or Nω-nitro-L-arginine methyl ester (L-NAME) (0.3 mM) significantly reduced the relaxation at flow rates of 5–30 μl/min. This effect was partially reversed by 1 mM L-arginine. These inhibitors had no effect on the flow-induced relaxation of endothelium-denuded vessels. L-NNA did not influence the relaxation to 1 and 3 μM papaverine. Exposure to 10 μM aspirin, 10 μM indomethacin, or 300 nM tetrodotoxin had no effect on the flow-induced relaxation of either endothelium-intact or -denuded vessels (n=6). Flow-induced relaxation was attenuated, but not abolished, by removal of the cerebrovascular endothelium. This reduction was not statistically significant.

Conclusions: These results show that intraluminal flow caused relaxation of a branch of the rabbit middle cerebral artery, in part through a mechanism sensitive to inhibitors of nitric oxide synthase, most likely the generation of nitric oxide from the vascular endothelium. The major component of the relaxant response is independent of the endothelium and of nitric oxide synthase through an L-NNA– or L-NAME–sensitive mechanism. The relaxation does not involve cyclooxygenase products nor neurogenic mediators. These results suggest that pial arteries, like those of the rabbit ear, exhibit a novel mechanism for the flow-induced relaxation of agonist-induced tone that is intrinsic to the tissues of the vascular wall subjacent to the endothelium. (Stroke 1993;24:105–110)

Key Words • blood flow velocity • cerebral arteries • endothelium • vasodilation • rabbits

In vivo experiments have shown in a number of vascular beds that changes in intraluminal flow can influence vascular tone (for summary, see Reference 1). Subsequent in vitro studies have demonstrated that the intraluminal infusion of physiological saline through small rabbit ear arteries of the order 200–300 μm o.d. results in vasoconstriction or vasodilation, depending on the level of arterial wall tone.1,2 In 1988 it was observed that the flow-induced relaxation of a branch of the rabbit ear artery3 persisted, although somewhat diminished, after removal of the vascular endothelium. The implication was that flow-induced shear stress on the wall of the blood vessel after endothelium removal can influence vascular smooth muscle tone independently of its ability to cause relaxation through the endothelium.4

The object of this study was to examine flow-induced relaxation of a branch of the rabbit middle cerebral artery (MCA) to clarify the involvement of the vascular endothelium in the relaxant response of the cerebrovascular bed and the role of some possible mediators. Previous studies in several vascular beds by others5–6 have implicated cyclooxygenase products and the L-arginine nitric oxide synthase pathway in the relaxant response to flow. For these reasons, inhibitors of the cyclooxygenase pathway and Nω-nitro-L-arginine (L-NNA) and Nω-nitro-L-arginine methyl ester (L-NAME), inhibitors of endothelial nitric oxide synthase,7,8 were employed in the experimental analysis.
Tetrodotoxin was used to exclude the possibility of neurogenic involvement in the response.

Materials and Methods

New Zealand White rabbits weighing 2.5–3 kg were anesthetized with 30 mg/kg i.v. pentobarbital sodium and exsanguinated. The brain was removed and placed in cold physiological buffer (PSS) of the following millimolar composition: 130 NaCl, 14.9 NaHCO3, 4.7 KCl, 1.6 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, and 11 glucose. Part of the MCA and its branches was dissected free, and two adjacent segments (200–250 μm unstretched external diameter, 2 mm length) were mounted on resistance artery myographs between two intraluminal parallel wires of 34 μm diameter to measure changes in isometric force. The arteries were maintained in PSS bubbled with 95% O2, 5% CO2 at 37°C, pH 7.35–7.45 for 20 minutes before application of the optimal resting force of 100 mg. After a 30-minute equilibration period the arteries were precontracted with a submaximal concentration of histamine (10 μM), which elicited 82.4±3.3% of the histamine-induced maximum response. Integrity of the endothelium was established by observing the relaxation elicited by the addition of 1 μM acetylcholine. The arteries were then washed at 1-minute intervals over 10 minutes.

An infusion cannula made from a glass micropipette and connected to an infusion pump was inserted into the lumen of each artery. The cannula system contained PSS with 10 μM histamine and 1 μM cimetidine, which was identical in composition to the external bath solution employed during the flow experiments. The endothelium was removed from some tissues by intraluminal perfusion with air for 2–3 minutes while immersed in PSS, followed by perfusion with PSS for 1 minute. The arteries were then washed and left to equilibrate for a further 30 minutes before preconstriction with 10 μM histamine. The artery was assumed to be denuded of endothelium if the subsequent addition of 1–100 μM acetylcholine did not cause relaxation. The complete absence of endothelium was verified later by scanning electron microscopy (see below). The arteries were washed and again left to equilibrate for 30 minutes before further experimentation. Cimetidine (1 μM) was present throughout the experiment to block the relaxant action of histamine mediated through H3 receptors.

Flow Responses

The arteries were precontracted with 10 μM histamine and allowed to attain a constant wall force before the commencement of intraluminal perfusion. The perfusate was identical to the bath solution in its composition of ions and agonists. The arteries were perfused for 1 minute at 3-minute intervals using a model 22 constant-flow Harvard infusion pump (South Natick, Mass.). Perfusion rates of 5, 10, 20, and 30 μl/min were used in these studies because in preliminary experiments in which the flow–response range was determined these rates caused dilation of approximately 20–100% of the maximum response. After obtaining consistent relaxant responses, the artery was incubated with L-NNA for 10 minutes and alterations in flow-induced relaxation were observed. A flow rate of 20 μl/min elicited a submaximal relaxation in both endothelium-denuded and -intact preparations. For this reason this flow rate was used as a standard to investigate the effects of 0.3 mM L-NAME, 10 μM aspirin, 10 μM indomethacin, and 300 nM tetrodotoxin.

Upon obtaining two reproducible responses to flow at 20 μl/min, one of the above agents was added to the tissue and allowed to incubate for a minimum of 25 minutes. The time cycle of flow stimulation was maintained throughout this period until two reproducible responses were obtained. At the end of each experiment, the arteries were washed with fresh PSS and allowed to relax to baseline tension. Control experiments were carried out in the absence of drugs to determine time-related effects on the flow-induced responses. In a number of experiments endothelium loss was confirmed by fixing the tissues at the termination of each experiment, cutting them longitudinally, and pinning them open before postfixing in 2% osmium. They were then critical-point dried and sputter-coated with gold platinum, and the entire luminal surface was scanned with a Cambridge Stereoscan 100 (Cambridge, Mass.) at magnifications of up to ×1,500 as previously described.3

The relaxant responses were expressed as a percentage of the baseline histamine-induced tone, and the effects of each test agent were expressed as the change from the mean of the initial responses. The data were found to be normally distributed, and statistical analysis was carried out with raw data using Student’s t test for paired and unpaired data and analysis of variance followed by the Newman-Keuls test where applicable. Significant differences were assumed at p<0.05.

The agents used were acetylcholine chloride, L-arginine hydrochloride, cimetidine, histamine, indomethacin, L-NNA, L-NAME, and tetrodotoxin and were obtained from Sigma Chemical Co., St. Louis, Mo. All drugs were made up in distilled water at a stock solution of 10 mM with the exception of indomethacin, which was made up in 10% ethanol at a stock solution of 1 mM.

Results

Endothelium-Intact Cerebral Artery Segments

In endothelium-intact MCA segments, 10 μM histamine elicited a contraction of 354±57.5 mg (n=6), which 1 μM acetylcholine relaxed by 81.4±5.1% (n=6). In these preparations, intraluminal perfusion with PSS caused a flow rate–dependent relaxation of histamine-induced tone (Figure 1). Following exposure to 100 μM L-NNA for 30 minutes, the histamine-induced contraction increased by 18.1±2.7% to 418±66.2 mg (n=6); this change was not significant. The acetylcholine-induced relaxation of the rabbit MCA was reduced to 7.4±4.5% of the new level of histamine-induced tone (n=6) by L-NNA. The dilation to 10–6 M papaverine was unchanged by L-NNA. At 10–4 M papaverine, mean relaxation was 13.6±3.4% and 15.9±2.9% before and after L-NNA, respectively, and at 3×10–6 M papaverine relaxation was 32.0±5.9% and 29.8±6.3%, respectively (n=6).

Exposure to L-NNA attenuated the flow-induced relaxation of endothelium-intact preparations (Figure 1, top; p<0.05, n=6). This effect was reversed by incuba-
tion with 1 mM l-arginine, a competitive antagonist of the effects of L-NNA (p<0.05, n=6; Figure 1, top). At all flow rates studied the dilation was not significantly different from the control response. These effects of L-NNA were mirrored by 0.3 mM L-NAME. This drug reduced the dilation induced by 1 μM acetylcholine to 6.7±4.2% of the control level. L-NAME did not alter the magnitudes of the histamine-induced contraction or the papaverine-induced dilation. It significantly reduced the dilation to a 20-μl/min flow to 71.8% of the control value, an effect that was reversed by l-arginine.

Incubation with 10 μM aspirin, 10 μM indomethacin, or 300 nM tetrodotoxin did not significantly alter the histamine-induced contraction; when expressed as percent of the original histamine-induced tone, after 25 minutes responses to these agents were 101±4.2%, 93.2±4.6%, and 94.5±3.4%, respectively. Acetylcholine-induced relaxations were unaffected by these agents; responses were 89.2±5.6%, 90.4±4.2%, and 97.6±3.4% of the original, respectively. The relaxation in response to a flow rate of 20 μl/min was not influenced by these three agents (Figure 2).

Endothelium-Denuded Cerebral Artery Segments

In vessels subjected to intraluminal perfusion with air, 10 μM histamine elicited a contraction of 346±31.5 mg (n=8), which was not significantly different from that observed in endothelium-intact vessels. Endothelium-denuded vessels were not relaxed by (1–100 μM acetylcholine (n=8), suggesting complete removal of the endothelium, which was verified by scanning electron microscopy. In three of the eight preparations, 100 μM acetylcholine caused a further increase of histamine-induced tone, an effect that was found to be statistically nonsignificant.

Intraluminal infusion of the endothelium-denuded vessels resulted in relaxation of the histamine-induced tone (Figure 1, bottom); the relaxation was flow-rate-dependent. There was no significant difference in the loss of wall force between endothelium-intact and -denuded vessels at any flow rate used (Figure 1, bottom). Incubation with 100 μM L-NNA, 0.3 mM L-NAME, 10 μM aspirin, 10 μM indomethacin, or 300 nM tetrodoxin did not affect the histamine-induced tone as percent of the original value; after a 25-minute incubation the relaxations were 98.1±3.4%, 94.9±4.6%, 97.2±1.4%, 95.4±3.2%, and 95.8±2.4%, respectively, of the pretreatment responses. The endothelium-independent relaxation to flow was not altered by any of these agents (Figure 2).

In time control experiments (adding saline in volumes equivalent to the drug additions), the histamine-induced tone was found to fade over 45 minutes to 92.1±4.3% and 94.6±5.8% of the original responses in endothelium-intact and -denuded preparations, respectively (n=6). The relaxant response to an intraluminal flow of 20 μl/min was 92.4±4.5% and 98.5±6.2% of the original response after 30 minutes (n=6) in endothelium-intact and -denuded preparations, respectively.
**Discussion**

This study shows that the intraluminal infusion of PSS through a ring segment of a branch of the rabbit MCA causes reversal of agonist-induced tone similar to that previously observed in resistance vessels from the vasculature of the rabbit ear. When the magnitudes of flow-elicited dilations in these pial artery segments were matched using the two tied-in cannula technique with automatic registration of diameter change, wall shear stress varied between 3 and 5 dynes/cm² (J.L. García-Roldan and J.A. Bevan, unpublished data). This is comparable to values determined to occur in vivo under resting conditions (for example, see Reference 10).

Various procedures have been used to inhibit selectively the role of endothelium-derived relaxing factor (EDRF) and to remove or destroy the vascular endothelium in the study of flow-induced relaxation. Based on these procedures, the endothelium has been claimed to mediate exclusively the relaxant or dilator responses to flow in major conduits and resistance arteries. Endothelium-dependent relaxation to flow in small arteries is mediated by cyclooxygenase products in rat cremaster muscle and by an endogenous nitrosodilator in rabbit ear arteries. A large endothelium-independent component has been observed in the flow-induced relaxation of agonist-induced tone in small arteries in the circulation of the rabbit ear. The dilator response to flow in pressurized rabbit cerebral arteries exhibiting myogenic tone was also observed to be independent of the vascular endothelium.

EDRF, first described by Furchgott and Zawadzki, has recently been found to be a nitric oxide–containing moiety generated enzymatically from L-arginine. Both L-NNA and L-NAME are potent inhibitors of the production of this nitrosocompound. For this reason they were used to determine if the relaxation observed to flow involved the production of an endogenous nitrosodilator in the branch of the rabbit MCA used in this study. The two agents were used in concentrations just short of those that exert nonspecific effects on histamine-induced contraction and papaverine-induced dilation (i.e., in the highest concentrations that can be considered functionally specific). These concentrations were effective in preventing dilation in response to 10⁻⁴ M acetylcholine, an effect that was reversed by L-arginine.

In endothelium-intact preparations, intraluminal flow caused a rate-dependent relaxation of histamine-induced tone that was attenuated by incubation of the vessel with L-NNA and L-NAME. This attenuation was reversed by incubation with L-arginine, which is known to displace these agents from nitric oxide synthase. These findings suggest that the endothelial component of flow-induced relaxation involves the production of a nitrosodilator from L-arginine, a conclusion that is underwritten by the finding that the two agents had no effect on flow-induced dilation after endothelium removal. This is similar to other observations in perfused rabbit ear arteries and in coronary arteries. It is consistent with the observations of Katusic et al who emphasize the similarities in the responsiveness of canine basilar artery to EDRF and nitric oxide.

The cyclooxygenase inhibitors aspirin and indomethacin had no effect on flow-induced relaxation, suggesting that production of vasodilator prostaglandins by the vascular endothelium or tunica media are not involved. Both these drugs were used in concentrations considered to be effective on adult vascular smooth muscle, including that of the cerebral circulation (for examples, see References 18 and 21–24).

In endothelium-intact preparations at the flow rate that caused half-maximal flow-induced dilation, approximately 35% of the response was influenced by incubation with L-NNA and L-NAME. This component presumably originated from the endothelium because, upon removal of this layer the relaxation induced by intraluminal flow was insensitive to their effects. Somewhat surprisingly, there was no significant difference between the relaxations induced by flow in endothelium-intact and -denuded preparations. It is hypothesized that the production of EDRF by flow is a consequence of shear stress on endothelial cells. It seems likely that transmission of this stress to the muscle layers through the extracellular matrix can directly stimulate relaxant mechanisms within the vascular smooth muscle and its immediate environment. It could be that the shear stress exerted on the inner surface of endothelium-denuded vessels would be greater for a given flow rate than in endothelium-intact vessels. The extent to which the endothelium normally attenuates the mechanical transferral of flow-related shear forces to the intima is not known. Furthermore, mechanical removal of the endothelium would involve disruption of the myoendothelial junctions and expose them directly to shear, an effect that might augment the endothelium-independent component of flow-induced relaxation.

Flow-mediated dilation in the basilar arteries of anesthetized rats has been recently investigated using a cranial window technique. Mean blood velocity increased after unilateral and further after bilateral carotid artery occlusion. Both changes were associated with dilation. None of the topically applied pharmacological interventions used by these investigators (tetrodotoxin, N-nitro-L-arginine, tetraethylammonium, glibenclamide, SKF 525A, and ouabain) influenced the dilation. There are several similarities and differences between the study by Fujii et al and the present results. In neither case did tetrodotoxin or indomethacin influence the flow-induced change. In the in vivo rat study L-NMMA had no effect. In our study, the two inhibitors of nitric oxide synthase reduced the dilation, and this action was linked to the endothelial layer. One possible explanation for this discrepancy is that with topical application a concentration gradient of drug occurs across the artery wall that is lowest at the intima because the drug concentration in circulating blood approaches zero. The findings of Fujii et al that ouabain had no effect is consistent with our observations after a 30-minute exposure of a resistance branch of the rabbit ear to this glycoside. Fujii et al found no reason to implicate vascular smooth muscle potassium channels in the dilator response.

The inability of tetrodotoxin to influence relaxation discounts any role of voltage-activated sodium channels in the flow response. This eliminates the possibility that flow-induced shear stress stimulates action potential-mediated release of vasodilators from nerves within the blood vessel wall. It also emphasizes that the recently described sodium-dependency of flow-induced dilation does not involve a fast sodium channel.
Flow-induced relaxation is an important adaptive mechanism in the cerebral circulation. For example, during focal increases in cerebral metabolism and blood flow, it is essential that large arteries upstream dilate to preserve distal perfusion. Because resistance of large arteries is surprisingly high, if large cerebral arteries failed to dilate during focal, distal hyperemia, the distal vessels would be susceptible to a steal phenomenon. This complicated concept is discussed in previous papers.1,2

What is the stimulus for dilatation of large cerebral arteries during focal, distal hyperemia? It appears that increases in velocity of blood flow in some way initiate dilatation of large arteries. Most evidence indicates that endothelium senses increases in velocity of flow and plays a central role in initiating dilatation, or relaxation, in cerebral and other arteries.3

The study by Gaw and Bevan indicates that, after mechanical removal of endothelium, flow-induced relaxation is largely preserved in a branch of the rabbit
Flow-induced relaxation of the rabbit middle cerebral artery is composed of both endothelium-dependent and -independent components.
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