Release of Endothelium-Derived Relaxing Factor From Human Umbilical Vessels

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The ability of human umbilical endothelial cells to release relaxing substance(s) in response to different agonists was investigated. Endothelium-denuded aortic rings of rats were used for the bioassay and tension recording. After precontraction, this preparation showed no response to histamine, acetylcholine, A23187, or adenosine triphosphate while serotonin elicited further contraction. Superfusion of the precontracted preparations with the perfusate from umbilical veins and arteries stimulated with histamine (10^{-2}-10^{-5} M), A23187 (10^{-5}-10^{-4} M), or adenosine triphosphate (10^{-2}-10^{-4} M) elicited a relaxation. No relaxation was obtained with acetylcholine (10^{-4}-10^{-5} M) or serotonin (10^{-4}-10^{-5} M). The relaxation of bioassay aortic rings under the influence of the perfusate from histamine-stimulated umbilical vessels was inhibited by mepyramine (10^{-5} M) but not by cimetidine (10^{-4} M) suggesting the involvement of H_{1}-receptors. The relaxation was also inhibited by increasing the transit time between the donor and the detector preparation, by methylene blue (5 \times 10^{-5} M), and by nordihydroguaiaretic acid (5 \times 10^{-5} M) but not by indomethacin (5 \times 10^{-5} M), characteristics which have been reported for endothelium-derived relaxing factor. The involvement of umbilical endothelial cells in the relaxation response was further confirmed by studying precontracted, rubbed rat aortic rings seeded with cultured endothelial cells from human umbilical veins. Such preparations relaxed in response to histamine (10^{-2}-10^{-4} M) in contrast with the control preparations. No relaxations of these preparations were observed in response to acetylcholine (10^{-2}-10^{-4} M). We conclude that the endothelial cells of the human umbilical blood vessels release an endothelium-derived relaxing factor in response to histamine, acting via H_{1}-receptors, but not in response to acetylcholine.

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The original observations of Furchgott and Zawadzki have clearly demonstrated that the endothelial cells have an important influence on the vascular effects of certain vasoactive agents. Indeed, removing the endothelium abolishes the vasodilator responses to a variety of substances and potentiates the effect of vasoconstrictor substances. The original sandwich techniques and later the cascade-transfer experiments have demonstrated that the endothelium releases, in response to a variety of stimuli, a chemical mediator called endothelium-derived relaxing factor (EDRF), which induces a relaxation of the vascular smooth muscle cells. In most cases, however, the ability of vascular in vitro preparations to show endothelium-mediated effects varies widely with the stimulus, the species, and the blood vessel under study.

Materials and Methods

Umbilical cords, obtained after normal vaginal deliveries, were ligated at the placental and fetal ends and placed in sterile containers filled with cord buffer (composition in mM: NaCl 137, KCl 4, glucose 11, HEPES 10 [pH 7.4 at 37°C] supplemented with antibiotics [penicillin 100 U/ml, streptomycin 100 μg/ml, amphotericin B 2 μg/ml]. The cords were stored at 4°C and used within 24 hours.

Bioassay

The capacity of human umbilical arteries and veins to release relaxing substances was assessed using a bioassay that has been described elsewhere. The vessels were carefully dissected out of the cord. During the preparation they were continuously kept in Krebs-Ringer bicarbonate solution (composition in mM: NaCl 135, KCl 5, NaHCO₃ 20, glucose 10, CaCl₂ 2.5, MgSO₄·7 H₂O 1.3, KH₂PO₄ 1.2, EDTA 0.026) at room temperature. A 4-cm piece of artery or vein was cannulated at both ends and fixed in an organ chamber containing Krebs-Ringer bicarbonate solution maintained at 37°C and gassed with 95% O₂ and 5% CO₂. A polyethylene tube (PE 160) of the same length was mounted parallel to these vessels in the organ chamber. These “donor” preparations were continuously per-
fused at a constant flow (2.5 ml/min) by means of a roller pump, with prewarmed (37° C) Krebs-Ringer bicarbonate solution gassed with 95% O₂ and 5% CO₂. A rat aortic ring from which the endothelium had been removed was suspended directly below the organ chamber with two hooks passing through its lumen. One of the hooks was connected to an isometric force-transducer (Gould Statham, UC 2-cell, Oxnard, Calif.), which enabled registration of the tension of the aortic ring. This “detector” preparation was continuously superfused by one of the perfusion lines. Care was taken to keep the distance between the donor and detector preparations as short as possible. The construction of the whole system allowed one to change the perfusion system superfusing the detector preparation by gently rotating the organ chamber. The detector ring was first superfused for at least 2 hours with the control solution perfusing the polyethylene tube. During that interval the ring was stretched regularly until the basal tension reached 0.5 g. Then norepinephrine (10⁻⁴ M) was added to the perfusion solution to induce contraction of the detector preparation. When the contraction level became steady, increasing doses of the substances under study were selectively added to the perfusion fluid superfusing the detector preparation at that moment.

Preparation of Rubbed Rat Aortic Rings

Male rats weighing 300-400 g were killed, and the thoracic aortas were excised and dissected free of surrounding tissue. Ring segments of 2-3 mm were prepared and the intimal surface denuded of endothelial cells by rubbing with a roughened polyethylene tube (PE 90). In all experiments, the efficiency of the rubbing was confirmed by the addition of histamine or acetylcholine. These substances do not have relaxation effects on precontracted, rubbed preparations whereas the effect is seen on intact rings.5

Culture of Endothelial Cells

Endothelial cells were obtained from human umbilical veins according to the method described by Jaffe et al. 12 The umbilical vein was cannulated at both ends and flushed with a warmed (37°C) sterile Ca²⁺- and Mg²⁺-free phosphate-buffered saline solution (composition in mM: NaCl 171, KCl 3.4, NaH₂PO₄ 10, KH₂PO₄ 1.8). The vein was filled with phosphate-buffered saline containing 0.1% collagenase and incubated at 37°C for 15 minutes. Cells from the collagenase digest and washings (30-ml culture medium without serum) were pooled, centrifuged for 10 minutes at 1,000 rpm, and resuspended in a fresh culture medium (composition: culture medium M 199, 10% fetal calf serum, 10% human serum, 2 mM L-glutamine, penicillin 100 U/ml, streptomycin 100 µg/ml, and amphotericin B 2 µg/ml). The suspended cells were seeded into T-25 flasks (25 cm²) coated with gelatin and grown at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. The culture medium was changed 1 day after the initial culture set-up and then twice a week. Confluency was reached in 3-12 days. Only primary cultures were used in the experiments. The homogeneity of the cultures was verified by morphological criteria. The identity of some of the cultures was checked by indirect staining with FITC-labelled factor VIII antibody.

Seeding Endothelial Cells Onto Rat Aortic Rings

Cells from primary confluent cultures were suspended by brief trypsinization (trypsin 0.05%–EDTA 0.02%). The cells were washed and suspended with a density of 10⁶ cells/ml in culture medium containing indomethacin (10⁻⁵ M). Rat aortic rings denuded of their own endothelial cells were added to this suspension, incubated at 37°C, and gently shaken every 30 minutes. After 4 hours of incubation, the aortic rings were carefully removed from the cell suspension, rinsed several times with Krebs-Ringer bicarbonate solution to remove the culture medium and the nonadhering endothelial cells, and fixed isometrically in a 20-ml muscle chamber with a tension of 0.5 g. The preparations were equilibrated under tension for 1 hour before the start of the experiment. Changes in tension were measured with a force-displacement transducer. Indomethacin was present throughout this procedure in a concentration of 10⁻⁵ M.

Drugs

The drugs used were acetylcholine chloride, adenosine 5'-triphosphate, histamine dihydrochloride, calcium ionophore A23187 calcium salt, 5-hydroxytryptamine creatinine sulfate complex, mepyramine maleate, norepinephrine bitartrate, nordihydroguaiaretic acid (NDGA), and indomethacin crystalline (Sigma Chemical Co., St. Louis, Mo.), methylene blue (Merck, Dormstadt, FRG), cimetidine (gift from Smith Kline & French Laboratories, Philadelphia, Penn.), fetal calf serum (Gibco Labor, Grand Island, N.Y.), culture medium M 199, human serum, amphotericin B, streptomyacin sulfate, sodium benzilpenicillin, L-glutamine (Flow Labor, McLean, Vir.), and factor VIII antibody (Atab, Scarborough, Maine).

All solutions were freshly prepared and solubilized in Krebs-Ringer bicarbonate solution. NDGA, indomethacin, and A23187 were first dissolved in ethanol and then added to the Krebs-Ringer solution. All concentrations are expressed as the final concentration in the solution. The results are expressed as means ± standard error of the mean. The statistical significance of the changes in response to various antagonists was evaluated by means of Student’s t test for paired data.

Results

Bioassay Experiments With Different Agonists

The perfused umbilical vein, umbilical artery, or polyethylene tube was exposed to increasing concentrations of histamine (10⁻⁵-10⁻⁴ M), acetylcholine (10⁻⁴-10⁻³ M), A23187 (10⁻⁸-10⁻⁴ M), ATP (10⁻⁴-10⁻³ M), or serotonin (10⁻⁴-10⁻³ M). Figure 1 illustrates the influence of the perfusates on the precontracted (norepinephrine 10⁻⁵ M) rubbed rat aortic rings after addition of histamine, A23187, and ATP.
Histamine, acetylcholine, A23187, and ATP elicited no effect when passed through the polyethylene tube before superfusing the assay ring while serotonin elicited a contraction. However, when the umbilical artery or vein was perfused with histamine before superfusing the assay ring, marked relaxation effects were observed. Analogous results were obtained using A23187. The relaxation effects elicited by ATP were much less pronounced, particularly with the perfusate from the umbilical vein. With acetylcholine no relaxation was observed. The addition of carbachol (10^-8-10^-5 M) also did not induce an effect on the detector preparation. The addition of serotonin to the perfusate of the umbilical vessels and the polyethylene tube were completely analogous, indicating that the direct stimulation of the detector preparation by serotonin was not modified by the release of a relaxing substance from the umbilical vessels. The order of the agents and donors tested was carefully changed during the experiments. Ethanol, the solvent for A23187, was tested in two experiments; however, no relaxation effect was observed.

An original recording of an experiment is shown in Figure 2. In the first part of this experiment, the assay ring was superfused through the polyethylene line. The addition of histamine elicited no relaxation of the detector preparation. In the second part of the experiment, the organ chamber was rotated so that the perfusate of an umbilical vein superfused the detector preparation. The addition of the same concentrations of histamine then elicited dose-dependent relaxation responses of the rubbed aorta. After washing, the effect of increasing doses of acetylcholine was tested on the same preparation. No relaxation occurred, suggesting that there was no release of relaxing substance(s) from the umbilical vein in response to acetylcholine and confirming the efficient rubbing of the detector preparation.

**Type of Receptor Involved in the Histamine-Induced Release of Relaxing Substance(s)**

The type of receptor (H₁ or H₂) in the umbilical veins and arteries, which on stimulation by histamine produces substance(s) able to relax a contracted rubbed rat aorta, was determined from the dose-response curves to histamine before and after the perfusion of the vessels for 30 minutes with the H₁-antagonist mepyramine (10^-3 M) or the H₂-antagonist cimetidine (10^-4 M). The results (Figure 3) show the inhibitory effect of mepyramine but not of cimetidine, suggesting the involvement of H₁-receptors. Analogous results were obtained in both vessel types.

**Reactivity of Rubbed Rat Aortic Rings Seeded With Cultured Endothelial Cells**

Rubbed rat aortic rings, which were seeded with cultured human umbilical vein endothelial cells as described in the “Materials and Methods” section, were precontracted with norepinephrine (10^-7 M) and then exposed to increasing doses of histamine and acetylcholine. The rings were mounted in parallel with rubbed rat aortic rings incubated in culture medium without endothelial cells. The latter preparations did not respond to histamine (10^-7-10^-4 M) or acetylcholine (10^-9-10^-6 M). In contrast, the rings seeded with umbilical vein endothelial cells responded in most cases (14 of 18 tested preparations) with dose-dependent relaxations in response to histamine (10^-7-10^-3 M). The preparations failed to relax in response to acetylcholine (10^-6-10^-4 M), however (Figure 4).

**Nature of the Relaxing Factor(s) Released by Human Umbilical Vein and Artery in Response to Histamine**

To verify whether the relaxation of the rubbed rat aorta elicited by the perfusate from a perfused histamine-stimulated umbilical vein can be attributed to the release of EDRF, dose-response curves to histamine were established before and after the addition of indomethacin, NDGA, and methylene blue to the perfusion solution. In addition, the influence of an increased transit time between the donor and detector preparation using a polyethylene tube (PE 160) was investigated. The results, illustrated in Figure 5, demonstrate that the relaxation effects are inhibited by the presence of NDGA (5 x 10^-5 M, 5 minutes) and methylene blue (5 x 10^-5 M, 20 minutes) and by an increase of the transit time between donor and detector preparation by only 6 seconds. They were not inhibited by the presence of indomethacin (5 x 10^-5 M, 30 minutes), which significantly increased the relaxation response.

Analogous results on the relaxation effect elicited by the perfusate from a histamine-stimulated umbilical artery were obtained with indomethacin and NDGA.
Discussion

The present study reports the ability of some substances to induce the release of an endothelium-derived relaxing factor (EDRF) from human umbilical arteries and veins. Most of the results were obtained using a bioassay technique in which denuded rat aortic rings were superfused with the perfusate of umbilical arteries or veins (donors). In a precontracted state, this aortic preparation, which serves as a "detector," responds to relaxing substances released from the perfused preparations.

When the rubbed bioassay ring was superfused with the perfusate from a polyethylene tube, the addition of histamine, acetylcholine, A23187, or ATP to the perfusion fluid elicited no relaxation, which confirmed the efficient rubbing of the detector preparations. The addition of serotonin resulted in further constriction. However, when the detector preparation was superfused with the perfusate from an umbilical vein or artery, dose-dependent relaxation effects were obtained in response to histamine, A23187, and ATP, which demonstrated the release of relaxing substance(s). Almost no relaxation was observed in response to acetylcholine, while serotonin again showed only a constricting effect.

The absence of a relaxing effect with acetylcholine was somewhat surprising since acetylcholine releases EDRF in most mammalian blood vessels. This absence is not an enzymatic destruction of acetylcholine because no effect was also observed with carbachol. It may indicate that endothelial cells lack the appropriate sensitive muscarinic receptors leading to EDRF release. The calcium ionophore A23187, which is in general the most effective agent for the stimulation of an EDRF-dependent relaxation response, is also very potent in releasing EDRF from the umbilical vessels.

From the endogenous substances tested, histamine clearly had the strongest potency in releasing EDRF from both the umbilical artery and vein. This effect of histamine is the result of the stimulation of an H₁-receptor mechanism. The involvement of H₁-receptors in histamine-induced EDRF-release is in accordance with previous findings on rat aorta.

To ensure that the relaxation elicited by histamine results from the stimulation of endothelial cells of the perfused vessels rather than from other types of cells or structures and based on the work of Cocks et al., we incubated endothelium-denuded rat aortic rings in suspensions of cultured endothelial cells from human um-
bilical veins so that the cells could adhere to the aorta. After incubation, these rings were rinsed several times with Krebs-Ringer bicarbonate solution to remove the culture medium and the nonseeded cells. Morphological study has shown that with this seeding procedure endothelial cells adhere to the internal surface of the artery. Such treated rings again showed relaxation responses to histamine but not to acetylcholine, which confirms our bioassay results and illustrates the involvement of endothelial cells.

Endothelial cells of the human umbilical vein repeatedly have been reported to produce large quantities of prostanoids. Moreover, histamine was reported to induce an enhanced release of the potent vasodilator prostacyclin from human umbilical vein endothelial cells, an effect that is also mediated by H₁-receptors. However, the relaxing substance(s) released in our experiments were not blocked by indomethacin, a cyclooxygenase inhibitor, when used in concentrations that block the synthesis of prostacyclin almost com-

**Figure 4.** Original recording of an experiment showing effect of increasing doses of histamine and acetylcholine on norepinephrine (10⁻⁷ M)-contracted rat aortic ring denuded of its endothelium and seeded with cultured human umbilical vein endothelial cells.

**Figure 5.** Relaxation, expressed as percent of the preexisting tone, of norepinephrine (10⁻⁷ M)-contracted detector preparations elicited by the perfusate from human umbilical veins stimulated with increasing doses of histamine before (control) and after perfusion with indomethacin (5 x 10⁻⁵ M, 30 minutes), NDGA (5 x 10⁻⁵ M, 5 minutes), or methylene blue (MB, 5 x 10⁻⁵ M, 20 minutes) and after increasing transit time between donor and detector preparation by 6 seconds (n = 4; *p<0.05; **p<0.01).
pletely (Weksler et al.17 and our measurements). In fact, the relaxation effect was increased. The relaxation effect was inhibited in the presence of NDGA and methylene blue and also by a small increase in transit time between the donor and the detector preparation. These findings correlate well with the characteristics reported for EDRF, a nonprostanoid arachidonic acid metabolite whose formation is inhibited by NDGA, which is a very labile compound whose effect is halved in about 6 seconds of delay6,14 and which increases cGMP levels of vascular smooth muscle cells, an effect considered to be at the origin of the biochemical mechanism that produces relaxation and can be inhibited by methylene blue.18,19

The physiologic role of an EDRF-release in response to histamine by the umbilical vessels is unclear. Human umbilical blood vessels repeatedly have been reported to contract in response to histamine.20–22 Only one study mentions a transient dilation followed by a constriction after the addition of histamine to perfused umbilical arteries.23 In our tests, no relaxation effect could be obtained with histamine on umbilical preparations mounted for isometric tension recording in the presence or absence of indomethacin and contracted with serotonin. However, Clymann et al.24 reported marked increases in cGMP levels in the human umbilical arteries in response to low concentrations of histamine, an effect that is also mediated by an H1-receptor mechanism, while they obtained only moderate increases in cGMP in response to serotonin and acetylcholine at high concentrations. This would indicate that EDRF also induces increased cGMP levels in the umbilical vessels but that there is only a poor response of these vessels to cGMP in contrast to other vascular smooth muscle cells.

We conclude that histamine, acting on H1-receptors, and A23187 are potent releasers of EDRF(s) from human umbilical vessel endothelial cells, either in the umbilical vessels themselves or from primary tissue culture.

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References


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