Abluminal Release and Asymmetrical Response of the Rabbit Arterial Wall to Endothelium-Derived Relaxing Factor

Eberhard Bassenge, Rudi Busse, and Ulrich Pohl

A marked functional polarity of endothelial cells as well as an asymmetry of the vascular wall in response to vasoactive compounds is well established. Therefore, we investigated the polarity of endothelium-derived relaxant factor (EDRF) release from native endothelial cells, its diffusion characteristics, and its dilator effects on inner and outer muscle layers of the vascular wall in isolated rabbit arteries. Following exposure of rabbit aortae (with intact endothelium) to the EDRF stimulators acetylcholine, A23187, or thimerosal, a humoral dilator compound could be assayed in the adventitial superfuse of the vessels. The vasodilator effects were blocked by the EDRF inhibitors hemoglobin, dithiothreitol, and gossypol. Penetration of the dilator through the arterial wall following stimulation by acetylcholine, A23187, or thimerosal was observed only when dilator EDRF activity in the luminal perfusate was maximal. Luminal administered EDRF, released from cultured endothelial cells, did not cross the aortic wall in detectable amounts. EDRF (from cultured cells) elicited significantly smaller dilations (9 ± 4%) when applied to the adventitial side of endothelium-denuded rabbit aorta or femoral arteries as compared with luminal application (92 ± 7%). In contrast, sodium nitroprusside was equieffective by both routes of administration. We conclude that EDRF in native endothelial cells is released in both luminal and abluminal directions and can penetrate the entire vascular wall. However, the lengthy diffusion time and the decreasing sensitivity of outer smooth muscle layers suggest that mechanisms other than EDRF diffusion contribute significantly to the propagation of endothelium-mediated vasodilation through the arterial wall. (Circulation Research 1987;61(suppl I):II-68-II-73)

A large number of vasoactive compounds, as well as certain physicochemical conditions, stimulate endothelial cells to release a humoral factor that relaxes vascular smooth muscle cells. Relaxation by this endothelium-derived relaxing factor (EDRF), which can directly activate soluble guanylate cyclase, is likely attributable to an increase of cyclic guanosine 3',5' monophosphate (cGMP) formation in the vascular smooth muscle cells. It has been shown by several investigator groups that EDRF released from native and cultured endothelial cells is a labile nonprostanoid substance that, because of its humoral character, can be transferred to bioassay preparations. Although these studies demonstrate a luminal release of EDRF, they provide no direct information as to the processes involved in the signal transfer from endothelial cells to the vascular smooth muscle. Endothelial cells in situ exhibit a pronounced morphologic and functional polarity of their luminal and abluminal surfaces, which may also include release of EDRF and other signals. The different layers of vascular smooth muscle in the arterial wall also show a marked asymmetry in response to certain vasoactive compounds. Therefore, information on the mode of signal transmission between endothelium and vascular smooth muscle during endothelium-mediated vasomotion cannot simply be deduced by analogy from transfer experiments with luminally released EDRF.

In the present study, the possibility of a significant abluminal release and transfer of EDRF was investigated by studying its penetration through the arterial wall. Whether there was an asymmetry in the sensitivity of vascular smooth muscle layers across the vascular wall in response to EDRF was also investigated.

Materials and Methods

EDRF was obtained from either endothelium-intact rabbit aortic segments or cultured bovine endothelial cells. Endothelium-denuded rabbit arteries were used as detectors of EDRF in our bioassay technique. The vessels were obtained from rabbits of either sex (2.5-3.5 kg) that had been killed by decapitation.

Donor Segments

Rabbit aortae were dissected free from adherent adventitial tissue, and all side branches were carefully ligated. Special care was taken during preparation to avoid damage to the endothelium. The segments were fixed between 2 steel cannulas and placed into a small organ chamber that was tightly closed by a cover (Figure 1). The segments were simultaneously perfused and superfused by separate routes (intraluminal perfusion and adventitial perfusion). Oxygenated Tyrode's solution (pH 7.4, 37°C) was used as perfusate and had the following composition (in mM): Na⁺ 144.0, K⁺ 2.2, Cl⁻ 95.0, HCO₃⁻ 25.0, H₂PO₄⁻ 1.1, Mg²⁺ 1.0, CaCl₂ 1.25, and glucose 5.5. The perfusion rate was 20 ml/min and the vessel pressure was kept constant at 70-80 mm Hg. The adventitial superfuse (3 ml/min) was passed through a reservoir containing 10% hemoglobin in Tyrode's solution at 4°C. The reservoir was stirred continuously. The vessels were stimulated by acetylcholine, A23187, or thimerosal.
Then, about 250 μl of the beads with about 3-4 × 10^6 cells were poured into the barrel of a 1-ml plastic syringe covered by a cannulated plunger. This cell column was kept in a water jacket and perfused continuously (0.67 ml/min) with oxygenated Tyrode’s solution. Release of EDRF from the cell column was elicited by addition of thimerosal (5 μM) or bradykinin (10-50 nM) to the perfusate.

**Protocols**

**Series 1.** Ach (0.1–3.0 μM), the calcium ionophore A23187 (0.5 μM), or thimerosal (5 μM) were added to the luminal perfusate for 15–30 minutes to stimulate EDRF release in endothelium-intact aortic segments. In 5 experiments, the donor segments were then exposed for 30 minutes to the polyphenolic antioxidant gossypol (5 μM), a potent inhibitor of EDRF production, and the endothelial stimulation was repeated 40–50 minutes after control responses.

In 8 experiments, endothelial stimulation was repeated in the presence of an inactivator of released EDRF (hemoglobin, 1 μM, or dithiothreitol, 0.2 mM) instead of gossypol. The inhibitor was administered distal to the donor segment. To test for the effects of time and repeated endothelial stimulation on EDRF release, the stimulation was repeated without administration of inhibitors in 6 experiments.

**Series 2.** Using the same bioassay setup, the permeability of the aortic wall to exogenously applied EDRF was studied. The effluent from a stimulated endothelial cell column containing EDRF was passed through an aortic segment (intraluminal perfusion). The presence of EDRF in the intraluminal perfusate was verified by vasodilation of the downstream luminal detector. Penetration of the aortic wall (of endothelium-intact as well as endothelium-denuded segments) by EDRF derived from cultured cells could be assayed by means of the adventitial detector. Similar experiments were performed using the hydrophilic arachidonic acid metabolite PGF_2α instead of EDRF.

**Series 3.** The dilator effects of EDRF (released from cultured endothelial cells stimulated with bradykinin or thimerosal) administered via different routes (i.e., at the intimal or adventitial side of endothelium-denuded detectors) were compared to examine for a potential asymmetry of vascular smooth muscle to EDRF. In contrast with the setup used in Series 2, the detectors were exposed directly to EDRF without interposition of another vascular segment. Paired segments of the same vessel type (aorta or femoral artery) were used as detectors. One segment was perfused in everted configuration (inside out), while the other segment was perfused in normal configuration. Thus, either the luminal or the adventitial surface was exposed to the EDRF-containing perfusate. The same type of experiments were performed using sodium nitroprusside instead of EDRF.

**Drugs**

ACh, thimerosal, nordihydroguaiaretic acid (NDGA), dithiothreitol (DTT), indomethacin, gossypol acetic acid, bovine hemoglobin, and prostaglandin F_2α (PGF_2α) were purchased from Sigma Chemical Co.
Norepinephrine (NE) (Arterenol) was obtained from Hoechst (Frankfurt, F.R.G.). ACh, thimerosal, NE, hemoglobin, and PGF\textsubscript{2\alpha} were dissolved in distilled water. Oxyhemoglobin was prepared as described by Martin et al\textsuperscript{23} and diluted with Tyrode's solution. Indomethacin was dissolved in ethanol 0.1 M NaHCO\textsubscript{3} (1:3, vol/vol) and diluted with Tyrode's solution. Gossypol, NDGA, and NE were dissolved directly in Tyrode's solution. Once prepared, NDGA solutions were used for no longer than 1 hour. All concentrations of drugs refer to the respective free bases or acids.

Statistics

Dilation was expressed as the percent of the contractile response to NE (EC\textsubscript{50}). A paired or unpaired Student's t test was used to evaluate statistical significance of the data. Differences were considered to be significant at \( p<0.05 \). All values are reported as mean ± SEM.

Results

When endothelium-intact aortic segments were stimulated intraluminally with ACh, a dilation of the detector segment assaying the intraluminal aortic perfusate (intraluminal detector) was observed. This dilation occurred in a dose-dependent manner and was sustained over the whole stimulation period (Figure 2). Maximal responses were reached at ACh concentrations between 1 and 3 \( \mu \)M. In contrast, no dilator response could be detected in the adventitial perfusate of the aorta at ACh concentrations below 1 \( \mu \)M. A dose dependency of these vasomotor responses could not be detected in this range. The onset of the dilation was delayed by 242 ± 25 seconds compared with the onset of dilation in the intraluminal detector.

Using either thimerosal (5 \( \mu \)M) or A23187 (0.5 \( \mu \)M) as the endothelial stimulus, a dilator response (11.3 ± 2.1%) of the adventitial detector could be induced in 5 of 11 perfusion experiments, while dilations of the intraluminal detector occurred in all experiments. Again, the onset of dilation was delayed compared with the onset of dilation of the intraluminal detector.

In all segments exhibiting penetration of the wall by EDRF, penetration of PGF\textsubscript{2\alpha} (1–10 \( \mu \)M) added to the intraluminal perfusate of the aorta could also be demonstrated by contraction of the adventitial detector. In the other experiments, which showed no detectable amounts of EDRF in the adventitial superfusate, penetration of PGF\textsubscript{2\alpha} of the aortic wall was observed in only 33%. Addition of hemoglobin (1 \( \mu \)M) (n = 2) or dithiothreitol (0.2 mM) (n = 4) to the perfusates distal to the donor segments abolished the dilation of both detector segments following endothelial stimulation with ACh (also see Figure 4) and A23187 (n = 2). Exposure of the donor segments to gossypol (5 \( \mu \)M, 30 minutes) also abolished dilation of both detectors following en-
endothelial stimulation with ACh (n = 5). No significant attenuation of dilatation was observed in control experiments after repeated endothelial stimulation.

To obtain further information about the permeability of the aortic wall to EDRF, aortic segments (n = 6) were intraluminally perfused with EDRF-containing effluent from stimulated cultured endothelial cells. This effluent induced a 100% vasodilation of the intraluminal detector in all experiments, indicating the presence of high amounts of EDRF. However, in no case was a dilation of the adventitial detector observed. PGF$_2$alpha (1–10 μM) administered into the intraluminal perfusate of the same segments elicited a contraction of both the luminal and the adventitial detectors in all experiments.

To test for a difference in the sensitivity of intimal and adventitial smooth muscle layers to EDRF, the dilator responses of everted and normal aortic (n = 9) segments (without endothelium) were compared during perfusion with EDRF released from cultured cells. The dilatation in response to EDRF was significantly smaller in the everted segments as compared with the noneverted segments (Figure 5a). In contrast, this dilator response in the everted segments was not changed when EDRF was superfused (i.e., EDRF applied at the everted intimal layer) (data not shown). The segments that had been tested in the everted configuration were studied again after reversion. The dilator response to EDRF after reversion did not significantly differ from that in the noneverted control segments (Figure 5a). To exclude the potential contribution of a diffusion barrier (formed by the adventitia) to the asymmetric dilator response to EDRF, the thickness of adventitial remnants was examined histologically. In approximately one half of the sites examined, the adventitia was absent, while in the remaining half, most of the remnants were below 10 μm (Figure 5b). In no case was a continuous adventitial layer observed. However, adventitial remnants were obtained in everted and noneverted vessels during intraluminal perfusion with sodium nitroprusside (10–100 nM). Identical experiments were performed in femoral arteries (n = 3) and yielded the same results (data not shown).

Discussion

The present study demonstrates the penetration of a nonprostanoid endothelium-derived relaxant factor (EDRF) (released by ACH, thimerosal, or A23187) through the walls of rabbit aortic segments. Three effective (although unspecific) inhibitors of EDRF (hemoglobin, dithiothreitol, and gossypol)12–24 abolished the vasodilatory activity of both luminal and adventitial perfusates. This suggests an identical nature of the EDRF detected at the adventitial and luminal sides during endothelial stimulation.

The appearance of EDRF in the adventitial superfusate probably represents an abluminal release from native endothelial cells. A rediffusion of an exclusively luminally released EDRF (which may be an alternative source of adventitial EDRF) would have to occur against a diffusion gradient. Furthermore, detectable adventitial EDRF activity was not diminished during augmented luminal perfusion rates (data not shown). Since this resulted in dilution of luminally released EDRF, rediffusion into the wall (and therefore the amount of EDRF to penetrate the wall) should also have diminished in the absence of an abluminal EDRF release. On the other hand, augmented perfusion in such a range did not elicit a detectable relaxation in endothelium-intact segments, thus arguing against an increased EDRF release attributable to increased flow4,6 (which could have compensated for a dilution effect).

The low EDRF activity in the adventitial superfusate
Asymmetric contractile responses of blood vessels to exogenous norepinephrine and serotonin have also been shown in several studies. Part of this asymmetry can be explained by neuronal uptake mechanisms for biogenic amines located preferentially in the adventitial layers of the wall. However, since this difference is still observed after uptake blockade, real differences in sensitivity to vasoconstrictors between inner and outer smooth muscle layers seem to exist. Regarding asymmetric responses to vasodilators, no further data are available at present. There was no difference between the amplitudes of dilation observed with intimal and adventitial application of sodium nitroprusside. However, sodium nitroprusside is a stable compound, and an approximately homogenous distribution can be assumed irrespective of the mode of application. This may mask a difference in sensitivity across the vascular wall, while it would become evident in response to EDRF, whose short activity half-life would result in a steep concentration gradient over the entire media. This asymmetry in EDRF sensitivity may have important functional implications. The signal transmission of endothelium-mediated dilation across the smooth muscle layers of the media does not appear to be solely attributable to EDRF diffusion. Direct relaxant effects of EDRF on smooth muscle cells in the outer wall layers should play only a minimal role because of the low EDRF sensitivity and the decreased EDRF concentration in these layers. It is conceivable, however, that an electrical signal (i.e., hyperpolarization) generated by EDRF in the inner smooth muscle layer is propagated by myogenic coupling into the outer layers of the wall. Propagation of this signal is likely to be much faster than the diffusion of the relaxant factor across the wall. Although only suggestive, two observations support this view. First, the initial phase of dilation induced by EDRF is too fast to fit the concept of a pure diffusive propagation of dilator response compared with the response characteristics of other dilators. Second, in endothelium-denuded arteries, EDRF-induced dilation (EDRF released from cultured endothelial cells) was significantly less in the presence of depolarizing concentrations of potassium than in the presence of NE or PGF2α.  

In conclusion, the present study demonstrates that EDRF is released by endothelial cells in situ at both their luminal and abluminal sides. This relaxant factor does reach and can penetrate all layers of the arterial wall by diffusion. However, the lengthy diffusion time and the low sensitivity of the outer smooth muscle layers of the media are suggestive of additional mechanisms other than EDRF diffusion participating in the propagation of the endothelium-mediated relaxation within the arterial wall.

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