Shear Stress–Induced Release of Nitric Oxide From Endothelial Cells Grown on Beads

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An in vitro bioassay system was developed to study endothelium-mediated, shear stress–induced, or flow-dependent generation of endothelium-derived relaxing factor (EDRF). Monolayers of aortic endothelial cells were grown on a rigid and large surface area of microcarrier beads and were packed in a small column perfused with Krebs bicarbonate solution. The perfusate was allowed to superfuse three endothelium-denuded target pulmonary arterial strips arranged in a cascade. Fluid shear stress caused a flow-dependent release of EDRF from the endothelial cells. The action of EDRF was abolished by oxyhemoglobin and methylene blue, and the generation of EDRF in response to shear stress was markedly inhibited or abolished by N\textsuperscript{\textcircled{O}}-nitro-L-arginine, by N\textsuperscript{\textcircled{O}}-amino-L-arginine, by calcium-free extracellular medium, and by depleting endothelial cells of endogenous L-arginine. Addition of L-arginine to arginine-deficient but not arginine-containing endothelial cells rapidly restored the capacity of shear stress and bradykinin to generate EDRF. These observations indicate that fluid shear stress causes the generation of EDRF with properties of nitric oxide from aortic endothelial cells and that the bioassay system described may be useful for studying the mechanism of mechanochemical coupling that leads to nitric oxide generation. (Hypertension 1991;17:187–193)

There are two components that make up the total force applied to the intimal surface of blood vessels during blood flow. One is a perpendicular pressure component and the other is a tangential component called the wall shear stress, a frictional force produced when the blood flows across the endothelial surface. The perpendicular pressure component is not appreciable at the endothelial surface because the force is borne primarily by structural proteins in the blood vessel wall. However, wall shear stress falls entirely on the endothelial cell layer. Thus, fluid shear stress refers to the mechanical forces generated at the endothelial cell surface by blood flowing under pressure.

The vascular endothelium is capable of modulating the tone of the underlying smooth muscle in conduit and resistance vessels of the arterial bed in response to local changes in shear stress, pressure, and other mechanical factors.\textsuperscript{1–10} The purpose of this local regulation by the endothelium is to adapt blood vessel diameter to sudden changes in tissue perfusion demands. The concept of endothelium-mediated local regulation of vascular tone in response to changes in blood pressure and flow is not new.\textsuperscript{11–13}

A sudden increase in steady flow as well as the introduction of pulsatile flow enhances the release of prostacyclin not only from endothelial cells in culture\textsuperscript{14–17} but also from endothelium of perfused arteries.\textsuperscript{1–2,6,7,18} However, the role of prostacyclin in flow-dependent vasodilation in vivo has not been shown. Instead, endothelium-derived relaxing factor (EDRF) may be primarily responsible for the flow-dependent, endothelium-mediated vasodilation observed in coronary and femoral arteries in vivo, at least in the canine species.\textsuperscript{1,2,6,9,19}

The objective of the present study was to develop an in vitro model, using aortic endothelial cells grown on microcarrier beads, to study the factors influencing mechanically induced generation of EDRF. Monolayers of endothelial cells grown on a rigid surface in the form of large surface area microcarrier beads packed in a narrow column offer a convenient and reproducible method of studying the effects of fluid shear stress on endothelial cell function. Because shear stress increases as a function of the product of flow rate and fluid viscosity, the shear stress applied at constant fluid viscosity to the endothelial cells is a function of the column flow rate.
Bioassay Cascade

The bioassay superfusion technique used in this laboratory for the perfusion of intact blood vessels has been described in detail. Several modifications have been made to accommodate the perfusion of small columns of vascular endothelial cells. Briefly, 1 ml microcarrier beads containing 10–30×10⁶ bovine aortic endothelial cells (see below) was packed into a disposable plastic column (0.4 cm in diameter) that was perfused in a closed system with Krebs bicarbonate solution gassed with a 10% O₂, 5% CO₂, and 85% N₂ mixture at 37°C. The perfusion rate or flow of fluid through the column was regulated from 0 to 3 ml/min by a perfusion pump (Harvard Apparatus, South Natick, Mass.). The perfusate was allowed to superfuse three helical strips of endothelial-denuded bovine pulmonary artery arranged in a cascade, separated in flow time by 2–3 seconds. A second perfusion line was directed over the strips for the delivery of Krebs bicarbonate solution at a constant rate of 6 ml/min. Chemical agents to be tested for their effects on EDRF action were added to the medium supervising the strips. Indomethacin (10 μM) was added to both perfusion and superfusion media to prevent the formation of prostacyclin and other vasodilator prostaglandins. Responses of target arterial strips were measured and recorded as described previously. Arterial strips were precontracted to a steady submaximal (60–70% of maximum) level of tone with a mixture of phenylephrine (10 μM final) plus U46619 (3 μM final) added to the medium perfusing the strips. Glyceryl trinitrate was superfused over the strips for 1 minute, and the direct relaxant responses on the arterial strips were used to standardize the strips as reported previously. When bradykinin was tested, it was perfused through the column of endothelial cells for 3 minutes. In each experiment, the column of endothelial cells was perfused for 20 minutes without allowing the perfusate to superfuse the strips. This procedure was used to allow the cells to equilibrate and prevent accumulation of EDRF and other substances. At the appropriate times, as indicated by the closed bars in Figures 1, 2, 4, and 6, the perfused column of cells was positioned over the strips and relaxation was observed. Similarly, at the appropriate times corresponding to the end of the superfusion interval, the perfused column was positioned away from the strips, and an increase in smooth muscle tone to control levels was observed. In experiments where breaks occur in column perfusion over the strips to change the flow rate, perfusion medium, or add test agents, the column of cells was continually perfused without superfusion directly over the strips.

Cultured Aortic Endothelial Cells

Endothelial cells were harvested from two to three bovine thoracic aortas by standard protease-free, collagenase-incubation techniques, taking care to avoid scraping the tissue to avoid contamination by smooth muscle cells. After washing the cells twice in Dulbecco’s Modified Eagle’s Medium containing 10% fetal calf serum and a mixture of penicillin, streptomycin, and gentamicin, the cells were incubated in 75 cm² culture flasks for 24 hours to allow cell adherence. Nonadherent cells were removed by washing twice with cultured medium, and the cultures were continued for an additional 48 hours. Adherent cells were then removed from the flasks with 0.1% trypsin-EDTA in phosphate-buffered saline at 25°C, washed three times with culture medium, and incubated with 0.5 g Cytodex 3 microcarrier beads coated with a fine film of collagen. The beads, cells, and medium were transferred into a stirrer flask and stirred (20 rpm) for 1 minute and left undisturbed for 29 minutes, and this cycle was repeated for 4 hours; after this time the mixture was stirred while it was incubating until confluence was attained (3–4 days as assessed by phase contrast microscopy). Multiple passaged cells (up to six passages) have been used successfully in this study. Cells were a single population of endothelial cells, as characterized by their cobblestone appearance (30–50 μm in diameter), and intense granular staining with a fluorescent low density lipoprotein (1,1’-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate) performed as described.

Chemicals and Solutions

Bradykinin triacetate, phenylephrine HCl, indomethacin, L-arginine, p-arginine, hemoglobin (human), methylene blue, N⁶-nitro-L-arginine, Cytodex 3 microcarrier beads, and EGTA were purchased from Sigma Chemical Co., St. Louis, Mo. The Upjohn Co., Kalamazoo, Mich., provided U46619 ([15S]-hydroxy-11α, 9α(epoxymethano)prosta-5Z, 13E-dienoic acid), which was dissolved in absolute ethanol at a concentration of 10 mg/ml. Dilutions were prepared in cold distilled water to a final concentration of 0.1 mM and stored frozen. Glyceryl trinitrate (10% wt/wt triturated mixture in lactose) was a gift from ICI Americas, Wilmington, Del. Oxyhemoglobin was prepared from hemoglobin by reduction with sodium dithionite in oxygenated Krebs bicarbonate solution at 4°C as described. The highly water-soluble HCl salt of N⁶-nitro-L-arginine was prepared by dissolving the free base in 3N HCl, followed by rotary evaporation of the solvent and by subjecting the residue to a high vacuum until completely dry. The dried crystalline solid was stored desiccated under nitrogen at −20°C. N⁶-Amino-L-arginine acetate was synthesized as described previously. Krebs bicarbonate solution consisted of (in mM): NaCl 118, KCl 4.7, CaCl₂ 1.5, NaHCO₃ 25, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 11, disodium EDTA 0.023.

Results

Flow-Dependent Release of EDRF From Endothelial Cells

Increasing the perfusion rate of Krebs bicarbonate solution through the column of endothelial cells in
FIGURE 1. Representative record and line graph showing dependence of endothelium-derived relaxing factor release on rate of perfusion of Krebs bicarbonate solution through a column of aortic endothelial cells grown on microcarrier beads. Perfusion rate was varied as shown, and perfusate from endothelial cells was superfused over three endothelium-denuded pulmonary arterial strips (designated 1, 2, and 3) arranged in a cascade. Solid horizontal bars signify time interval during which perfused column of cells was allowed to superfuse the strips. Control responses to superfused gyceryl trinitrate (GTN) are shown. A plot of perfusion rate vs. percent relaxation of upper arterial strip (designated 1) is shown in bottom panel. Data are expressed as mean±SEM from six separate experiments.

FIGURE 2. Representative record of effects of oxyhemoglobin (Hb) on relaxant action of endothelium-derived relaxing factor released by perfusing a column of aortic endothelial cells grown on microcarrier beads at a flow rate of 3 ml/min. Perfusion from endothelial cells was superfused over three endothelium-denuded pulmonary arterial strips (designated 1, 2, and 3) arranged in a cascade. Solid horizontal bars signify time interval during which perfused column of cells was allowed to superfuse the strips, and open bar signifies time interval during which oxyhemoglobin was superfused over the strips. Control responses to superfused gyceryl trinitrate (GTN) are shown. Values under upper tracing represent mean±SEM of percent relaxation from six separate experiments.

FIGURE 3. Bar graph illustrating effects of perfused N\(^{\circ}\)-amino-L-arginine (N-NH\(_2\)-Arg) and N\(^{\circ}\)-nitro-L-arginine (N-NO\(_2\)-Arg) on release of endothelium-derived relaxing factor provoked by perfusing (3 ml/min) a column of aortic endothelial cells grown on microcarrier beads in absence (control) and presence of perfused bradykinin as indicated. Data are expressed as mean±SEM from eight separate experiments. *p<0.01 between responses obtained in presence and absence of either N-NH\(_2\)-Arg or N-NO\(_2\)-Arg (Student's t test for comparison of paired values).

the presence of 10 \(\mu M\) indomethacin caused a flow-dependent increase in the release of EDRF, as assessed by the characteristics of relaxation of the endothelium-denuded arterial strips arranged in a cascade (Figure 1). The decrement of magnitudes of relaxation of the three arterial strips, each separated by a 2-second time delay, is attributed to the short half-life of EDRF released from the endothelial cells. A linear correlation was observed between percent relaxation and the logarithm of the flow rate over the range of 0–3 ml/min. Increasing the perfusion rate of fluid through an endothelial cell-free column packed with only microcarrier beads produced no effects on the cascade arterial strips (not shown).

Inhibition of Flow-Dependent EDRF Release by Agents That Interfere With the Actions or Biosynthesis of Nitric Oxide

A perfusion rate of 3 ml/min of fluid through the column of endothelial cells caused a marked release of EDRF that was abolished in the presence of 1 \(\mu M\) oxyhemoglobin (Figure 2) or 10 \(\mu M\) methylene blue (not shown) added to the medium bypassing the cell column but superfusing the target arterial strips. Perfusion of 1 nM bradykinin through the column of cells caused an additional release of EDRF (Figure 3). In the presence of either 10 \(\mu M\) N\(^{\circ}\)-nitro-L-arginine or 10 \(\mu M\) N\(^{\circ}\)-amino-L-arginine perfused through the column of endothelial cells, the release of EDRF by control perfusion (3 ml/min) and by bradykinin was markedly inhibited (Figure 3). Extensive preliminary experiments showed that neither
Representative record of effects of L-arginine (L-Arg) on release of endothelium-derived relaxing factor provoked by perfusion (3 ml/min) with and without bradykinin (BKN) of normally grown aortic endothelial cells (top panel) and endothelial cells grown in medium in which L-Arg was replaced with D-arginine during final 24-hour period of growth on microcarrier beads (bottom panel). Perfusion from endothelial cells was superfused over three endothelium-denuded pulmonary arterial strips (designated 1, 2, and 3) arranged in a cascade. Solid horizontal bars signify time interval during which the perfused column of cells was allowed to superfuse the strips, and open bars signify time interval during which L-Arg was perfused through the column of cells. Control responses to superfused glyceryl trinitrate (GTN) are shown. Values under upper tracings represent the mean±SEM of percent relaxation from seven separate experiments.

Requirement of L-Arginine for Flow-Dependent EDRF Release

Perfusion of 100 μM L-arginine through a column of endothelial cells that had released EDRF normally in response to flow or bradykinin failed to alter the release of EDRF during 3 ml/min of control perfusion or perfusion with 1 mM bradykinin (Figure 4). Aortic endothelial cells that had been grown in medium in which L-Arg was replaced with D-arginine during the final 24-hour period of growth on microcarrier beads failed to release any EDRF in response to flow and released little or no EDRF on addition of bradykinin to the perfusion medium. However, addition of L-arginine to the medium perfusing the endothelial cells rapidly restored the capacity of flow and bradykinin to release EDRF (Figure 4).

Obligatory Role of Calcium in Flow-Dependent EDRF Release

Removal of calcium from the medium perfusing the column of endothelial cells nearly abolished EDRF release in response to either flow or bradykinin (Figure 5). Addition of calcium (1.5 mM) back to the perfusion medium rapidly restored the capacity of flow and bradykinin to release EDRF (Figure 5).

Release of EDRF From Endothelial Cells by Mechanical Stimulation

Experiments were conducted to determine whether electrical as well as mechanical stimulation could provoke the release of EDRF from endothelial cells. Although electrical field stimulation failed to provoke EDRF release, disturbing the column of endothelial cells during fluid flow through the column by insertion of platinum electrode wires caused a marked but transient release of EDRF (Figure 6). Just one pass of the wire across a distance equal to the inside diameter of the column caused a pronounced release of EDRF. The same manipulation with aluminum wire or narrow glass tubing produced similar results, and the amount of EDRF release was proportional to the duration of mechanical agitation. The release of EDRF by mechanical stimulation was abolished in the presence of 10 μM N\textsuperscript{G}-nitro-L-arginine perfused through the column of cells (Figure 6).
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platinum wire 1 cm into the bed of beads followed by passage of fusate from endothelial cells was superfused over three endo-

relaxing factor provoked by perfusion (3 ml/min) of a column of aortic endothelial cells grown on microcarrier beads. Per-

fusate from endothelial cells was superfused over three endo-

thelium-denuded pulmonary arterial strips (designated 1, 2, and 3) arranged in a cascade. MS signifies the insertion of a platinum wire 1 cm into the bed of beads followed by passage of the wire from one side of the column to the other (0.4 cm) and removal of the wire. EFS (30 V; 16 Hz, 0.2 msec square wave pulses) was applied for 30 seconds. Solid horizontal bar signifies time interval during which perfused column of cells was allowed to superfuse strips, and open bar signifies time interval during which NNA was perfused through column of cells. Control responses to superfused glycerol trinitrate (GTN) are shown. Values under upper tracing represent mean ± SEM of percent relaxation from five separate experiments.

FIGURE 6. Representative record of effects of mechanical stimulation (MS), electrical field stimulation (EFS), and N⁶-nitro-L-arginine (NNA) on release of endothelium-derived relaxing factor provoked by perfusion (3 ml/min) of a column of aortic endothelial cells grown on microcarrier beads. Per-

fusate from endothelial cells was superfused over three endothelium-denuded pulmonary arterial strips (designated 1, 2, and 3) arranged in a cascade. MS signifies the insertion of a platinum wire 1 cm into the bed of beads followed by passage of the wire from one side of the column to the other (0.4 cm) and removal of the wire. EFS (30 V; 16 Hz, 0.2 msec square wave pulses) was applied for 30 seconds. Solid horizontal bar signifies time interval during which perfused column of cells was allowed to superfuse strips, and open bar signifies time interval during which NNA was perfused through column of cells. Control responses to superfused glycerol trinitrate (GTN) are shown. Values under upper tracing represent mean ± SEM of percent relaxation from five separate experiments.

perfusion rate or flow of fluid through the rigid column of cells. The generation of EDRF from endothelial cells was found to be proportional to the logarithm of the perfusion rate or flow. Thus, the present observations indicate clearly that a homogeneous population of aortic endothelial cells possesses mechanoreceptors that sense changes in fluid shear stress and that these receptors are coupled in some manner to the formation and release of EDRF. The in vitro model system described here appears to be suitable for studying endothelium-mediated, flow-dependent generation of EDRF. In this manner, flow through the column of endothelial cells generates a fluid shear stress at the endothelial surface that appears to mimic that which is generated by blood flowing through rigid-walled blood vessels or fluid flowing through a glass capillary tube to which arterial endothelial cells were grown to confluence along the inside surface. This model system may be useful in studying the mechanism of mechanochemical coupling that leads to EDRF generation, including the possibility that transmembrane potassium or calcium channels are involved.

In the presence of fluid shear stress, additional mechanical manipulation of the endothelial cells provoked the release of EDRF, and this was blocked by pretreatment of the cells with N⁶-nitro-L-arginine. Mechanical manipulation also releases prostacyclin from vascular endothelium. In the present study, the forces involved in the gentle mechanical manipulation of endothelial cells with wire or glass tubing may be additional fluid shear stress generated at the surface of the endothelial cells by the rapid movement of a solid object through the fluid medium bathing the tightly packed endothelial cells. This may be true also for the release of prostacyclin from vascular endothelium, since prostacyclin release by increasing flow is well-documented in isolated endothelial cells and blood vessels. The mechanochemical coupling mechanism that leads to the generation of EDRF may be similar to or the same as that leading to the generation of prostacyclin.

Relaxation of target arterial strips was used to quantify EDRF release. Perhaps a more quantitative measure of flow-dependent EDRF release would be chemical analysis of the relaxing factor released. Unfortunately, the diazotization procedure, which detects nitrite anion as a measure of nitric oxide generation, is too insensitive to monitor nitric oxide release from endothelial cells as compared with large perfused blood vessels. A sensitive chemiluminescence procedure is under development in our laboratory to quantify the release of nitric oxide from endothelial cells subjected to fluid shear stress.

Nevertheless, the properties of EDRF released by fluid shear stress and mechanical manipulation of the endothelial cells were similar to the properties of authentic nitric oxide. Experiments were conducted in the presence of 10 μM indomethacin, and therefore, it is highly unlikely that prostacyclin or other prostaglandins were responsible for the biological actions of the...
EDRF observed. The relaxant effect of the endothelial cell factor was inhibited by chemical agents that are known to inhibit the actions or formation of nitric oxide, such as hemoglobin, methylene blue, N^O-nitro-L-arginine, and calcium chelating agents in a calcium-free extracellular environment. Furthermore, EDRF generation was entirely dependent on L-arginine, as has been described for endothelial cells and other cell types. These observations are consistent with the view that EDRF is nitric oxide or a labile nitroso precursor. Moreover, the data are compatible with the view that EDRF is S-nitroso-L-cysteine, because the pharmacological properties of S-nitrosothiols are attributed to nitric oxide, and the endogenous nitric oxide that would be required to form S-nitroso-L-cysteine would likely be derived from L-arginine.

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