SUMMARY Cultured bovine endothelial cells were grown on microcarrier beads. Columns (0.2 ml) packed with microcarriers were perfused with oxygenated (20% O₂) Tyrode's solution containing indomethacin (10 μM), and the effluent was passed through precontracted, endothelium-denuded detector arteries. When the endothelial cells were stimulated with bradykinin (3–100 nM), adenosine 5'-triphosphate (0.3–30 μM), or calcium ionophore A23187 (10–300 nM), they released dose-dependently a nonprostanoid compound that dilated the detector vessel. The factor, probably identical to the endothelium-derived relaxing factor of native endothelium, evoked dilations of the same magnitude in different types of detector vessels (rabbit thoracic aorta, rabbit femoral artery, canine coronary artery). However, this relaxant factor was significantly more effective in arteries precontracted by norepinephrine or serotonin than in arteries precontracted by potassium depolarization. Thus, its dilator action resembles that of the nitrovasodilators. The factor is labile, with an apparent half-life in the range of 20 to 30 seconds. Its dilator potency was inhibited by dithiothreitol (0.2 mM), metyrapone (0.2 mM), nordihydroguaiaretic acid (20 μM), and hemoglobin (1 μM), all of which apparently inactivated the factor. Synthesis or release (or both) of the relaxant factor was abolished by methylene blue (1 μM). High P₂O₅ levels (> 400 mm Hg) in the perfusate markedly reduced the release of the relaxant factor from the cultured cells. This study demonstrates that a vascular relaxant factor is released from endothelial cells in monoculture by adenosine 5'-triphosphate, bradykinin, and A23187 and establishes such a culture as a useful tool for analyzing the mechanisms of endothelium-dependent vasomotion. (Hypertension 9: 295–303, 1987)

KEY WORDS • cultured endothelial cells • endothelium-derived relaxing factor • adenosine triphosphate • bradykinin

CONSIDERABLE evidence suggests that the endothelium-dependent relaxation of vascular smooth muscle induced by acetylcholine, adenosine 5'-triphosphate (ATP), bradykinin, and various other vasoactive substances is mediated by a humoral signal.1,2 However, the chemical nature of this endothelium-derived relaxing factor (EDRF) has not been identified. In transfer experiments, EDRF has been characterized as a labile factor that is inactivated by either antioxidants or substances interacting with carbonyl groups.3 Release of EDRF from native endothelium has been observed under basal conditions and under stimulation with acetylcholine.3,5 A vascular relaxing factor was shown to be released from cultured endothelial cells6 in response to bradykinin and the calcium ionophore A23187 and is probably identical to the EDRF produced by the endothelium of intact vascular segments. In several recent studies, however, it was emphasized that the phenotypic expression of agonist-induced EDRF synthesis and release from cultured endothelial cells requires mixed cultures of endothelium and vascular smooth muscle.7,8 Therefore, the present study was designed to test whether endothelial cells in monoculture release EDRF in response to receptor stimulation. The action of this relaxant factor was characterized, and the inhibition of its synthesis or action by several differently acting compounds was analyzed. We report that ATP, in addition to bradykinin and A23187, elicits release of EDRF from cultured bovine endothelial cells. All three stimuli evoked the release of a transferable, nonprostanoid factor, identical in terms of lability and interaction with inhibitors and relaxing equally smooth muscle cells of different vessels and animal species. Furthermore, the study provides evidence that the
well-known inhibitory effect of methylene blue on the endothelium-dependent vasodilation is not only the consequence of an inhibition of guanylate cyclase at the level of vascular smooth muscle, but may also result from an inhibition of EDRF synthesis in endothelial cells.

Materials and Methods

Endothelial Cell Culture

Segments of adult or calf bovine thoracic aorta were obtained from the abattoir. The aortic segments were opened longitudinally, stripped of the adventitia, and incubated at 37°C with collagenase (CLS I Biochrom, Berlin, Federal Republic of Germany), 125 U/ml, for 20 minutes. Endothelial cells were then scraped off the intimal surface with either a rubber policeman or a cotton tip applicator. Harvested intimal cells were washed by centrifugation (100 g for 5 minutes) in Dulbecco's modified Eagle's/Ham F12 medium, seeded in standard culture dishes (Falcon, Oxnard, CA, USA) at densities of about 2 x 10⁴ cells/cm², and incubated at 37°C in a humidified 5% CO₂, 95% O₂ atmosphere. The culture medium contained 20% fetal calf serum (SeroMed, Munich, Federal Republic of Germany), endothelial cell growth factor prepared according to Maciag and Weinstein, streptomycin (50 mg/L), and penicillin (5000 U/L). After reaching confluence (3—4 days after seeding), approximately 6 x 10⁴ cells/cm², 5% CO₂, 95% O₂, and 200 rpm, the cells were detached by a short incubation in a Ca²⁺-Mg²⁺-free phosphate buffer solution containing trypsin (0.5 g/L) and EDTA (0.2 g/L) and then subcultured on dishes with a split ratio of 1:3. The identification of confluent cultured cells as endothelial cells was performed by examination for the typical homogenous "cobblestone" morphology using phase-contrast microscopy and, in some dishes, by indirect immunofluorescent staining for factor VIII antigen and angiotensin converting enzyme.

Preparation of Endothelial Cell Columns

Endothelial cells of second to fourth subculture were allowed to attach to the surface of solid plastic microcarrier beads (Biosilon, Nunc A/S, Roskilde, Denmark) with diameters ranging between 160 and 300 μm. The cells were cultured in stirring flasks (Techne Microcarrier System, Cambridge, UK) under continuous stirring (20 rpm). When the cells had reached confluence (3—4 days after seeding), approximately 250 μl of microcarrier beads (with about 4 x 10⁶ cells) were poured into the barrel of a 1-ml plastic syringe with a nylon mesh in the outlet conus. The plunger of the syringe, perforated by a thin Teflon tube, was carefully placed just above the level of beads in suspension. This cell column was kept in a water jacket (37°C) and perfused continuously (40 ml/hr) from bottom to top with Tyrode's solution (Po 120 mm Hg, pH 7.4). The perfusate was drained off from the column through the Teflon tube inserted into the plunger. The Tyrode's solution used in this experiment (also that bypassing the columns) always contained indomethacin (10 μM).

Bioassay System

The effluent from the endothelial cell columns was assayed for vasodilator factors using endothelium-denuded segments of rabbit or canine arteries. Segments of rabbit thoracic aorta or rabbit femoral artery were obtained from animals of either sex (2.5—3.5 kg) killed by decapitation. Mongrel dogs (25—39 kg) were killed with a high dose of sodium pentobarbital. The heart was removed, and the left anterior coronary artery was excised. Segments of these arteries (1—2 cm in length) were prepared free of connective tissue. The endothelium of femoral and coronary arteries was removed mechanically by gently rubbing the segments over a rough steel cannula. Deendothelialization of rabbit aortas was achieved by carefully skimming the tissue with a razor blade after inverting the segments. After ligation of the side branches, the segments were cannulated from both sides, stretched to their mean in situ length, and mounted within an organ bath containing Tyrode's solution (pH 7.4) of the following composition (mM): Na⁺, 144.0; K⁺, 4.0; Ca²⁺, 1.6; Mg²⁺, 1.0; Cl⁻, 140.0; HCO₃⁻, 11.9; H₂PO₄⁻, 0.36; calcium disodium EDTA, 0.025; glucose, 11.2. The Tyrode's solution, equilibrated with 95% O₂, 5% CO₂ and maintained at 37°C, was perfused through the organ bath at a rate of 50 ml/hr. The intraluminal perfusion of the segments (40 ml/hr) was also performed with Tyrode's solution (Po 120 mm Hg, pH 7.4). The transmural pressure of the segments was maintained hydrostatically at 60 mm Hg by adjusting the height of the outflow tubing. The outer vascular diameter was recorded continuously using a photoelectric device. After an equilibration period of about 60 minutes, the segments were precontracted by adding appropriate concentrations of norepinephrine (NE; 5 nM—1 μM) to the organ bath. In the coronary segments serotonin (0.1—0.5 μM) was used instead of NE. Depolarizing potassium solution had the same composition as the Tyrode's solution, except that the NaCl was partly replaced by an equimolar concentration of KCl.

The effectiveness of endothelium removal was confirmed by demonstrating the loss of dilator response to intraluminally perfused acetylcholine (1 μM). Furthermore, the segments were tested for vasomotor response to a direct application of any of the drugs used to stimulate endothelial cells or to inhibit EDRF-induced dilations. Segments exhibiting a direct response to a direct application of any of the drugs used to stimulate endothelial cells or to inhibit EDRF-induced dilations. Segments exhibiting a direct responsiveness were excluded as bioassay tissue. For the bioassay experiments, the outflow tubing from the cell column was connected to the inflow cannula of the detector segment, so that the column effluent entered the vessel after a transit time of 1 second. The following experimental manipulations could be performed by insertion of T connectors within the line between the column and the vessels:

1. The column effluent could be passed through delay coils of various lengths (transit time between 1 and 60 seconds; Figure 1).
2. Substances could be infused into the effluent distal to the column (see Figure 1).
EDRF FROM CULTURED ENDOTHELIAL CELLS/Lückhoff et al. 297

Figure 1. Experimental setup used for bioassay of EDRF released from cultured endothelial cells. The relaxant factor from the endothelial cell column was assayed by passing the effluent through a precontracted detector vessel. Drugs could be infused distal to the endothelial cell column. Transit time from the column to the vessel could be increased by passing perfusate through intervening tubing of different lengths. External diameter of the detector segment was recorded by a photodetector.

3. The column effluent could bypass the detector vessel, which was perfused separately with Tyrode's solution at this time.
4. The effluent could be passed alternately through two detector vessels arranged in parallel in two separate bath chambers. At the same time, the vessel not used for bioassay was perfused with Tyrode's solution, bypassing the column.

Stimulation of the endothelial cells was achieved by switching to a perfusion medium containing the particular stimulus. Cells were stimulated for 3 to 10 minutes and then allowed to recover for at least 20 minutes. A single superfusion experiment lasted up to 8 hours, depending on the viability of the endothelial cells.

Drugs and Solutions
Acetylcholine HCl, sodium nitroprusside (SNP), methylene blue, metyrapone, nordihydroguaiaretic acid (NDGA), indomethacin, dithiothreitol (DTT), melittin, and bovine hemoglobin were purchased from Sigma, Munich, Federal Republic of Germany. BAY k 8644 (methyl,1,4-dihydro-2,6-dimethyl-3-nitro-4-[2-trifluoromethylphenyl]-pyridine-5-carboxylate) was a gift of Bayer AG, Wuppertal, Federal Republic of Germany. SNP was dissolved in 10 ml of sodium acetate and diluted with Tyrode's solution. Acetylcholine, melittin, methylene blue, and metyrapone were dissolved in distilled water and diluted in Tyrode's solution. NE (Arterenol, Hoechst, Frankfurt, Federal Republic of Germany) was also diluted with Tyrode's solution. Hemoglobin was dissolved in distilled water. Oxyhemoglobin was prepared as described by Martin et al.14 and diluted with Tyrode's solution. Indomethacin was dissolved in ethanol/0.1 M NaHCO₃ (1:3 vol/vol) and diluted with Tyrode's solution. BAY k 8644 was dissolved in ethanol (0.2 mg/ml) and diluted with Tyrode's solution. DTT and NDGA were directly dissolved in Tyrode's solution at 37°C. Once prepared, NDGA solutions were used for no longer than 1 hour.

Figure 2. A. Dilator responses in a segment of rabbit thoracic aorta, precontracted with norepinephrine (2 μM) added to the organ bath medium, to EDRF released successively by 30 nM bradykinin (BK), 10 μM ATP, and 0.1 μM A23187, a calcium ionophore. D = external diameter. B. Dose-dependency of EDRF-induced dilations of precontracted rabbit aortic segments in response to agonist stimulation of cultured endothelial cells. Dilation is expressed as a percentage of the precontraction obtained by activation with 0.5 μM norepinephrine. Number in parentheses indicates number of experiments. This figure includes only data from cell batches in which the effects of all three stimuli were compared.

All concentrations of drugs given refer to the respective free bases or acids.

Statistics
Paired or unpaired Student's t test was used to evaluate statistical significance of the data. Differences were considered to be significant at a p value less than 0.05. All values are reported as means ± SEM.

Results
Release of a Nonprostanoid EDRF from Cultured Endothelial Cells by Various Stimuli
During stimulation with bradykinin, ATP, or the calcium ionophore A23187 in the presence of indomethacin (10 μM), cultured endothelial cells released humoral factor(s) that dilated the endothelium-denuded precontracted detector vessels (Figure 2A). The most potent agent for production or release (or both) of this EDRF was bradykinin (Figure 2B). The dose-effect curve for the EDRF-stimulus bradykinin was characterized by a steep increase of the slope within 1 log unit. Thus, the dose of bradykinin eliciting maximal dilation was only 10 times greater than the threshold concentration. Although substantial variation of the threshold concentration between 0.3 and 30 nM was observed between cell batches, the slope of the dose-effect curve remained virtually unchanged. The dilator response of the detector segments to the EDRF released by bradykinin was rapid in onset, but always only transient. Despite continuous stimulation of endothelial cells with bradykinin, the diameter of the
segments returned to the initial precontraction level (see Figure 2A). When the effluent from the cell column stimulated with bradykinin was switched to a second detector segment when the dilation of the first detector was waning, a further dilator response could not be elicited in the second segment. The responsiveness to maximal doses of bradykinin declined after repetitive challenges. Submaximal stimulation with bradykinin resulted in reproducible responses.

A graded concentration-dependent dilation also could be elicited by stimulation of endothelial cell columns with ATP in a threshold concentration of 0.3 μM. The maximum dilation was reached at 30 μM ATP and amounted to 75 ± 6% of the precontraction level (see Figure 2B). The dilator response persisted for as long as the stimulation was maintained and was rapidly and completely reversible when ATP was removed from the perfusate (see Figure 2A). As many as six to eight stimulations with ATP could be performed without any noticeable change in the response.

The calcium ionophore A23187, used in concentrations between 10 and 300 nM (see Figure 2B), was a strong stimulus for the production or release (or both) of EDRF from the cultured endothelial cells, as evidenced from the large and long-lasting dilation of the detector segments. The release of EDRF induced by calcium ionophore A23187 persisted longer than the actual stimulation (see Figure 2A). After repetitive maximal challenges with A23187, however, the ability of endothelial cells to release EDRF in response to any stimulus was greatly reduced.

The release of nonprostanoid relaxant factor also could be induced by melittin (1 μg/ml) and BAY k 8644 (1–10 nM) (see Figure 2B). While the latter compound elicited only a dilator response that was 11 ± 3% of the precontraction level (rabbit aorta, n = 4), melittin resulted in a complete reversal of the NE-induced contraction (rabbit aorta, n = 4). However, perfusion of the endothelial cells with melittin for 15 minutes led to striking changes in the cell shape, detectable by light microscopy, and to a partial detachment of the cells from the microcarrier beads.

Neither substance P (1–10 nM) nor acetylcholine (0.1–10 μM) induced any release of EDRF. Furthermore, no basal EDRF release from the cultured endothelial cells was detectable in our bioassay system.

**Lability of EDRF**

The EDRF released from cultured endothelial cells by bradykinin, ATP, or A23187 was labile, as seen in the experiments with the delay coils. If the transit time between endothelial cell column and detector vessel was experimentally increased (from 1 second up to 50 seconds), the dilator response decreased.

Two protocols were applied to demonstrate the decrease of the biological activity of EDRF with time. First, the effluent from a stimulated column (ATP or A23187) was passed through a delay coil before entering the detector. As soon as the dilation reached a stable plateau, the delay coil was bypassed. Under these conditions, the segment dilated further, reaching a new steady state. In the second protocol, successive stimulations (bradykinin, ATP, A23187) were performed with delay coils of various lengths. These responses were then related to a subsequent control response (without coil; Figure 3). Estimations of the half-life of EDRF released by the three agonists varied between 16 and 39 seconds.

**Reduction of EDRF Release by High Po2**

When endothelial cells were superfused with Tyrode's solution saturated with 95% O2, 5% CO2 and then stimulated with submaximal doses of bradykinin, the dilations elicited by EDRF in the rabbit aorta were reduced to 19 ± 3% (n = 8) of the control responses under 20% O2 (Figure 4A).

To differentiate between the Po2 effects on EDRF production and EDRF stability, we compared the attenuation of EDRF-induced dilations caused by increasing the transit time (from 1 to 30 seconds) under 95% O2 and 20% O2. The stimulus for EDRF release was bradykinin. The dilation under 95% O2, with a transit time of 1 second was brought to the same magnitude as the response under 20% O2 by increasing the concentration of bradykinin (Figure 4B). Under 20% O2, the reduction of the dilation after increasing the transit time to 30 seconds amounted to 47 ± 9% of the control response, and under 95% O2, to 39 ± 6% (rabbit aorta, n = 5, p < 0.2). Thus, the apparent half-life of EDRF in these experiments was only moderately decreased by high Po2. To exclude a possible interference of high intraluminal Po2 level (95% O2 in the perfusate) on smooth muscle responsiveness, precontracted aortic segments (n = 5) were perfused alternately with Tyrode's solution oxygenated with either 20% O2 or 95% O2. Neither the contractile response to NE nor the dilator response to SNP (10–100 nM) was significantly changed under the condition of intraluminal hyperoxia.

**Potency of the Effect of EDRF on Different Types of Arterial Segments**

All three types of arterial segments examined exhibited dilations in response to the effluent of stimulated endothelial cell columns. No significant differences in the dilator responses between rabbit thoracic aorta and rabbit femoral artery were found when both segments

![Figure 3. Effect of an increase in transit time on the EDRF-induced dilator responses in a precontracted rabbit femoral artery. The endothelial cell column was stimulated with 30 nM bradykinin. D = external diameter.](http://hyper.ahajournals.org/fig/3/3/v9n3p298f3.jpg)
were precontracted with NE and exposed alternately to the effluent of the same endothelial cell column stimulated with bradykinin (10 nM). Furthermore, the dilator responses of canine coronary artery (precontracted with serotonin) to EDRF did not differ significantly from the response observed in the rabbit thoracic aorta (Table 1). Similarly, EDRF released in response to A23187 (0.1 μM) caused identical dilations in both rabbit femoral artery and rabbit aorta (data not shown). A comparison between the dilator responses to EDRF released in response to ATP was not performed, since the rabbit femoral artery dilated directly in response to 1 μM ATP.

However, a differing capacity of EDRF to dilate vascular segments precontracted with either K⁺ or NE was found in our study. Paired segments of rabbit aorta, taken from the same animal, were precontracted to identical levels by either K⁺ or NE. In the segments precontracted with K⁺, the dilator response to EDRF was significantly smaller than that in NE-activated segments (see Table 1). The same finding was observed for the dilator action of SNP. SNP (0.1 μM) evoked a dilation of 62 ± 5% in the rabbit aorta (n = 5) precontracted with NE, whereas a dilation of 10 ± 4% was observed when precontraction was achieved with K⁺ (p < 0.001).

**Inhibition of EDRF-Induced Dilation**

Dilations observed in the detector vessels (rabbit thoracic aorta and femoral artery) when the endothelial cell column was exposed to the EDRF-releasing agents (bradykinin, ATP, and A23187) could be attenuated or abolished in two ways. First, by adding substances to the effluent from stimulated cell columns, an inhibitory effect on the dilator activity of the EDRF already released was demonstrated for hemoglobin, DTT, NDGA, and metyrapone (Table 2). Inhibition was completely reversible upon withdrawal of the inhibitors, with the exception of hemoglobin. The EDRF-induced dilation after washout of hemoglobin was attenuated by 68 ± 6% of the preceding control response (Figure 5). DTT, NDGA, and metyrapone added directly to the organ bath had no significant effect on the contractile response to NE in the rabbit aorta (n = 6). Preincubation of precontracted aortic segments (15 minutes) with DTT (n = 6), NDGA (n = 6), or metyrapone (n = 6) added to the organ bath neither significantly reduced the responsiveness to SNP (10 nM) nor affected EDRF-induced dilations.

Second, a complete and long-lasting inhibition of the ability of endothelial cells to release any dilating signal in response to bradykinin, ATP, or A23187 was observed after a short exposure (15 minutes) to methylene blue (1 μM). Following a washout period of at least 15 minutes (while the effluent of the column bypassed the detector), the effluent of the stimulated cell column failed to elicit vasodilation in a detector vessel, which, itself, was never in contact with methylene blue (rabbit aorta, n = 6). The capability of the detector vessel to dilate in response to other relaxant agents, such as SNP, was unchanged during this period. A partial recovery of the capacity of endothelial cells to elicit endothelium-mediated dilations was ob-

**Table 1. Precontraction Levels and EDRF-Induced Dilation of Different Detector Vessels**

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Activating agent</th>
<th>Precontraction (% of r.o.d.)</th>
<th>Dilation (% of precontraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit femoral artery (n = 7)</td>
<td>NE</td>
<td>0.18 ± 0.06 μM</td>
<td>19.5 ± 2.9</td>
</tr>
<tr>
<td>Rabbit thoracic aorta (n = 7)</td>
<td>NE</td>
<td>0.46 ± 0.12 μM</td>
<td>17.8 ± 2.6</td>
</tr>
<tr>
<td>Canine coronary artery (n = 5)</td>
<td>Serotonin</td>
<td>0.21 ± 0.10 μM</td>
<td>13.4 ± 3.1</td>
</tr>
<tr>
<td>Rabbit thoracic aorta (n = 6)</td>
<td>NE</td>
<td>0.39 ± 0.13 μM</td>
<td>14.5 ± 3.3</td>
</tr>
<tr>
<td>Rabbit thoracic aorta (n = 12)</td>
<td>K⁺</td>
<td>0.41 ± 0.15 μM</td>
<td>17.6 ± 2.8</td>
</tr>
<tr>
<td>Rabbit thoracic aorta (n = 12)</td>
<td>K⁺</td>
<td>78 ± 11 mM</td>
<td>15.8 ± 3.5</td>
</tr>
</tbody>
</table>

NE = norepinephrine; Serotonin = serotonin; r.o.d. = resting outer diameter.
*EDRF was derived from endothelial cells after stimulation with bradykinin (10 nM) and alternately passed through two different segments.
†p < 0.001, compared with dilation of rabbit aorta precontracted by NE.
served after 60 minutes. Following methylene blue exposure, the dilator response to bradykinin (30 nM) in the rabbit aorta was reduced to 17 ± 5% of the control response. Methylene blue (10 μM), added directly to the bath medium of the detector for 10 minutes, completely abolished any EDRF-induced dilation, while no inhibitory effect was observed at a concentration of 0.5 μM.

Synthesis or release (or both) of EDRF was also attenuated by raising extracellular K+. In these experiments the detector vessels (rabbit aorta, n = 14) were precontracted with K+ (70 mM). A control dilation was induced with EDRF released from endothelial cells stimulated with bradykinin (10 nM). Then, endothelial cells were superfused with high-K+ Tyrode's solution (70 mM K+). Under these conditions, stimulation with bradykinin (10 nM) elicited a release of EDRF that caused, in the same detector vessel, a significantly smaller dilation than that observed under identical conditions. The amplitude of this contraction could not be changed by varying the transit time between cell column and detector. After return to the normal endothelial superfusate, the dilator response to bradykinin (10 nM) did not significantly differ from the preceding control response (20.2 ± 3.9% vs 23.7 ± 4.5%).

Dependence of EDRF Release on Extracellular Calcium

Continuous superfusion of endothelial cells with a calcium-free Tyrode's solution attenuated in a time-dependent fashion the EDRF-mediated dilator response elicited by bradykinin or ATP. The calcium concentration of the effluent from the column was increased to the level of the normal Tyrode's solution by introducing CaCl2 before the effluent entered the detector. One minute after switching to the calcium-free solution the EDRF-induced dilation was not significantly diminished, compared with the control, but the dilator response elicited 15 minutes later amounted to 43 ± 9% (rabbit femoral artery, n = 7) of the control response. Ten minutes after restoring the normal calcium concentration in the endothelial superfusate, the dilator response increased to 77 ± 12% of the control response.

Discussion

In the present study, cultured endothelial cells were stimulated to release a relaxant factor that was transferred to, and bioassayed in, endothelium-denuded arterial segments. With the use of this approach, we were able to study separately the synthesis and the release of a relaxing factor in endothelial cells, the physicochemical properties of this factor, and its actions on smooth muscle cells.

The humoral relaxing signal released from cultured bovine endothelial cells in response to ATP, bradykinin, and A23187 is not a cyclooxygenase product, since the release was not inhibited by indomethacin, and it is labile, since its dilator potency decreased with increasing transit times. These findings extend those obtained by Cocks et al.,6 who reported the release of EDRF from pure cultures of multipassaged endothelial cells challenged with either bradykinin or A23187. Although the half-life of released EDRF was estimated to be 6 seconds by Cocks et al., a considerably greater half-life was obtained from our experiments. Although this discrepancy might imply differences in the chemical nature of the relaxant factors released, a more likely explanation is the imprecise manner in which such half-life estimations are performed (only 1 concentration of endothelial stimulation). An exact analysis would require complete dose-effect curves of endothelial stimuli in addition to varying the transit times. Furthermore, the half-life of EDRF may be a
function of the Po$_2$ in the superfusate to endothelial cells, which was different in both experimental systems. A decrease in the EDRF-induced responses depending on the Po$_2$ level of the perfusate was previously observed in bioassay experiments using intact vascular segments as EDRF donors. Similarly, the present experiments imply a reduction of EDRF stability during exposure to high Po$_2$, since a transit time of 30 seconds decreased EDRF-induced dilation slightly more when carbogen-saturated Tyrode’s solution was used instead of a medium aerated with 20% O$_2$. However, this moderate reduction of stability cannot account for the marked attenuation of dilator response by high Po$_2$ observed in experiments with short transit time (1 second). Therefore, we suggest that an inhibitory effect of Po$_2$ acts predominantly on EDRF synthesis or release (or both).

The protective action of superoxide dismutase on the stability of EDRF has recently been shown by several groups. It was suggested that superoxide anions and probably other oxygen-derived radicals play a decisive role in the destruction or the synthesis of EDRF. Superoxide anions can be generated enzymatically by various pathways in endothelial cells or nonenzymatically in aqueous solutions. Since increased oxygen concentration results in an enhanced intracellular generation of superoxide that cannot escape across the endothelial cell membrane, it is conceivable that this highly reactive radical interferes with the synthesis of EDRF and is the main reason for the dependence of EDRF release.

The failure of muscarinic agonists and substance P to elicit EDRF release from the cultured endothelial cells has been observed by other authors as well. Since these bovine aortic segments exhibited endothelium-dependent dilation to acetylcholine (unpublished observations, 1986), a loss of muscarinic receptors must occur in pure endothelial cell culture. Recently, it was shown that an elevation in intracellular cGMP after stimulation with purinergic (ATP) or muscarinergic agonists (metacholine) could be induced only in mixed culture of endothelial and vascular smooth muscle cells and not in endothelial or muscle cell culture alone. Therefore, it is conceivable that the full phenotypic expression of endothelial muscarinic and other receptors requires the presence of smooth muscle cells. Such an interaction between these two cell types might also be mediated by humoral factors.

Also, the failure of cultured endothelial cells to exhibit a detectable basal EDRF release may be due to the absence of smooth muscle cells, which might act as a stimulus or permissive factor for basal EDRF release. An alternative explanation may be the relatively low flow rate used in our experiments, since an increase of shear stress on the endothelial surface can stimulate EDRF release from native endothelial cells in situ or in vitro.

No significant differences in the maximum dilator response to EDRF could be seen in the different types of arteries precontracted by NE or serotonin to the same level. This finding argues against a greater sensitivity to EDRF in coronary or peripheral arteries than in large conduit arteries such as the aorta, as is sometimes discussed. The enhancement of endothelium-mediated relaxation as one proceeds from proximal to distal sections of a vascular bed, as well as the greater endothelial responsiveness in coronary arteries than in the aorta or other great arteries, might therefore reflect the greater endothelium to smooth muscle volume ratio in the peripheral arteries, rather than an increase in sensitivity to EDRF.

Compounds that inhibit endothelium-mediated vasodilation may have three principal modes of action. First, they may inhibit the synthesis or release of EDRF by endothelial cells. This requires contact with the endothelial cells to exert inhibitory effects. Second, they may interact with the released factor, thereby inactivating it. The inhibitor would then be effective in our system when added to the cell effluent rather than to the endothelial cells. Third, they may inhibit the relaxation of smooth muscle cells in response to EDRF. No direct evidence for such an action could be derived from our model.

The second possibility of inhibitor drug action has previously been demonstrated for the antioxidants NDGA and DTT. Both compounds inactivate EDRF released from rabbit aortic preparations stimulated with acetylcholine. The present results confirm this finding for the relaxant factor released from cultured endothelial cells stimulated with ATP, bradykinin, and A23187. Similarly, addition of hemoglobin or metyrapone (both compounds are reported as inhibitors of endothelium-mediated vasodilation) to the effluent of stimulated endothelial cells inhibited the EDRF-induced dilation in the detector vessels. One could argue that all these substances might affect smooth muscle responsiveness to EDRF rather than EDRF itself. This is unlikely, however, since NDGA, DTT, and metyrapone did not reduce smooth muscle relaxation elicited by SNP (which should use the same relaxing mechanism). Furthermore, when these compounds were applied extraluminally to the detector vessels, they did not affect significantly EDRF-induced dilations. Only the effects of hemoglobin may be attributed in part to an action at the smooth muscle level, since, in our study, the inhibition of EDRF-induced dilation by hemoglobin was not completely reversible and hemoglobin (10 μM) has been reported to abolish the relaxation induced by glycerol trinitrate and the concomitant rise of intracellular cGMP level in smooth muscle cells. Some of these EDRF-inactivating compounds, which probably act (as in the case of DTT, NDGA, and hemoglobin) by the generation of superoxide anions and other oxygen-derived radicals in saline solutions, may have additional inhibitory effects on EDRF production or release.

The inhibition of EDRF-induced dilation by metyrapone (an inhibitor of the cytochrome P-450 monoxygenase) at a level different from the site of EDRF production was unexpected. Indirect pharmacological evidence for the involvement of the cytochrome P-450 monoxygenase pathway in the production of EDRF in
the rabbit aorta has been presented by Singer et al., 25 who showed that the two inhibitors of cytochrome P-450, metyrapone and SKF 525A (β-dietilaminoethyl-diphenylylpropylacetate), partially suppressed ace-tylcholine-induced and A23187-induced relaxation. Cytochrome P-450 monooxygenase activity has been detected in the endothelium of rabbit arteries and pig aorta. 56, 27 Recently, Pinto et al. 28 were able to demon-strate that endothelium-dependent relaxations induced by arachidonic acid were potentiated after induction of cytochrome P-450 monooxygenase enzymes and attenuated after either depletion or inhibition of these enzymes. Although these data are consistent with the view that cytochrome P-450 is involved in the endothelium-mediated relaxant effect of arachidonic acid, further evidence is required to establish its role in the production of EDRF.

Methylene blue, described as a specific inhibitor of the soluble guanylate cyclase, 9 has been used as a pharmacological probe to study the correlation between arterial tissue levels of cGMP and endothelium-dependent relaxation. However, our study demonstrates that the inhibitory effect of methylene blue on the EDRF-induced relaxation 29 is not restricted to the level of smooth muscle but is the consequence of an inhibition at the endothelial cell level as well. Methy-lene blue is known to interact with the enzymes of the respiratory chain and may thus influence the energy-requiring synthesis or release (or both) of EDRF. Furthermore, methylene blue has been shown to generate superoxide anions, which can subsequently yield highly reactive hydroxyl radicals. 30 Recently, Kontos 31 has suggested that these radicals probably are involved in the inhibition of EDRF synthesis by methylene blue.

The mechanism(s) by which an increase in extracel-lular K+ suppresses the EDRF synthesis or release is unclear. Since the same magnitude of inhibition was observed in cells stimulated with A23187 or bradykinin, an interference at the receptor level is unlikely. In some experiments using K+-depolarized endothelial cells, a contraction instead of a dilator response was seen. Therefore, it is conceivable that the simultaneous release of a contracting factor under the conditions of K+ depolarization of endothelial cells counteracts the dilation induced by EDRF. The existence of such a stable polypeptide constrictor derived from cultured endothelial cells has recently been described. 32

A critical role for calcium in the initiation of EDRF production was proposed by Furchgott et al. 33 Singer and Peach 34 have found, in the rabbit aorta, that the endothelium-dependent relaxation by methacholine or A23187 is markedly attenuated after removal of calcium from the bath medium. The superfusion of cul-tured endothelial cells with a calcium-free solution does not induce an abrupt, complete abolition of recep-tor-mediated EDRF response, as was observed in cas-cade systems using superfused aortic segments. 35, 36 but rather a time-dependent decrease, probably cor-reponding to a slow washout of calcium from intra-cellular stores. Such a washout can also be seen by measurements of the intracellular free calcium concentration in endothelial cells with the fluorescent indicator quin 2. In the absence of extracellular cal-cium, ATP as well as bradykinin still raised intracellu-lar calcium concentration, although repeated chal-lenges were followed by decreasing responses. 37

Our observation that BAY k 8644 induced the re-release of EDRF from cultured endothelial cells might be taken as suggesting a possible involvement of voltage-operated calcium channel in the synthesis or release of EDRF, as was hypothesized by Rubanyi et al. 38 However, at present no electrophysiological data are available on endothelial cells that could substantiate such a hypothesis.

This study demonstrated that EDRF is more effec-tive in dilating rabbit aortic segments precontracted with NE than segments activated with KCl. Nitrovaso-dilators and 8-bromo cGMP 39 show virtually the same difference in relaxant effectiveness for both types of contractile activation. Calcium antagonists, on the other hand, are much less effective in reversing NE-in-duced than K+-induced contractions. 40 This finding might suggest that EDRF, like nitrovasodilators, does not act by inhibition of calcium influx. This finding lends further support to the hypothesis that nitrovaso-dilators and EDRF exert their relaxant effect through a common intracellular pathway, probably involving ac-tivation of soluble guanylate cyclase and a subsequent phosphorylation of certain myoplasmic proteins in-volved in the regulation of smooth muscle tone. 41

In conclusion, this study shows that endothelial cells in monoculture are able to synthesize and release EDRF. Because of the high ratio of endothelial cell surface to perfusate volume in the densely packed columns, higher concentrations of the labile EDFR can be achieved than in the effluent of intact arterial seg-ments. Furthermore, our findings support the concept of one common EDRF (rather than several different factors), since different substances uniformly inacti-vated the signal conveyed from cultured endothelial cells independently of the agonist used to stimulate the endothelial cells. Additionally, the study emphasizes that methylene blue suppresses EDRF-induced dilation, not by inhibition of the soluble guanylate cyclase in the vascular smooth muscle but rather by a complete abolition of EDRF production or release. This inhibi-tory effect may be causally related to the generation of superoxide anions in the endothelial cells. Similarly, the attenuation of the EDRF-induced response at high endothelial Po2 levels may be due to an interference by oxygen-derived radicals generated under those condi-tions with the production of EDRF.

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References


Characterization of vascular relaxant factor released from cultured endothelial cells.
A Lückhoff, R Busse, I Winter and E Bassenge

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