Nitric oxide-mediated arteriolar dilation after endothelial deformation

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IN THE LAST DECADE, studying isolated arterioles, we frequently observed dilation of vessels after agonist-induced constrictions. In vivo and in vitro studies of small vessels indicated that removal of the endothelium or inhibition of nitric oxide (NO) synthase enhances responses of vessels to various constrictor agonists (12, 13), and thus it was suggested that, parallel to a decrease in diameter, dilator factors, such as NO, may be released from the endothelium. The exact relationship between the action of constrictor agents and the release of NO, however, is still not elucidated. Several studies of large vessels suggested that many constrictor agents have receptors not only on smooth muscle but also on endothelial cells (3, 6, 15). Thus these agonists, by acting on endothelial receptors, could initiate the synthesis of NO (3). A recent study suggested that the cell-to-cell communication between the smooth muscle and endothelium may facilitate the release of endothelial NO concurrent with vessel constriction (7).

Early studies investigating arteriolar structure by electron microscopy revealed that, during constriction of arterioles, the endothelial cell layer undergoes folding, and the nucleus of endothelial cells becomes compressed (5, 9, 20, 25). In addition, the endothelial layer becomes thicker, and parts of the cells extrude through fenestrations at myoendothelial junctions. It is likely, therefore, that, regardless of the stimulus, the circumferential shape of endothelial cells changes during decreases in vessel diameter, even in the absence of significant changes of pressure, wall shear stress, or isometric wall tension, and this could lead to the release of vasoactive factors. Thus we hypothesized that changes in the shape of endothelium of arterioles during constriction itself can lead to the release of NO, which would be followed by dilation of the vessel.

To test this idea, changes in diameter of isolated arterioles in response to constrictor agents of different mechanisms of action and increases in extraluminal pressure in the presence of constant intraluminal pressure were followed as a function of time in the presence and absence of endothelium or inhibition of NO synthase. In addition, release of NO from cultured endothelial cells after deformation of their shape was also assessed.

MATERIALS AND METHODS

Preparation of isolated arterioles. All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the current guidelines of the National Institutes of Health and the American Physiological Society for the use and care of laboratory animals. Segments, ~1 mm in length, of arterioles isolated from the mesentery of anesthetized male Wistar rats (intra-muscular pentobarbital sodium, 50 mg/kg) were cannulated

Sun, Dong, An Huang, Fabio A. Recchia, Yanning Cui, Edward J. Messina, Akos Koller, and Gabor Kaley. Nitric oxide-mediated arteriolar dilation after endothelial deformation. Am J Physiol Heart Circ Physiol 280: H714–H721, 2001.—Previously, we frequently observed dilation of arterioles after agonist-induced constrictions. We hypothesized that deformation of the endothelium during decreases in diameter of isolated arterioles elicits the release of nitric oxide (NO). In isolated arterioles of rat mesentery, phenylephrine (PE, 10^{-7} M), U-46619 (10^{-7} M), and KCl (50 mM)-induced constrictions were followed by potent dilations. Inhibition of NO synthase with N\textsuperscript{\textdegree}-nitro-l-arginine (L-NNA, 2 \times 10^{-4} M) or removal of the endothelium significantly enhanced constriction and reduced the postconstriction dilation. In the presence of 80 mmHg of intraluminal pressure, an increase in extraluminal pressure (P_E) to 75 mmHg for 20 s and 1 and 2 min decreased vessel diameter. After release of P_E, arterioles dilated as a function of the duration of diameter reduction by P_E. Removal of the endothelium or administration of l-NNA significantly diminished the post-P_E dilations. In cultured mesenteric arteriolar endothelial cells (EC), PE, U-46619, or KCl did not increase, whereas ACh did increase, the production of NO, as measured by a fluorometric assay for nitrite. Furthermore, when EC, cultured on a stretched silicone membrane, were subjected to deformation by shortening the membrane to 50% of its original length, NO release increased significantly. Based on all of the above, we propose that deformation of EC per se elicits release of NO, a mechanism that modulates arteriolar constriction.

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with two glass pipettes in a vessel chamber (1 ml in volume). They were suffused (1 ml/min) with physiological salt solution (PSS), buffered with NaHCO$_3$ (24.0 mM) and 5% CO$_2$ plus ambient air, and maintained at a pH of 7.4 and a temperature of 37°C (22, 23). Intravascular pressure was maintained at 80 mmHg, and intraluminal flow was set at zero by pressure servo systems (Living-Systems). Arterioles were equilibrated for 1 h to develop spontaneous tone (55 ± 1% of passive diameter). Changes in diameter in response to phenylephrine (PE, 10$^{-7}$ M), U-46619 (10$^{-7}$ M), or KCl (50 mM) were then assessed. PE (10 µl), U-46619 (10 µl), or KCl (0.6 ml) was added to the vessel chamber directly, without stopping suffusion. KCl (50 mM) was prepared by substituting an equimolar amount of K$^+$ for Na$^+$. Changes in diameter in response to agonists were measured with an image-shearing monitor (model 907, IPM) and recorded with a chart recorder (Graphitec Multicorder MC6625). After control responses were obtained, the endothelium was removed by injection of air into the vessel lumen, as described previously (22). N$^\omega$-nitro-L-arginine (l-NNA, 2 × 10$^{-4}$ M) was added to the suffusion solution for 30 min to inhibit the synthesis of NO. Next, responses to PE, U-46619, and KCl were reassessed.

**Decrease in diameter by extraluminal pressure.** Mesenteric arterioles were isolated and cannulated in a 15-ml vessel chamber filled with MOPS-PSS. Temperature and pH of the solution were maintained at 37°C and 7.4, respectively. The chambers were sealed air tight. Thus intraluminal and extraluminal pressures could be controlled separately with two pressure servo systems. During experiments, vessels were first equilibrated at 80 mmHg of intraluminal pressure for 1 h to develop spontaneous tone. Next, extraluminal pressure was increased from 0 to 75 mmHg to reduce the diameter of arterioles for 20, 60, and 120 s. After control responses were obtained, removal of the endothelium or inhibition of NO synthase with l-NNA was performed. Next, the effects of extraluminal pressure were reassessed.

**Unidirectional deformation of cultured endothelial cells.** Primary and secondary passage cultures of endothelial cells isolated from mesenteric arterioles were grown on a pre-stretched elastic 1 in. × 1 in. × 0.01 in. glass/glass silicone membrane. One end of the membrane was anchored to the end of the culture chamber (1 in. × 2 in.) while the other end was attached to a bar that could be moved precisely and smoothly in the long axis direction. Two sides of the membrane with three attachments on each side were anchored to two sliding bars, which allowed the membrane to be stretched only in the longitudinal direction without shortening in the horizontal direction. The membrane was stretched to double its original length in the chamber and was coated with 1% gelatin two times by drying under a hood after each coating. Endothelial cells were cultured on the stretched membrane with medium 199 (M199). After the cells reached confluence, M199 was replaced with MOPS-PSS. Cells were washed two times and incubated at 37°C with room air for 1 h in 10 ml of MOPS-PSS. At the beginning and end of the incubation, 200-µl samples of MOPS-PSS were taken to serve as controls. The membrane was then shortened to 50% of its stretched length in the longitudinal direction by releasing the prestretched tension without any change in horizontal length. After 2, 5, and 10 min of shortening, additional 200-µl samples were taken at each time point.

**Chemicals.** All chemicals were obtained from Sigma Chemical except for l-NNA, which was purchased from Aldrich Chemical. l-NNA (10$^{-2}$ M) was dissolved in saline with sonication. All solutions and drugs were prepared on the day of the experiments by dilution with buffered PSS.

**Data analysis and statistics.** The area under the vasoconstrictor and dilator response curves was measured by a computer program (SigmaScan) from the original records. Basal tone of arterioles was expressed as percentage of passive diameter. Data are means ± SE; n denotes the number of vessels or the number of culture dishes or chambers. One vessel was isolated from a rat for each experimental group. Statistical significance was calculated by paired Student's t-test for changes in arteriolar diameter and one-way
ANOVA for time-dependent accumulation of nitrite concentration. Significance level was taken at $P < 0.05$.

RESULTS

Dilation after drug-induced constriction. Original records show that, in isolated, cannulated, and pressurized rat mesenteric arterioles, PE-, U-46619-, and KCl-induced constrictions were followed consistently and instantaneously by substantial dilations. Removal of the endothelium or inhibition of NO synthase with L-NNA did not affect the basal diameter (81 ± 5 vs. 79 ± 5 μm, $n = 13$, and 79 ± 7 vs. 80 ± 6 μm, $n = 13$, respectively) but essentially eliminated the postconstriction dilation. (We have also observed that KCl induced a transient endothelium-independent dilation before the constriction; Fig. 1A.) Summary data in Fig. 1B demonstrate that, after removal of the endothelium or L-NNA administration, postconstriction dilation was significantly reduced, and constrictions in response to all three agonists were enhanced significantly. Importantly, removal of the endothelium or L-NNA had no significant effect on the initial portion of the constrictions characterized by the peak change after PE (49 ± 2 vs. 54 ± 3 μm), U-46619 (51 ± 3 vs. 55 ± 4 μm), and KCl (54 ± 3 vs. 59 ± 5 μm) nor the rate of decrease in diameter after PE (3.1 ± 1.4 vs. 2.9 ± 1.2 μm/s), U-46619 (2.2 ± 0.4 vs. 2.2 ± 0.7 μm/s), and KCl (2.6 ± 1.4 vs. 2.3 ± 0.8 μm/s; examples before and after L-NNA).

Nitrite release from endothelial cells to agonists. To investigate whether the constrictor agents elicited a direct release of NO from endothelial cells, PE, U-46619, KCl, or ACh was applied on primary cultures of endothelial cells isolated from mesenteric arterioles. Figure 2 shows that the basal level of nitrite (decomposition product of NO) assessed by a fluorometric assay did not change significantly in response to administration of PE, U-46619, or KCl. In contrast, ACh significantly increased the production of nitrite.

Dilation after decrease in diameter by extraluminal pressure. In this group of studies, we further examined the question of whether the endothelium-dependent postconstriction dilation requires activation of smooth muscle cells. To this end, the diameter of isolated and pressurized mesenteric arterioles was reduced by increases in extraluminal pressure from 0 to 75 mmHg for 20 s, 1 min, and 2 min while intraluminal pressure was maintained at 80 mmHg. Increases in extraluminal pressure decrease the diameter of arterioles due to the reduction in transmural pressure (pressure gradient between the inside and outside of the vessel). Figure 3A depicts original tracings of changes in diameter of arterioles to an increase in extraluminal pressure in the presence and absence of the endothelium.
and before and after inhibition of NO synthesis with L-NNA. The average diameter decreased from 85 to 52 μm, which mimicked agonist-induced constriction. After extraluminal pressure was restored to control (i.e., atmospheric), the diameter of arterioles immediately returned to control levels and continued to increase further above the control level, exhibiting a dilator response. The peak changes in diameter and the duration of the dilation were proportional to the duration of the rise in extraluminal pressure. Removal of the endothelium or L-NNA administration greatly reduced dilations after the release of extraluminal pressure (Fig. 3A). Summary data of these experiments (Fig. 3B) demonstrate that the release of extraluminal pressure elicits significant dilations. In the absence of the endothelium or NO (arterioles treated with L-NNA), the dilations were attenuated significantly. Thus these results demonstrate that, without activation of vascular smooth muscle, a decrease in arteriolar diameter per se is sufficient to activate the synthesis of NO. The data also suggest that it is the endothelium itself, caused by its deformation during the process of reduction in ves-

Fig. 2. Nitrite formation as a function of time in response to PE (10^{-7} M, n = 7), U-46619 (10^{-7} M, n = 7), KCl (50 mM, n = 7), and ACh (10^{-7} M, n = 12) in the medium of primary cultured endothelial cells of mesenteric arterioles. Nitrite concentration is represented by arbitrary units of fluorescent intensity on the y-axis. *P < 0.05 vs. control.

Fig. 3. A: original tracings of extraluminal pressure-induced decreases in diameter, followed by dilations in control (EC+) and after endothelial removal (EC−) and before and after L-NNA administration in rat mesenteric arterioles. B: summary data of post-pressure-induced dilations, as indicated by the area under the response curves in control (EC+) and after endothelial removal (EC−) (n = 5) or after administration of L-NNA (2 × 10^{-4} M; n = 6). *P < 0.05 vs. control.
sel diameter, that is responsible for the modulation of the constrictor response by the release of NO.

Increased NO production induced by unidirectional deformation of cultured endothelial cells. To provide additional evidence that the NO-dependent postconstriction dilation is due to the deformation of endothelial cells, primary and secondary passages of cultured mesenteric arteriolar endothelial cells were isolated and grown on a prestretched elastic membrane. Figure 4, A and B, shows changes in the shape and size of the cells before and after shortening the membrane, respectively. The average maximal length of the cells in the direction of shortening and perpendicular to the shortening from three experiments, counting 100 cells in each, was 35.6 ± 1.6 and 30.7 ± 1.6 μm, respectively. After a 50% shortening of the membrane, the cell length in the two directions was 18.3 ± 0.9 (P < 0.05) and 33.5 ± 1.5 μm, respectively. Figure 4C shows the average of six standard curves of nitrite. The average regression coefficient (r²) was 0.988 ± 0.004. The sensitivity of detection of nitrite with the method used was ~20 nM (average readings for background and 20 nM of nitrite were 18.5 ± 0.7 and 21.5 ± 0.7 fluorescence units, respectively; P < 0.05). Figure 4D shows the production of nitrite by cultured endothelial cells in response to the experimental intervention. In control conditions, there is a low level of basal production of nitrite that does not change significantly with time (from time 0 to 1 h, fluorescence units increased from 26.8 ± 1.7 to 28.6 ± 1.8, corresponding to nitrite concentration of 50.0 ± 10.9 and 65.6 ± 12.3 nM, respectively; n = 13). In contrast, when the cells were deformed by shortening the prestretched membrane to ~50% of its initial length, nitrite production increased significantly within the first 2 min (126.7 ± 24.8 nM) and then increased continuously for up to 10 min (180.6 ± 33.6 nM).

DISCUSSION

The new findings of this study are that decreases in arteriolar diameter, regardless of whether they are induced by constrictor agents or increases in extraluminal pressure, lead to an endothelium-dependent NO-mediated dilation. This response is independent of changes in intraluminal pressure, wall shear stress, or isometric wall tension. In addition, unidirectional deformation of cultured endothelial cells, by shortening of
a prestretched elastic membrane, also resulted in a significant release of NO. Together, the results strongly support our hypothesis that the endothelial cells respond to the circumferential cellular deformation during decreases in arteriolar diameter, leading to the release of NO.

**Release of NO from arteriolar endothelium in response to reduction in diameter.** We found that removal of the endothelium or inhibition of NO synthesis did not significantly change the diameter of isolated arterioles, suggesting that, in the absence of intraluminal flow there is only minimum basal release of NO. These results are consistent with previous findings (14, 23). Despite the negligible basal release of NO, however, removal of the endothelium or inhibition of NO synthesis significantly increased agonist-induced constriction and decreased the postconstriction dilation of arterioles. These findings demonstrate that release of endothelium-derived NO is initiated during the constriction and, consequently, modulates the constriction by eliciting a dilator response (Fig. 1).

Further analysis of the data reveals that inhibition of NO synthesis or removal of the endothelium did not change the rate or the peak decrease in arteriolar diameter to any of the constrictor agents used. In contrast, the magnitude of arteriolar constriction as indicated by the area under the response curves was significantly increased after removal of the endothelium or administration of L-NNA (mainly due to significant increases in the duration of constriction; data not shown). Thus endothelium-derived NO does not affect the initial decreases in diameter but only reduces the magnitude of the constrictions. These findings are consistent with our hypothesis that the release of NO is initiated only after and because of the reduction in arteriolar diameter, during which the endothelium is subjected to deformation. Still, one cannot exclude the possibility that the dilation of arterioles provides an additional stimulus for the endothelial release of NO.

Although a response of an arteriole even to the simplest intervention is the result of complex, multiple mechanisms, the phenomenon revealed in the present study seems to be different from those postulated previously. One mechanism that has been proposed recently (7) should especially be differentiated from our findings. It is known that, during constriction of arterioles to various vasoactive substances or an increase in transmural pressure, intracellular Ca\(^{2+}\) in vascular smooth muscle increases. Dora et al. (7) suggested that, parallel with the increase in intracellular Ca\(^{2+}\) in arteriolar smooth muscle, there is an increase in endothelial Ca\(^{2+}\) as well. They hypothesized a transfer of Ca\(^{2+}\) from smooth muscle to the endothelium during constriction that consequently could, via a rise of Ca\(^{2+}\) in endothelial cells, thought to be required for NO synthesis (11, 15), lead to an enhanced synthesis of endothelial mediators. In the present study, we found, however, that by increasing external pressure a passive reduction of vessel diameter is also followed by dilation (Fig. 3). In this experimental condition, the transmural pressure is decreased, and consequently one would expect that intracellular Ca\(^{2+}\) in smooth muscle, if anything, will also decrease (19, 24). Still, we found that after releasing the extraluminal pressure an endothelium-dependent, NO-mediated dilation occurred, ruling out the possible role of intercellular Ca\(^{2+}\) trafficking in the response. Furthermore, the failure of the constrictors to release NO from cultured arteriolar endothelial cells also demonstrates the lack of a direct effect of these agents.

One could argue that in our studies, as a consequence of increased extraluminal pressure, transmural pressure decreases, leading to a decreased myogenic tone, which might contribute to the dilation of the vessel after the release of extraluminal pressure. However, inhibition of the response (60–80%, Fig. 3B) by removal of the endothelium or L-NNA administration indicates that the major component of the postconstriction dilation cannot be attributed to a myogenic response. In addition, previous studies have already demonstrated that myogenic responses of arterioles are independent of the endothelium and that endothelial mediators do not significantly modify arteriolar tone in the absence of intraluminal flow (23, 24). Therefore, the significantly reduced post-extraluminal pressure-induced dilation after removal of the endothelium or administration of L-NNA is ipso facto not due to a decrease of myogenic tone but rather to an endothelium-dependent, NO-mediated dilation.

Previous studies using various experimental techniques demonstrated that changes in physical forces affecting the endothelium could elicit the release of endothelial factors (4, 10, 11, 14). In all of these studies, wall shear stress and/or intraluminal pressure changed and was likely responsible for the release of vasoactive substances. It is important to emphasize that in the present study there was no increase in intraluminal pressure or wall tension (such as in vascular ring studies), which distinguishes the phenomenon revealed by the present study from those of others. Finally, although one can hypothesize that NO release could be due to the parallel stimulation of receptors on the endothelium during administration of constrictor agonists (1, 3, 6), we found NO release in cultured arteriolar endothelial cells only to ACh but not to the constrictor agents (Fig. 2). In our experimental setup, isolated arterioles were secured on two cannulas, and the length of the vessels remained constant throughout the experiments. During constriction, the diameter of arterioles decreased, and endothelial cells were subjected to deformation primarily in a circumferential direction, perpendicular to the vessel wall. Thus, based on the present findings together with earlier morphological data (5, 25), we propose that it is the deformation of endothelial cells during decreases in arteriolar diameter that is responsible for the release of NO.

**Release of NO to deformation of endothelial cells.** To provide further evidence for the idea of deformation-induced NO release and to exclude the possible role of vascular smooth muscle in the initiation of the endothelium-mediated dilator response, we designed experiments using cultured endothelial cells on prestretched...
elastin membranes. Endothelial cells were isolated from rat mesenteric arterioles, the very same vessels that were used for the experiments with constrictor agents. In response to ACh but not in response to vasoconstrictor agents, these cells synthesized NO, indicating that cellular integrity was maintained. By releasing the stretch, cell length was changed primarily in the direction of membrane shortening as shown on a two-dimensional surface (Fig. 4, A and B). Because the cellular volume cannot be reduced, an additional cellular deformation must occur in the direction perpendicular to the membrane. This deformation is likely to be similar to what takes place in endothelial cells during decreases in arteriolar diameter (5, 25). After a single release of the stretch, while the cells were kept in a deformed condition, nitrite measurements show significantly increased NO production (Fig. 4D). These data demonstrate conclusively that unidirectional shortening of endothelial cells is in itself sufficient to increase NO synthesis.

We speculate that shape changes of endothelial cells during constriction could affect their cytoskeletal structure, known to be involved in the synthesis of NO (10, 11, 21) and consequently the maintenance of arteriolar tone (21). The exact mechanisms by which arteriolar endothelial cells sense and respond to various physical forces and/or shape changes are not yet fully characterized. It is likely, however, that deformation of the endothelium during changes in diameter (the novel hypothesis of the present study) could be one of the important stimuli provoking the release of NO.

Physiological importance. Endothelial modulation of arteriolar constriction has been demonstrated to originate either from smooth muscle by providing signals to the endothelium (7) and/or from the endothelium per se by sensing a deformation in cellular shape, as demonstrated in the present studies. In this context, endothelial NO has been shown to be released to a cyclic strain that also upregulates NO synthase (4). It has also been demonstrated that compression-induced cutaneous vasodilatation is mediated by an axon reflex, although whether endothelium-derived NO is involved in the response has not been assessed (8). Collectively, it is tempting to speculate that perhaps multiple and functionally redundant mechanisms influence the regulation of arteriolar diameter during constrictions.

Our findings and those described by others suggest that the modulation of vasoconstriction by endothelin-derived factor(s) is a general phenomenon that could well contribute to the local regulation of blood flow. Severe vasoconstriction induced by constrictor agonists may happen only rarely in physiological conditions, but decreases in vessel diameter induced by increases in extraluminal pressure can be observed frequently in many circulatory beds, such as in skeletal and cardiac muscle. The existence of the endothelium-dependent mechanism described in this paper may also provide an alternative explanation for early findings by Mohrman and Sparks (18) showing that, after tetanic contraction, there was a vasodilator response in skeletal muscle that, however, occurred too suddenly to have been caused by changes in oxidative metabolism. Thus this newly described endothelium-dependent vasodilator mechanism could, along with neural, humoral, and other local factors, contribute to hyperemic responses.

In conclusion, we found that deformation of endothelial cells during decreases in diameter of arterioles elicits the release of NO that results in dilation. This newly defined mechanism may be important in the maintenance of endothelial cell shape and arteriolar diameter and consequently may contribute to the local regulation of blood flow and vascular resistance.

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