Endothelium-Derived Relaxing Factor in Cultured Cells

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SUMMARY Many vasoactive agents stimulate release of an endothelium-derived relaxing factor (EDRF). EDRF stimulates cyclic guanosine 3',5'-monophosphate (cGMP) accumulation and relaxation of vascular smooth muscle in a manner similar to that produced by sodium nitroprusside. Endothelium and vascular smooth muscle were isolated from porcine, bovine, and rat thoracic aorta. The capacity of sodium nitroprusside to stimulate cGMP accumulation in cultured bovine, porcine, and rat vascular smooth muscle was found to increase with time in culture to a maximum of 12 to 14 days after plating. In addition, bovine and porcine vascular smooth muscle, but not rat vascular smooth muscle, lost the sodium nitroprusside-stimulated cGMP response after the fifth passage. Cultured endothelial cells did not respond to endothelium-dependent vasodilators or sodium nitroprusside with increased cGMP levels. Vascular smooth muscle cells responded only to sodium nitroprusside. Mixed cultures of porcine and bovine endothelium and vascular smooth muscle and bovine endothelium and rat vascular smooth muscle responded to endothelium-dependent vasodilators with increased cGMP levels. Short-term (4 hours) coculture experiments using bovine endothelium grown on microcarriers to assess the need for long-term contact between the two cell types produced similar results. Release of EDRF from bovine endothelium was studied by loading endothelium-covered microcarrier beads into a column superfused with physiological buffer. Treatment of the column with bradykinin, the calcium ionophore A23187, melittin, and arachidonate released EDRF from the column as measured by cGMP changes in denuded aortic rings and vascular smooth muscle cells and by relaxation of rings when bathed in column effluent. The time course of cGMP changes and relaxation were similar and could be reversed by hydroquinone. These findings demonstrate that EDRF can be released from cultured cells and define a variety of cell culture techniques that can be used in the further study of EDRF. (Hypertension 9 [Suppl III]: III-186–III-192, 1987)

KEY WORDS • cell culture • vascular smooth muscle • endothelium • cyclic GMP • relaxation

ONE of the primary advantages of using cell systems to study the phenomenon of endothelium-dependent relaxation is that large numbers of cells with defined characteristics can be used in each experiment. Cultured endothelial cells grown on microcarrier beads can supply much greater quantities of endothelium-derived relaxing factor (EDRF) than can be released from isolated vessels. In addition, cell culture techniques allow the endothelial or vascular smooth muscle (VSM) cells to be treated or exposed to agonists or inhibitors of EDRF separately. In the intact vessel, endothelium is always in intimate contact with the underlying smooth muscle. It is well known that the endothelium and smooth muscle are able to modulate each other’s morphology. It also has been suggested that guanylate cyclase activity may be modulated in cultured cells when the muscle and endothelial cells are tested for cyclase activity separately.

In order to assay the presence of EDRF where relaxation cannot be measured, a biochemical parameter that correlates with relaxation must be used. The most appropriate biochemical parameter appears to be the stimulation of guanylate cyclase activity and subsequent accumulation of intracellular cyclic guanosine 3',5'-monophosphate (cGMP). This activity has been shown to be correlated with EDRF-induced responses with all indomethacin-insensitive, endothelium-requiring relaxing agonists studied to date. Using a transfer-superfusion technique, we found that EDRF can be transferred from cultured endothelial cells to

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produce endothelium-dependent relaxation and to induce an increase in cGMP levels in cultured smooth muscle and arterial rings or strips.

Materials and Methods

All cultured cells used in these studies (regardless of the source) were isolated as previously described for porcine aortic endothelium and smooth muscle or rat aortic smooth muscle.9 Bovine cells were grown in Waymouth’s medium (Gibco), and porcine and rat cells in medium 199 (M199, Gibco) in a 95% air, 5% CO₂ incubator. Both of these media were supplemented with fetal calf serum (Hyclone) plus penicillin (100 U/ml) and streptomycin (100 μg/ml).

For microcarrier culture, dispersed endothelial cells (2 x 10⁶) were seeded onto Cytodex 3 microcarrier beads (Pharmacia) in a 2-L roller bottle containing Waymouth’s medium (100 ml) and 20% serum, and aerated with 95% air, 5% CO₂. The culture was stirred every 30 minutes for 2 hours. The bottle of cells was rolled at 1 rpm, and the volume of the culture increased to 200 ml after 2 days. The cultures were fed twice per week. When confluent (after about 1 week), aliquots of cells on microcarriers were removed and washed with Krebs-Ringer buffer to remove culture medium in preparation for study.

Mixed cultures consisted of confluent VSM cells onto which suspensions of endothelial cells were plated (1 x 10⁵/cm²; Figure 1C). After 3 to 5 days, the cultures were used in experiments. Coculture experiments were initiated by placing washed endothelial cells grown on microcarrier beads directly onto confluent VSM monolayer cultures, which also had been washed with Krebs-Ringer buffer, as shown in Figure 1B. The number of endothelial cells added was calculated to be equivalent to the number of VSM cells in the dish. After 4 hours, the cocultures were challenged with various endothelium-dependent and -independent vasodilators. For both mixed and cocultures, 1.5 minutes after addition of drugs the medium was aspirated and 0.1 N HCl added to extract cyclic nucleotides. Previous experiments have shown this time to be optimal for the accumulation of cGMP after stimulation with sodium nitroprusside (SNP).

For transfer experiments, columns of cells were prepared as shown in Figure 1A. To make up the column, endothelial cells on microcarrier beads were placed into the barrel of a 20-ml syringe. Nylon mesh (100 μm) was fixed in the base of the column to prevent the beads from washing out. The cell column was superfused with oxygenated (95% O₂, 5% CO₂) Krebs buffer (3 ml/min), and the column temperature was maintained at 37°C. Relaxant activity in the column effluent was determined by bioassay using an endothelium-denuded rabbit thoracic aortic ring. In a typical experiment, the bioassay ring was mounted below the cell column prior to the addition of cells and was superfused with buffer as described above. The ring was allowed to equilibrate at 2.0 g resting tension for 1.5 hours. Phenylephrine (0.1 μM) was added to the buffer reservoir to constrict the ring. Drugs usually were infused into the superfusion system just prior to the cell column to give the desired final concentration in the superfusate for 1 minute. Endothelial cells were then added to the column and allowed to equilibrate before the effects of vasoactive agents on EDRF release were tested.

EDRF release from the column also was determined with cultured VSM cells using multiwell plates of confluent rat aortic VSM or rat aortic rings. The VSM cultures were preincubated in Krebs buffer for 1 hour prior to testing. Immediately before testing, the preincubation medium was aspirated from one well at a time, and column effluent was collected into the well for 1 minute. After another 30 seconds, the superfusate was removed and 0.1 N HCl added to extract cyclic nucleotides. Thus, the multiwell plate could be used to collect 1-minute fractions of column effluent prior to and during infusion of agonists through the endothelial cell column.

Aortic ring cGMP levels were determined using HCl extracts of aortic homogenates. For these studies, rings were suspended in tissue baths without an applied resting tension and incubated for 2 hours. After adminis-
tration of phenylephrine to constrict the rings, they were removed from the bath and placed into the column effluent path at appropriate times after drug infusion through the column. After a 90-second exposure to the column effluent, each ring was rapidly frozen in acetone-dry ice slush. The frozen rings were stored at −80°C until homogenization in 2 ml of ice-cold 0.1 N HCl. Each homogenate was centrifuged and the supernatant containing cyclic nucleotides was stored until radioimmunoassay. The protein pellet was solubilized in 0.2 N NaOH and assayed as described below.

Tissue cyclic nucleotide levels were measured by radioimmunoassay (RIA Kits, New England Nuclear, Boston, MA, USA) of HCl extracts of cell cultures. Protein content of the cells or aortic rings was determined using the Coomassie Blue method. Phenylephrine, melittin, methacholine, hydroquinone, SNP, and bradykinin, (all from Sigma, St. Louis, MO, USA) were dissolved in Krebs buffer. The calcium ionophore A23187 (calimycin; Sigma) was dissolved in dimethyl sulfoxide prior to dilution with buffer. Arachidonic acid (Sigma) was dissolved in hexane under nitrogen and stored at −80°C. Before use, the hexane was evaporated under nitrogen and the arachidonic acid dissolved in 0.1 N NaOH, diluted with buffer, and kept in ice.

Results

Detection and assay of EDRF using a response in cultured cells requires that the cell type bioassayed respond to organic nitrates as well as to EDRF with an increase in soluble guanylate cyclase activity and subsequent accumulation of intracellular cGMP. However, growing VSM cells appear to modulate this response over time. Figure 2 shows the increase over basal levels of SNP-induced cGMP in porcine VSM cells, second passage. The muscle cells reached confluence between Days 6 and 8 after plating, but the cyclase did not become maximally responsive to SNP stimulation until after Day 12. This time course of response probably was due to post-growth-phase cytodifferentiation of cultured VSM cells. This behavior of the cyclase was not limited to porcine VSM cells, but also was present in VSM cells derived from bovine and rat aorta (data not shown) and may be analogous to the time-dependent phenotypic expression of other differentiated characteristics in cultured VSM cells. Not only did cultured VSM cells require a certain postconfluence period to express the response to SNP, but the maximal response was also affected. For example, guanylate cyclase in bovine VSM cells lost the ability to respond to SNP after multiple passaging (Figure 3). Porcine aortic VSM cells also demonstrated a diminished response to SNP with number of passages, although not as quickly as bovine VSM cells. Rat aortic VSM, however, did not appear to lose the ability to respond to SNP even after multiple passages (up to 30).

When pure cultures of VSM or endothelial cells from rat, bovine, or porcine sources were exposed to endothelium-dependent vasodilators, there were no significant elevations in cGMP levels. However, when these same endothelial cells were plated directly onto confluent VSM cell monolayers for 3 to 5 days, the addition of certain endothelium-dependent vasodilators induced increases in cGMP concentrations. Figure 4 demonstrates this phenomenon in mixed cultures from porcine aortic VSM and endothelial cells, bovine aortic VSM and endothelial cells, and bovine aortic endothelial cells grown with rat aortic VSM cells. The lack of methacholine response appears to be due to the loss of cholinergic receptors on bovine endothelium, since adenosine 5'-triphosphate (ATP) and bradykinin treatment induced increases in cGMP in bovine aortic endothelium grown with rat aortic VSM.

Short-term cocultures of endothelial and VSM cells were made to determine whether the EDRF response required prolonged contact between the cell types. For these experiments, endothelial cells cultured to confluence on microcarrier beads were introduced onto confluent VSM monolayers for 4 hours. The total number of endothelial cells added was calculated to be equivalent to the number of VSM cells in the well (approximately 6 x 10³ cells). Figure 5 shows that long-term culture of the cells together was unnecessary to demonstrate EDRF release by the endothelial cells. In cocul-
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Figure 4. Evidence for EDRF production in mixed cultures. A. Porcine endothelial and porcine vascular smooth muscle (VSM) cells. B. Bovine endothelial and bovine VSM cells. C. Bovine endothelial and rat VSM cells. Endothelial cells were plated onto confluent VSM cell cultures. Three to five days later, mixed cultures were challenged with agonists. Drugs used were: methacholine (MCH, $10^{-5}$ M), ATP ($10^{-4}$ M), melittin (MEL, 3 μg/ml), arachidonic acid (AA, $10^{-4}$ M), calcium ionophore (A23187, $10^{-5}$ M), and sodium nitroprusside (SNP, $10^{-6}$ M). Asterisk indicates significant increase in cGMP level compared to control cultures (paired t test).

Figure 5. Release of EDRF after short-term coculture. Bovine aortic endothelial cells ($6 \times 10^9$) grown on microcarrier beads were introduced onto confluent monolayers of bovine (A) or rat (B) vascular smooth muscle (VSM) cells. After 4 hours, cultures were challenged with drugs or vehicle. MCH = methacholine; BK = bradykinin; SNP = sodium nitroprusside; AA = arachidonic acid; MEL = melittin.

Tissues in which bovine endothelium on microcarriers was incubated with either bovine or rat VSM, ATP, bradykinin, and melittin produced significant elevations in cGMP levels.

Since cultured endothelial cells clearly could be demonstrated to mediate an increase in intracellular cGMP concentrations in both mixed and cocultures, we next sought to determine whether the release of relaxing factor could be shown to affect tone or cGMP levels in endothelium-denuded arterial tissue or cultured VSM cells separated by some distance from the endothelium. For these experiments, a cell column superfused with Krebs buffer was mounted over a rabbit aortic ring from which the endothelium had been removed (see Figure 1), as described in Materials and Methods. The representative tracings shown in Figure 6 depict typical responses of the bioassay ring prior to, during, and after the addition of endothelial cells to the column. Prior to the addition of cells to the column, maximally effective doses of methacholine, A23187, bradykinin, clonidine, melittin, and arachidonic acid had minimal effects on tone of the rabbit aortic ring preconstricted with phenylephrine. The slight relaxation induced by A23187 is due to the dilator effect of the vehicle, dimethyl sulfoxide (0.1% final concentration). The addition to the column of cells grown on microcarriers ($5 \times 10^7$) invariably resulted in a relaxation of the bioassay ring that usually lasted for about 10 minutes. In some cases, tone did not return completely to the control level. This may have been due to basal or continuous release of EDRF from the endothelial cells. One-minute infusions of endothelium-dependent vasodilators through the column produced relaxations of the bioassay vessel (see Figure 6B). Although these agents were active at lower doses, the responses shown are to maximally effective concentrations of vasodilator. The responses were not affected by treatment with $2.8 \times 10^{-4}$ M indomethacin. The vasodilators of Figure 6 had no effect on the tone of the bioassay vessel when VSM cells on microcarriers were added to the column rather than endothelial cells.
To ascertain whether the relaxations seen in the bioassay vessel were in fact due to the action of EDRF, we examined the effect of the cell column effluent on cGMP production by cultured rat VSM and rat aortic rings (without endothelium). As shown in Figure 7, A23187, ATP, and bradykinin stimulated the release of a substance from the column that induced cGMP accumulation in rat aortic rings and in the VSM cell cultures. Bradykinin, which produced a transient relaxation in the bioassay ring, also produced a transient stimulation of cGMP production in both rings and VSM cells. In contrast, A23187, which induced prolonged relaxation of the bioassay ring, also appeared to induce prolonged release of the stimulus for cGMP production in VSM cells. Following exposure to A23187, the column effluent continued to contain enough EDRF to sustain elevated cGMP levels for at least 10 minutes. Clonidine, at a dose that caused endothelium-dependent relaxation of the bioassay ring, had no effect on cGMP or cyclic adenosine 3',5'-monophosphate concentrations.

When the known EDRF inhibitor hydroquinone was infused through the cell column during an A23187-induced vasodilation, the relaxation was rapidly reversed. This reversal of EDRF-induced relaxation also was correlated with a reversal of the cGMP response seen in rat aortic rings and in VSM cell cultures, as shown in Figure 8.

**Discussion**

The study and identification of EDRF has been hampered by the short half-life and elusive nature of the compound. The relatively small size of the endothelial cell population in isolated segments of vessels makes this problem of characterization even more profound. In the intact vessel wall, it is impossible to expose either cell type independently to drugs. We found that once the proper bioassay conditions are established, cultured endothelial cells can be demonstrated to produce EDRF, and the actions of EDRF on VSM cells can be studied both biochemically and functionally. We observed that the maximal capacity of VSM cells to respond to SNP with an increase in cGMP was delayed for several days after the cultures reached confluence. It is possible that in VSM cells the guanylate cyclase activity that is sensitive to nitrovasodilators is expressed most strongly in nongrowing, more differentiated cells. Many other differentiated cellular characteristics known to be present in vivo are expressed in cultured cells only after the cells stop growing. In addition, the loss of differentiated characteristics has been shown to occur in VSM as a function of time in culture.3 In fact, many types of cells in culture have been found to lose rapidly the soluble guanylate cyclase that responds to sodium azide, SNP, or EDRF.
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and only express the particulate form of this cyclase. The loss of the muscarinic stimulus for EDRF release in the bovine endothelium is another such example. In our experiments, bovine endothelial cells maintained their capacity to synthesize and release EDRF, and rat aortic VSM cells retained their sensitivity to EDRF.

Our findings on the transfer of EDRF from cultured endothelium as measured by relaxation of a bioassay vessel are consistent with findings from other laboratories. However, it is important to stress that relaxation may be caused by other agents in addition to or in conjunction with EDRF. Although clonidine appears to induce endothelium-dependent relaxation, the relaxation may not be related to EDRF because in all cases examined to date the actions of EDRF have been coincident with activation of guanylate cyclase. The mediator of the clonidine response remains to be determined.

The measurement of cGMP enabled us to verify that the relaxation seen in the column perfusion experiments was due to EDRF and to determine whether prolonged relaxations were due to continued EDRF release or a prolonged action of the relaxant factor or factors on the muscle. Since 1-minute fractions of column effluent released up to 10 minutes after a 1-minute stimulus of the column with A23187 were very active in stimulating the accumulation of cGMP in the bioassay, the prolonged relaxation probably was due to continued release of EDRF from the endothelium. Decreasing the flow rate or lengthening the dead space between the column and bioassay reduced or eliminated any vasodilator effect present in the column effluent. These findings confirm that the vasodilator released was very labile and similar to that seen by others.

The large increases in cGMP levels seen in bioassayed tissues (rings and cells) exposed to cell column effluent in comparison to that seen in intact rings suggests that the large amount of EDRF released from the column may be able to drive the guanylate cyclase to produce cGMP in excess of what is required for relaxation. For this reason, the cGMP response may be more accurate than relaxation as an indicator for EDRF.

The short half-life of EDRF in vitro, combined with its lability in serum or protein-containing solutions, suggests that EDRF is an autacoid that probably acts locally (where it is produced) rather than at a distant site. This is why the study of mixed and cocultures of endothelial and smooth muscle cells is important. Mixed cultures may most accurately simulate the environment of the blood vessel wall, allowing prolonged, intimate contact between the two cell types and the study of their interactions. However, no regulation or control of the ratio of VSM cells to endothelial cells is possible. The short-term coculture system does permit control of the cell ratio, which has several advantages. By altering the ratio of VSM to endothelial cells, one can model different types of vessels and perhaps their responses to EDRF. Small vessels have VSM to endothelial cell ratios close to 1, whereas large vessels can have very high ratios. Studies in which the cell ratios are altered may help clarify some of the reasons behind the disparate EDRF responses reported for large vessels versus small ones.

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