Release of Different Relaxing Factors by Cultured Porcine Endothelial Cells

C. Boulanger, H. Hendrickson, R.R. Lorenz, and P.M. Vanhoutte

Experiments were performed to determine the effect of ouabain on the release of relaxing factor(s) from cultured endothelial cells, and its action on the effect of the relaxing factor(s) on arterial smooth muscle. A column of porcine aortic endothelial cells grown on microcarrier beads in suspension culture was perfused with modified Krebs-Ringer bicarbonate solution. The release of relaxing factor(s) by the endothelial cells was detected under bioassay conditions by measuring the relaxing activity of the perfusate overflowing a ring of canine coronary artery (without endothelium) contracted with prostaglandin F\textsubscript{2}\textalpha. Incubation of the endothelial cells with ouabain did not affect the relaxation of the bioassay ring under basal conditions or upon stimulation of the endothelial cells with ADP but impaired the relaxation induced by bradykinin or the calcium ionophore A23187. Incubation of the bioassay ring with ouabain reduced the relaxation under basal conditions as well as the relaxation induced by ADP but did not affect the relaxation observed upon stimulation with bradykinin and A23187 and the endothelium-independent relaxations induced by nitric oxide. These experiments suggest that cultured porcine aortic endothelial cells release two endothelium-derived relaxing factors; one is released under basal conditions and upon stimulation with adenosine diphosphate and the other (which presumably is nitric oxide) upon stimulation with bradykinin and the calcium ionophore A23187. (Circulation Research 1989;64:1070-1078)

Endothelial cells play a key role in the modulation of the reactivity of vascular smooth muscle by their ability to synthesize and release endothelium-derived relaxing factor(s) in vitro,\textsuperscript{1,2} as well as under culture conditions.\textsuperscript{3-7} The relaxing factor released upon stimulation of cultured endothelial cells with bradykinin cannot be distinguished from nitric oxide.\textsuperscript{8} In the canine mesenteric artery, no changes in electrical properties of the smooth muscle could be observed during relaxations evoked by nitric oxide.\textsuperscript{9} Electrophysiological experiments have shown that the endothelium of the canine femoral artery releases a relaxing factor that induces transient hyperpolarization of the vascular smooth muscle, the action of which is inhibited by ouabain.\textsuperscript{10} The purpose of the present experiments was to determine whether or not cultured porcine endothelial cells release ouabain-sensitive and insensitive relaxing factors under basal conditions and upon stimulation with ADP, bradykinin, and the calcium ionophore A23187.

Materials and Methods

Culture of Endothelial Cells From Porcine Aorta

Porcine aortas were obtained from pigs killed at a local slaughterhouse less than 30 minutes after the death of the animals and were placed in Earle's balanced salt solution containing 100 units/ml penicillin G, 100 units/ml streptomycin, and 0.25 µg/ml fungizone kept on ice for transport. The thoracic aortas were cleaned of connective tissue, washed seven times with cold, sterile transport buffer, and cut longitudinally. The intimal surface was incubated for 10 minutes at 37° C in a Petri dish with 25 ml of collagenase solution from Clostridium histolyticum (0.5 mg/ml medium 199) and washed with culture media (composition: medium 199 supplemented with 10% fetal bovine serum, 20 µg/ml heparin, 100 units/ml penicillin G, 100 units/ml streptomycin, and 0.25 µg/ml fungizone). The intimal layer was then gently scraped with a rubber policeman, and the suspension obtained, as well as the collagenase solution, were collected and centrifuged at 250g for 7 minutes.\textsuperscript{11,12}
FIGURE 1. Identification of porcine endothelial cells with low density lipoprotein: fluorescence emitted by endothelial cells (grown on a glass coverslip coated with fibronectin) that had been incubated with acetylated low density lipoprotein coupled to the fluorescent probe 1,1'-dioctadecyl 3,3',3',3'-tetramethyl-indocarbocyanine perchlorate.

The pellet of cells was washed twice in culture media and collected again by centrifugation. The final suspension obtained in culture media was inoculated onto plastic Petri dishes (60 mm diameter; coated the day before with fibronectin by incubation for 4–6 hours with 10 µg fibronectin/ml medium 199). Endothelial cells obtained from one aorta were routinely seeded onto Petri dishes with a total surface area of 175 cm². The culture media was replaced routinely every 48 hours. Confluent monolayers of endothelial cells obtained after 4–5 days at 37°C in a 5% CO₂ incubator were washed with phosphate balanced salt solution (PBS) without calcium and magnesium and incubated for 5–10 minutes with a trypsin-EDTA solution (0.5 g trypsin and 0.2 g EDTA/l of PBS buffer) at room temperature. The suspension obtained was spun down at 250g for 7 minutes and the resulting pellet washed twice in culture media. The number of cells was determined by using a Neubauer device, and 6.5 x 10⁶ endothelial cells were seeded onto 2.5 x 10⁵ Cytodex 3 microcarrier beads. Microcarrier beads were maintained in suspension by continuous shaking of the 250-ml culture flasks at 60 rpm. Using this seeding, the microcarrier beads were coated with a confluent cellular monolayer within 3–4 days and were used for bioassay experiments in the next 3–4 days.

Besides the development of contact inhibition when reaching confluence, identification of endothelial cells in each primary culture was performed in the presence of acetylated low density lipoprotein coupled to a fluorescent probe and factor VIII antibody. Primary cultures were incubated with acetylated low density lipoprotein or with nonacetylated low density lipoprotein (used as control), which were both coupled to a fluorescent probe (1,1'-dioctadecyl 3,3',3'-tetramethyl-indocarbocyanine perchlorate). Controls were obtained by incubating endothelial cells with the nonacetylated low density lipoprotein coupled to the same fluorescent probe. Increased uptake of the acetylated low density lipoprotein showed positive identification of endothelial cells in the primary culture (Figure 1).

Cells for indirect immunofluorescence were fixed in 70% ethanol, then rehydrated in PBS buffer and incubated for 1 hour at 25°C with the monoclonal mouse related antibody to pig factor VIII (first antibody; designated as WI-23, courtesy of Dr. D.N. Fass, Mayo Clinic). Fluorescence was observed after incubation with the goat antimouse α-immunoglobulin coupled to fluoresceine. Controls were obtained by omitting the incubation with the first or the second antibody (Figure 2).
Bioassay of Relaxing Factors Released by Porcine Endothelial Cells

Left circumflex coronary arteries were taken from anesthetized (30 mg/kg i.v. sodium pentobarbital) and exsanguinated mongrel dogs (15–35 kg) of either sex. Excised tissues were placed in cold modified Krebs-Ringer bicarbonate salt solution (millimolar composition: NaCl 118.3, KCl 4.7, CaCl$_2$ 2.5, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, NaHCO$_3$ 25.0, Ca-EDTA 0.026, and glucose 11.1; control solution) cleaned of connective tissue, and cut into rings (4 mm length). The endothelium of a proximal ring was removed by gently rolling the luminal surface of the bioassay ring with the tip of a wet cotton swab. The bioassay ring was mounted between two stirrups, one anchored to a steel plate, the other connected to a strain gauge transducer (model FT03C, Grass Instrument, Quincy, Massachusetts) for the recording of isometric force. The setup consisting of bioassay ring, stirrups, and force transducer could be moved freely below two vertically mounted polypropylene chromatographic columns (internal diameter, 7 mm; Evergreen Scientific, Los Angeles, California) maintained at 37° C by a glass water jacket. This allowed the bioassay ring to be superfused with the perfusate from either a column filled with microcarrier beads without endothelial cells (direct superfusion) or with the perfusate of a column containing microcarrier beads coated with a confluent monolayer of porcine endothelial cells (endothelial superfusion). Each column contained approximately $1.2 \times 10^5$ sedimented microcarrier beads and was perfused at constant flow (3 ml/min) by means of a roller pump (Gilson Minipuls, Middleton, Wisconsin) with control solution oxygenated with a 95% O$_2$-5% CO$_2$ gas mixture (pH 7.4, 37° C). The transit time between the outlet of the column and the bioassay ring was less than one second. Any difference between direct and endothelial superfusion indicated the presence in the perfusate of factor(s) released from the endothelial cells. Drugs injected above the column of microcarrier beads without endothelial cells interacted only with the bioassay ring, while drugs injected above the column of microcarrier beads with endothelial cells interacted with both endothelial cells and the bioassay ring (Figure 3).

Experimental Protocol

The bioassay ring was stretched under direct superfusion in a stepwise manner until the response to 20 mM KCl was optimal (average optimal tension, 9 g). The removal of the endothelium from the bioassay ring was demonstrated by the absence of...
relaxation to acetylcholine (10⁻⁶ M) during contraction evoked by prostaglandin F₂α. All experiments were carried out in the presence of indomethacin (10⁻⁵ M) to inhibit the synthesis of vasoactive prostanoids.

In one set of experiments, the endothelial cells were incubated with ouabain (5×10⁻⁶ M) in the culture media for 45 minutes before packing them into the column. This column was connected to the bioassay system, allowing the endothelial cells to be washed for at least 20 minutes while the contraction of the bioassay ring was evoked by prostaglandin F₂α (4×10⁻⁶ M) under direct superfusion.

In the second set of experiments, only the bioassay ring was incubated under direct superfusion with ouabain (5×10⁻⁶ M) for 45 minutes. Because incubation of the bioassay ring with ouabain caused an increase in tension, different concentrations of prostaglandin F₂α (2×10⁻⁶ or 4×10⁻⁶ M) were chosen to match the contractions obtained in the control and the ouabain-treated ring. Prostaglandin F₂α contraction started just after the infusion of the ouabain was stopped at least 20 minutes before moving the bioassay ring below the endothelial superfusion.

The relaxing activity of the perfusate from endothelial cells was tested under basal conditions (basal release of endothelium-derived relaxing factors) or upon stimulation with bradykinin, ADP, or calcium ionophore A23187 (stimulated release of endothelium-derived relaxing factor[s]). At the end of each experiment, sodium nitroprusside (10⁻⁵ M) infused during direct superfusion induced relaxations below the baseline. In each experimental set, control responses under basal conditions or upon stimulation were obtained in parallel by perfusing a second bioassay ring with a column of endothelial cells from the same culture. The infusion of bradykinin, ADP, and A23187 through the endothelial line induced transient relaxations of the bioassay ring that peaked within 2 minutes (Figure 4); therefore, each concentration of endothelium-dependent vasodilators was administered during 2 minutes; dose-response curves were obtained in a noncumulative fashion (intervals of 20 minutes). Preliminary experiments showed that the effect of a submaximal concentration of bradykinin (10⁻⁸ M) or ADP (10⁻⁴ M) caused reproducible endothelium-dependent relaxations if they were applied at 20-minute intervals or more (data not shown).

Preparation of Nitric Oxide

A gas bulb fitted with a silicone rubber injection septum was filled with nitric oxide from a cylinder (Union Carbide, Chicago, Illinois). Appropriate volumes (100 and 1,000 µl) were removed with a syringe and injected into another gas bulb filled with 100 ml distilled water, which had been gassed with helium for approximately 3 hours, giving stock solutions of nitric oxide of 4×10⁻⁵ and 4×10⁻⁶ M. Noncumulative concentration response curves to nitric oxide were obtained under bioassay conditions by injecting a bolus of stock solution of nitric oxide on top of a column of microcarrier beads without endothelium.
Chemicals and Media for Cell Culture

Acetylated and nonacetylated low density lipoproteins were purchased from Biomedical Technologies, Stoughton, Massachusetts; collagenase and fibronectin from Boehringer Mannheim Biochemicals, Indianapolis, Indiana; fetal bovine serum from Hyclone Laboratories, Logan, Utah; heparin, trypsin and EDTA from Sigma Chemical, St. Louis, Missouri; microcarrier beads Cytodex 3 from Pharmacia, Piscataway, New Jersey; medium 199, Earle's balanced salt solution, phosphate balanced salt solution and antibiotics were obtained from M.A. Bioproducts, Walkesville, Maryland; all plastic flasks and dishes for cell culture were purchased from Falcon Lincoln Park, New Jersey.

Drugs

The following drugs were used: acetylcholine chloride, ATP, bradykinin, the calcium ionophore A23187, 5-hydroxytryptamine creatinine sulfate (serotonin), indomethacin, ouabain, prostaglandin F2α, sodium nitroprusside, theophylline all from Sigma Chemical; and UK 14304 (from Pfizer Limited, Sandwich, UK). Stock solutions of indomethacin were prepared in equimolar (10^-3 M) concentrations of Na2CO3 before further dilution with control solution or medium 199. The calcium ionophore A23187 (10^-4 M) was dissolved in pure dimethylsulfoxide, and further dilutions were obtained in distilled water. All other drugs were prepared in distilled water. All concentrations are expressed as molar concentrations in the solution superfusing the bioassay ring and the column of endothelial cells.

Statistical Analysis

Results are expressed as the means±SEM of the percentage of relaxation to sodium nitroprusside (10^-5 M) relative to the contraction just before administration of the agonist. In all experiments, n equals the number of dogs from which left circumflex coronary arteries were taken and N equals the number of different cultures of porcine endothelial cells. Each group tested with ouabain (endothelial cells or bioassay ring) was compared with its own control by statistical analysis performed with Student's t test for paired observations. When there were nonsignificant differences as determined by analysis of variance for unpaired observations, control values were pooled. Differences were considered significant at p<0.05.

Results

Vascular Smooth Muscle

The contractions of the bioassay rings evoked by prostaglandin F2α were not significantly different in the experimental groups (controls, bioassay ring treated with ouabain, endothelial cells treated with ouabain) (data not shown). Under direct superfusion, sodium nitroprusside (10^-5 M) induced relaxations of the bioassay rings below baseline, which were not significantly different among the experimental groups (data not shown).

Basal Release

A relaxation of bioassay rings contracted with prostaglandin F2α was observed upon switching from direct to endothelial superfusion with 10 out of 14 cultures of porcine endothelial cells (Figure 4). Incubation of the endothelial cells with ouabain (5×10^-6 M) did not significantly modify the response of the bioassay rings to the basal release of the relaxing factor(s) (Figure 5). However, the relaxations observed under basal conditions were significantly smaller after the bioassay rings had been incubated with ouabain (Figure 5).

Stimulated Release

ADP. When infused during direct superfusion, ADP (3×10^-7-3×10^-4 M) induced relaxation of the bioassay rings that were not significantly different between the experimental groups (controls, endothelial cells treated with ouabain, bioassay rings treated with ouabain) (Table 1). Infusion of ADP (3×10^-7-3×10^-4 M) through the column of endothelial cells caused transient relaxations of the bioas-
TABLE 1. Direct Effect of ADP on the Smooth Muscle of the Bioassay Ring in the Absence or Presence of Theophylline (3×10^{-3} M)

<table>
<thead>
<tr>
<th>ADP</th>
<th>Ouabain on bioassay ring</th>
<th>Control</th>
<th>Ouabain on endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>3×10^{-4} M</td>
<td>1.7±0.9</td>
<td>8.0±3.6</td>
<td>5.7±2.6</td>
</tr>
<tr>
<td>3×10^{-3} M</td>
<td>11.9±4.6</td>
<td>18.1±6.2</td>
<td>15.9±5.2</td>
</tr>
<tr>
<td>3×10^{-4} M</td>
<td>25.8±8.7</td>
<td>28.4±4.2</td>
<td>26.8±4.1</td>
</tr>
<tr>
<td>ADP (+theophylline, site 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3×10^{-4} M</td>
<td>-9.1±4.4</td>
<td>-6.1±1.0</td>
<td>. .</td>
</tr>
<tr>
<td>3×10^{-3} M</td>
<td>-5.8±5.1</td>
<td>2.4±6.2</td>
<td>. .</td>
</tr>
<tr>
<td>3×10^{-4} M</td>
<td>10.9±7.0</td>
<td>6.9±5.0</td>
<td>. .</td>
</tr>
</tbody>
</table>

Results are expressed as means±SEM of the percentage of the relaxation to sodium nitroprusside (10^{-3} M) relative to the contraction just before the administration of ADP. Positive numbers are taken as relaxation of the bioassay ring (site 2) underneath the bioassay ring treated with ouabain. Data shown as mean±SEM. *Effect of ouabain was statistically significant (p<0.05).

*Control experiments for the treatment of the bioassay ring with ouabain (5×10^{-6} M).
†Control experiments for the treatment of the endothelial cells with ouabain (5×10^{-6} M).

 Relaxation of coronary artery bioassay rings to relaxing factor(s) released from cultured endothelial cells stimulated by ADP. The responses in all experimental groups represent the difference between the relaxation of the bioassay ring observed when ADP was infused through the direct line (beads without endothelial cells) with the relaxation observed when ADP was infused through the endothelial line (beads with endothelial cells). Comparison with control (C) of the response to ADP after exposure of either the cultured endothelial cells (C) or the bioassay rings (■) with ouabain. For clarity, controls obtained for each treatment with ouabain (endothelial cells or bioassay ring) were pooled. The contractions to prostaglandin F_{2α} (expressed in grams) and the relaxations to sodium nitroprusside (expressed in percent of the contraction to prostaglandin F_{2α}) were, respectively: 7.8±0.5 g and 129.5±3.2% (controls; n=16); 8.0±0.6 g and 129.6±4.9% (n=9, bioassay ring treated with ouabain); 6.9±0.6 g and 141.4±6.5% (n=7, endothelial cells treated with ouabain). Data shown as mean±SEM. *Effect of ouabain was statistically significant (p<0.05).
prostanoid relaxing factors upon exposure to increasing concentrations (10^{-7}-10^{-4} \text{ M}) of acetylcholine, 5-hydroxytryptamine, or the selective \( \alpha_2 \)-adrenergic agonist UK 14,304 (data not shown).

**Endothelium-Independent Relaxation to Nitrergic Oxide**

Bolus injections of nitric oxide during direct superfusion induced transient relaxations of the bioassay rings that were not significantly modified by incubation of the bioassay ring with ouabain (5 \( \times \) 10^{-6} \text{ M}, for 45 minutes) (Figure 10).

**Discussion**

The present study using cultured endothelial cells demonstrates that ouabain: 1) reduces the effect of but not the liberation of endothelium relaxing factor released under basal conditions or upon stimulation with ADP; 2) blocks the release but not the effect of endothelium-derived mediators during stimulation with bradykinin or the calcium ionophore A23187; and 3) does not modify the relaxations evoked by nitric oxide.

Perfusion of cultured endothelial cells on microcarrier beads allows the measurement of the generation of vasoactive substances, using vascular smooth muscle as a detector.5,19-21 Under these bioassay conditions, porcine aortic endothelial cells release endothelium-relaxing factor(s), and prostanoid upon mechanical stimulation and when exposed to bradykinin and the calcium ionophore A23187.6 The relaxing activity due to endothelium-derived relaxing factor(s) alone is estimated by incubating the endothelial cells with indomethacin, which inhibits cyclooxygenase and thus the synthesis of prostacyclin.6 When studying the effect of drugs on the release of relaxing factor(s) from endothelial cells, the use of cell cultures eliminates the action of the tested drugs on other components of the blood vessel wall, which complicates the interpretation of experiments performed with intact blood vessels or in vivo; however, cultured porcine aortic endothelial cells do not release endothelium-derived relaxing factor(s) upon stimulation with acetylcholine (see also Gryglewski et al9), although endothelium-dependent relaxation to acetylcholine can be observed in the pig aorta in vitro.22

The present experiments demonstrate that incubation of the bioassay ring with ouabain differentiates between the effect on the smooth muscle of at
least two endothelium relaxing factor(s) released from the cultured endothelial cells. Indeed, the effect of the mediator(s) released under basal conditions and upon stimulation with ADP was sensitive to ouabain while that of the factor(s) released by bradykinin and the calcium ionophore was not. As endothelial-dependent relaxations to ADP were observed in the presence of a P1-purinergic receptor antagonist (theophylline) and were impaired by incubation of the bioassay rings with ouabain, it is unlikely that adenosine is the ouabain-sensitive relaxing factor released from cultured endothelial cells by the adenine nucleotide. The effect of ouabain on the relaxations observed under basal release or upon stimulation with ADP resembles the inhibition that our laboratory has reported of the hyperpolarization induced by endothelium-relaxing factor(s) released from canine femoral arteries stimulated with acetylcholine. Likewise, earlier work in the canine femoral artery indicates that potassium-free solutions inhibit the action of relaxing factor released from the endothelium upon stimulation with acetylcholine. The effect of ouabain could be due to direct inhibition of Na+-K+ pumping; alternatively, ouabain could shift the K+ equilibrium potential to a less negative level, reducing or abolishing a hyperpolarization due to opening of K+ channels. By contrast, the relaxations mediated by nitric oxide and the factor released from cultured endothelial cells by bradykinin and the calcium ionophore A23187 were not sensitive to the incubation of the bioassay rings with ouabain. Endothelial cells in culture release nitric oxide upon stimulation with bradykinin. Work from our laboratory has demonstrated that nitric oxide relaxes canine vascular smooth muscle without modifying its membrane potential. Thus, the present finding that ouabain differentially affects the response to endothelium-derived relaxing factor(s) released by adenosine diphosphate or shear stress (basal release) on the one hand, and by bradykinin and A23187 on the other hand, suggests that porcine endothelial cells can liberate two different endothelium-derived relaxing factors, one of which is not nitric oxide.

So far, no evidence that cultured endothelial cells can liberate different nonprostanoid relaxing factors by different mechanisms of release has been reported. In the present study, incubation of the...
endothelial cells with ouabain before stimulation decreased the relaxations induced by bradykinin and A23187. As the release of endothelium-derived relaxing factor(s) upon stimulation with the calcium ionophore is not receptor-mediated, ouabain is not likely to interact with the activation of membrane receptors for bradykinin triggering the increase of intracellular calcium in the endothelial cells.25,26 Cultured endothelial cells possess an electrogenic Na⁺-K⁺ pump, the inhibition of which by ouabain induces membrane depolarization, increase of Ca²⁺ influx and decrease of Ca²⁺ efflux.27-30 The present data do not allow us to link the mechanism of inhibition by ouabain of the release of relaxing factor(s) by bradykinin and A23187 to inhibition of this Na⁺,K⁺-ATPase pump although this is a likely site of action for the glycoside. Irrespective of its mechanism of action, since ouabain does not affect the response of the endothelial cells under basal conditions and upon stimulation with ADP, the use of the glycoside allows the distinction between at least two different mechanisms of release of relaxing factor(s) from cultured endothelial cells. The present study does not exclude that the two mechanisms of release of the different relaxing factors may operate in different types of endothelial cells growing together.

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