Evidence for Endothelium-Derived Relaxing Factor in Cultured Cells

ALEX L. LOEB, GARY K. OWENS, AND MICHAEL J. PEACH

SUMMARY Intracellular cyclic GMP concentration was used as a biochemical indicator of endothelium-dependent and organonitrate-induced responses to these vasodilators in cultured porcine aortic smooth muscle and endothelial cells. Sodium nitroprusside (10^{-6} M) caused a rapid increase in cyclic GMP levels in confluent smooth muscle cell cultures but not in confluent endothelial monolayers. Adenosine triphosphate (10^{-4} M) and methacholine (10^{-5} M), two agents that elicit endothelium-dependent relaxation in intact vessels, failed to raise cyclic GMP concentrations in muscle or endothelial cultures alone. When the cell types were grown together in mixed culture, however, treatment with adenosine triphosphate or methacholine induced an elevation in intracellular cyclic GMP levels. These findings suggest that mixed cultures of arterial smooth muscle and endothelial cells can be used to study the phenomenon of endothelium-dependent responses in arterial smooth muscle.

(Hypertension 7: 804-807, 1985)

KEY WORDS • endothelium-dependent relaxation • smooth muscle-endothelial cell coculture • sodium nitroprusside • methacholine • ATP • cyclic GMP

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N 1980, Furchgott and Zawadzki demonstrated that acetylcholine-induced arterial vasodilation was dependent on an intact layer of endothelial cells. Since that initial report many other vasodilator compounds have been shown to act by a similar mechanism. Since removal of the endothelial cells from an arterial segment prevented the vasodilation response, it was postulated that the endothelium transmitted a signal to the vascular smooth muscle (VSM) that induced muscle relaxation. Several laboratories have reported transfer of an endothelium-derived relaxing factor from one preparation to another. By varying the distance between the source of the factor (an intact vascular segment) and a bioassay vessel without endothelium, the half-life of endothelium-derived relaxing factor has been estimated to be about 6 seconds.

Rapoport and Murad have demonstrated that vasodilation induced by endothelium-derived relaxing factor can be correlated with an increase in cyclic GMP concentrations in rat aorta. Acetylcholine, histamine, and the calcium ionophore A23187 have been shown to produce large increases in cyclic GMP levels in intact vascular strips but to have no effect in those tissues devoid of endothelium.

These studies clearly established that intracellular cyclic GMP levels in arterial smooth muscle can be used as a biochemical marker for the action of endothelium-derived relaxing factor. To obtain large quantities of cells producing endothelium-derived relaxing factor, we have performed studies on endothelium-dependent responses in cultured cells using the cyclic GMP levels as an indicator of a smooth muscle response to endothelium-derived relaxing factor.

Materials and Methods

The VSM and endothelial cells were obtained from the thoracic aortas of young Yorkshire pigs killed by intravenous pentobarbital sodium overdose. After aseptic removal, the aorta was placed into cold medium 199 (M199; Gibco Laboratories, Grand Island, NY, USA). Loose connective tissue was removed, and the interior of the aorta was rinsed with fresh M199. The aorta was opened longitudinally and rinsed with fresh M199.

Endothelial cells were obtained by lightly scraping the intimal surface with a scalpel blade. The cells adhering to the blade were rinsed off into 35-mm culture dishes containing 2 ml of M199 supplemented with 20% fetal calf serum (HyClone, Hyclone Laboratories, Logan, UT, USA) plus penicillin (100 μg/ml) and streptomycin (100 μg/ml). Cells were allowed to grow in a 95% air, 5% CO₂ incubator until islands of endotheli-
um appeared. With the use of cloning rings, small numbers of endothelial cells were removed by trypsin treatment and plated into 2-cm² titer wells in M199 with 10% fetal calf serum. Confluent cells were harvested with trypsin and plated into 60-mm dishes.

To obtain smooth muscle cells from aorta, the intima was removed and the adventitia and outer third of the media were peeled away from the inner media and discarded. The remaining inner media consisting of arterial smooth muscle was minced into 1- to 2-mm pieces and incubated in collagenase type I (1 mg/ml; Worthington Biochemicals Corp., Freehold, NJ, USA), elastase type III (0.0125 mg/ml; Sigma Chemical Co., St. Louis, MO, USA), soybean trypsin inhibitor (0.375 mg/ml; Worthington), and bovine serum albumin (2 mg/ml; Sigma) in Hanks balanced salt solution (Gibco) at 37 °C for 17 hours. The dispersed cells were plated into tissue culture dishes in M199 with 10% fetal calf serum. At confluency, cells were detached from dishes using trypsin (0.01% VMF trypsin, Worthington; 0.02% ethylenediamine tetraacetic acid) and passaged by plating at a density of 1 × 10⁴ cells/cm².

Mixed cultures of endothelium and smooth muscle were obtained by letting endothelial cell cultures contaminated by smooth muscle become overgrown by the smooth muscle. Identification of endothelial cells in homogenous or mixed cultures was performed in the presence of factor VIII antigen and the fluorescent probe Dil-acetylated low density lipoprotein (Dil, Biomedical Technologies Inc., Cambridge, MA, USA), which is taken up preferentially into endothelial cells, and by examination of cellular morphology by phase contrast microscopy. Dil-acetylated low density lipoprotein at a concentration of 10 μg/ml in normal medium was incubated with the cells for 4 hours at 37 °C. The cells were washed with fresh medium to eliminate extracellular Dil-acetylated low density lipoprotein and visualized using a fluorescent-phase contrast microscope. Smooth muscle cells in mixed culture were identified using a smooth muscle-specific isoactin monoclonal antibody. The isoactins were also identified by two-dimensional isoelectric focusing/sodium dodecyl sulfate gel electrophoresis.

Cells for antibody staining were washed with phosphate buffered saline (pH 7.4), fixed for 5 minutes in 2% paraformaldehyde in phosphate buffered saline, and permeabilized with methanol (4 °C). Staining with either factor VIII antibody or the anti-actin antibody (designated B4, compliments of Dr. James Lessard, University of Cincinnati, or CGA7, compliments of Dr. Allen Gown, University of Washington) was done using an avidin-biotin peroxidase procedure (Vector Laboratories, Inc., Burlingame, CA, USA). Cyclic nucleotides were extracted from the cells into 0.1 N HCl. After removal of the acid extract, protein was removed from the dish by adding hot 0.2 N NaOH and scraping the dish with a rubber policeman. Cyclic nucleotide samples and protein samples were frozen and stored at −20 °C until assayed, using radioimmunoassay for cyclic AMP and cyclic GMP and the Bradford technique for protein.

**Results**

Porcine VSM cells were used at least 8 days after plating, when the cells were confluent. Drugs were added directly to the cells in culture medium. The time course of cyclic GMP increases after the addition of 10⁻⁶ M sodium nitroprusside or 10⁻⁵ M methacholine to pig aortic VSM is shown in Figure 1. The concentration of cyclic GMP was found to be increased 15 to 30 seconds after the addition of sodium nitroprusside and remained elevated at least 120 seconds. Addition of methacholine had no effect. The effect of 10⁻⁴ M ATP, 10⁻⁵ M methacholine, and 10⁻⁶ M sodium nitroprusside on monolayers of VSM or endothelium are shown in Figure 2. In pure cultures of either VSM cells or endothelial cells, ATP and methacholine had no effect on cyclic GMP levels. While sodium nitroprusside increased cyclic GMP concentrations in the VSM cells, it had no effect on cyclic GMP levels in endothelial cells. In contrast to these findings in homogeneous cultures, treatment of mixed endothelial–VSM cultures (up to the fifth passage, grown together since primary isolation) with either methacholine or ATP significantly increased the cellular concentrations of cyclic GMP (p < 0.05), as shown in Figure 3. Treatment with these agents did not change cyclic AMP levels from basal (1–20 pmol/mg protein; data not shown).

Figure 4 demonstrates the presence of endothelial cells in a mixed culture using the fluorescent probe Dil-acetylated low density lipoprotein.
FIGURE 2. Effect of the vasoactive agents methacholine (Mch), ATP, and sodium nitroprusside (SNP) on cyclic GMP (cGMP) accumulation in cultured porcine aortic endothelium (top panel) and smooth muscle (bottom panel). Cyclic GMP accumulation was measured after 30 (Mch) or 90 seconds (SNP, ATP). Values are means ± SEM from at least three separate experiments. * Significant difference from control (p < 0.05), using Dunnett's test for multiple comparisons to a control group.

FIGURE 3. Stimulation of cyclic GMP (cGMP) accumulation in mixed (endothelium-smooth muscle) cultures from porcine aorta at the fifth passage. Methacholine (Mch) and ATP induced rapid increases in cyclic GMP after 30 and 90 seconds respectively. Values are from at least three separate experiments. Statistics were done as stated in Figure 2.

FIGURE 4. Identification of cell type(s) in mixed culture. A. Phase contrast field of a mixed endothelium-smooth muscle culture (x 650). B. Same field as in Figure 4A demonstrating presence of fluorescent endothelial cells labeled with Dil-acetylated low density lipoprotein. The VSM cells are not fluorescent. C. Endothelial cell culture with contaminating VSM cells decorated with an anti-smooth muscle actin monoclonal antibody (x 700). Original magnification of Figure 4 (A–C) stated here has been reduced moderately for publication.
Dil-acetylated low density lipoprotein. This probe is selectively accumulated by and incorporated into membranes of the endothelial cells. Figure 4C shows a representative endothelial culture, sparsely contaminated by VSM, that has been stained with a smooth muscle cell-specific monoclonal antibody (designated B4) to smooth muscle actin.

**Discussion**

These findings demonstrate the feasibility of using cultured cells to study endothelium-dependent relaxation. Analogous to the intact tissue preparation, only mixed cultures of endothelium and VSM responded to methacholine and ATP with an increased accumulation of cyclic GMP. Cultures of a single cell type (VSM or endothelium) did not respond to methacholine or ATP with increased cyclic GMP levels. Studies are presently underway to determine 1) whether cell-cell (endothelium-VSM) contact is necessary for the stimulation of cyclic GMP accumulation seen in the cultured cells or 2) whether the VSM or endothelial cells release a conditioning factor(s) into the medium that enables the mixed cultures to respond with an increase in cyclic GMP following stimulation with agonists (e.g., methacholine or ATP) that cause endothelium-dependent vasodilation.

Several studies have shown that VSM cells in culture may not respond to stimuli in precisely the same way as arterial smooth muscle in vivo (see ref. 13). Dividing VSM cultures do not synthesize much smooth muscle-specific \( \alpha \)-actin, and replicating endothelium do not produce prostacyclin synthetase. Such phenotypic modulation must be considered when examining our data. In addition, it is known that endothelium and smooth muscle cells in coculture modify the growth behavior of each cell type. Although unlikely, it is possible that a third minor cell type thrives in the mixed cell cultures only and accounts for the results that are obtained. In any event, the present investigation suggests that mixed cultures of endothelium and VSM may be very useful not only in studying the phenomenon of endothelium-dependent relaxation in arterial smooth muscle, but also for the production of large amounts of endothelium-derived relaxing factor for isolation and characterization of this substance.

**Acknowledgments**

The authors thank George Vandenhoff for assistance with the cyclic GMP assay and Diane Singer for assistance with the cell culture portion of this study.

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Evidence for endothelium-derived relaxing factor in cultured cells.
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*Hypertension*. 1985;7:804-807
doi: 10.1161/01.HYP.7.5.804

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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