**N^G-Monomethyl L-Arginine Inhibits Endothelium-Derived Relaxing Factor–Stimulated Cyclic GMP Accumulation in Cocultures of Endothelial and Vascular Smooth Muscle Cells by an Action Specific to the Endothelial Cell**

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The effect of \( N^G \)-monomethyl L-arginine (LNMMA), an analogue of L-arginine (a proposed precursor of endothelium-derived relaxing factor [EDRF]), on EDRF release from bovine pulmonary artery endothelial cells was investigated using endothelial cell–vascular smooth muscle cocultures and a superfused column containing endothelial cells grown on microcarrier beads. Cocultures were stimulated with control buffer, ATP, bradykinin, melittin, A23187, or nitroprusside in the presence and absence of varying concentrations of LNMMA (30–300 \( \mu \)M). LNMMA caused significant, concentration-dependent decreases in cyclic GMP accumulation in response to the endothelium-dependent dilators bradykinin, ATP, melittin, and A23187 but had no effect on control or nitroprusside-stimulated cocultures. The inhibitory effect of LNMMA on cyclic GMP accumulation was partially reversed by treatment with L-arginine, but was unaffected by D-arginine. To determine the specific site of action of LNMMA, endothelial cells on microcarrier beads were placed in a column and superfused with buffer. The effluent from the column was collected in 30-second (1.5-ml) fractions into 2-cm\(^2\) monolayer wells of vascular smooth muscle cells before and after addition of agonists (bradykinin, A23187) to the column inflow. The cyclic GMP content of each well of smooth muscle cells was determined as an index of EDRF activity. LNMMA superfused through the endothelial cell column inhibited cyclic GMP accumulation in vascular smooth muscle cells induced by bradykinin and A23187. LNMMA introduced into the effluent from the endothelial cell column had no effect on smooth muscle cyclic GMP levels. We conclude that LNMMA is an effective, specific inhibitor of EDRF production or release, and its action is specific to the endothelial cell. (Circulation Research 1990; 67:979–985)
formed in intact vessels or in vivo preparations and have not demonstrated specifically that LNMMA antagonizes vasodilator or relaxation responses via an action on the endothelial cell.

We have studied the effects of LNMMA on EDRF production/action in two cell culture models. Cocultures of bovine pulmonary endothelial and rat vascular smooth muscle cells were used to study the concentration dependence and specificity of LNMMA-induced inhibition of EDRF-mediated cyclic GMP accumulation. In a second experimental model, the effluent from a column containing endothelial cells grown on microcarrier beads was collected sequentially into culture wells of vascular smooth muscle cells, and the muscle cells subsequently were analyzed for cyclic GMP content. By these procedures, we hoped to be able to localize the site of action of LNMMA (endothelial versus muscle cells) and to demonstrate the efficacy and specificity of LNMMA in cultured cells.

Materials and Methods

Cell Culture Techniques

Bovine pulmonary endothelial cells were prepared for culture by lightly scraping the intimal surface of the main pulmonary artery. The cells adhering to the blade were rinsed onto a 35-mm tissue culture dish and grown in medium 199 supplemented with 20% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). A pure culture of endothelial cells was obtained by fluorescent-activated cell sorting using diI-acyl–low density lipoprotein as the fluorescent marker. Identity of the endothelial cells was further confirmed by staining for factor VIII antigen; by demonstrating by Northern blot a single mRNA for actin in endothelium, while cultures of vascular smooth muscle express two actin mRNAs; and by observation of characteristic cobblestone morphological appearance. The endothelial cells were subsequently placed in microcarrier culture by seeding 2×10^6 cells onto 0.6 g Cytodex 3 microcarrier beads (Pharmacia, Uppsala, Sweden) (4,600 cm² surface area/g beads) in 200 ml medium 199 containing 20% fetal calf serum and maintained in 2-l roller bottles. All endothelial cells used in experiments were 2–8 days postconfluent and in passages 8–15. Rat aortic smooth muscle was isolated, cultured, and maintained as described previously.

Coculture Experiments

Endothelial cells on microcarrier beads were washed with serum-free medium and placed into monoculture wells (2 cm²) containing vascular smooth muscle that also had been washed with serum-free medium. The number of each cell type was calculated to provide a 1:1 ratio of endothelial to smooth muscle cells in each coculture. The cocultures were incubated at 37°C for 3–4 hours before experimentation, after which time the medium was replaced with phosphate buffered saline (pH 7.4) containing isobutylmethylxanthine (2×10^{-4} M). The cocultures were stimulated with the following agonists: bradykinin (1×10^{-8} M), ATP (1×10^{-4} M), melittin (2 μg/ml), A23187 (1×10^{-6} M), sodium nitroprusside (1×10^{-6} M), and buffer as a blank control. These concentrations of agonist were shown previously to maximally stimulate cyclic GMP accumulation. Each of these agonists was studied in the presence and absence of three concentrations of LNMMA (3×10^{-5}, 1×10^{-4}, and 3×10^{-4} M). LNMMA was added to culture wells 5 minutes before agonist stimulation. In some experiments, L-arginine (3×10^{-4} M) or D-arginine (3×10^{-4} M) was added to culture wells 5 minutes before the addition of LNMMA (1×10^{-4} M). Forty seconds after the addition of agonists (shown in preliminary studies to be the optimal time for detecting cyclic GMP changes under the current experimental conditions), the media in the culture well was aspirated, and 0.5 ml of 0.1N HCl was added to each well to extract cyclic GMP.

Column Transfer Experiments

A cell column for transfer of EDRF was prepared by placing endothelial cells grown on microcarrier beads (0.12 g, or approximately 4×10^7 cells) into the barrel of a 5-ml syringe with 100-μm nylon mesh fixed in its tip to prevent the beads from washing out. The cell column was superfused with oxygenated (95% O₂-5% CO₂) modified Krebs' buffer at 3 ml/min and maintained at 37°C C by a peristaltic pump. The column effluent was assayed for EDRF by collecting 1.5-ml (30-second) fractions into culture wells (2 cm²) containing confluent vascular smooth muscle cells. Fifteen seconds after collection of each fraction, the buffer from that well was aspirated and replaced with 0.5 ml of 0.1N HCl to extract cyclic GMP. The cell columns were stimulated to release EDRF by infusing bradykinin (1×10^{-4} M) or A23187 (1×10^{-4} M) into the inflow to the cell column for 30 seconds. The effluent from the cell column was collected using vascular smooth muscle culture wells like a fraction collector (30-second fractions) for a total of 5 minutes beginning 30 seconds before agonist infusion. In some experiments, LNMMA (1×10^{-4} M) was superfused through the column of endothelial cells, while in other experiments, LNMMA (1×10^{-4} M) was introduced into the effluent exiting the column so that only the smooth muscle cells were exposed to the compound. In both cases, the LNMMA infusion was begun 2 minutes before agonist stimulation of the endothelial cell column and continued through the duration of the experiment.

Radioimmunoassay of Cyclic GMP

Vascular smooth muscle cyclic GMP concentrations were determined by radioimmunoassay of acetylated HCl extracts by the method of Harper and Broker, as modified by Patel and Linden using an automated radioimmunoassay.

Drugs and Chemicals

Bradykinin, melittin, ATP, A23187, sodium nitroprusside, L-arginine, D-arginine, and isobutylmethylxanthine
L-arginine (L-ARG; 3x10^-4 cell-vascular smooth muscle cocultures. Drug dosage and combinations are shown in the figure. Each data point represents the mean of four experiments of four culture wells each. *Significantly different from control (p<0.05). Data are expressed as mean±SEM.

Statistical Analysis

Comparisons between groups were made using one-way analysis of variance followed by Dunnett’s test or the Newman-Keuls test when significant differences were present. Values of p<0.05 were accepted as significant. Values are expressed as mean±SEM.

Results

The results of the coculture studies are presented in Figures 1–6. Only the highest concentration of LNMA (3x10^-4 M) altered basal cyclic GMP concentration; however, treatment with L-arginine (3x10^-4 M) increased cyclic GMP levels above basal, and this increase was inhibited by LNMA (1x10^-4 M) (Figure 1). D-Arginine (3x10^-4 M) had no effect on basal or stimulated cyclic GMP accumulation in cocultures. In a concentration-dependent manner, LNMA significantly inhibited cyclic GMP accumulation in cocultures stimulated by the EDRF-releasing agents bradykinin (Figure 2), ATP (Figure 3), melittin (Figure 4), and A23187 (Figure 5). L-Arginine (3x10^-4 M) alone did not significantly potentiate agonist-induced cyclic GMP accumulation; however, it did partially reverse the inhibitory effect of LNMA (1x10^-4 M) in bradykinin-, ATP-, and melittin-stimulated cocultures (Figures 2–4, respectively). D-Arginine (3x10^-4 M) was ineffective in reversing the blockade of agonist responses with LNMA (1x10^-4 M) (Figures 2–5). In contrast to the attenuation of cyclic GMP accumulation in cocultures stimulated by endothelium-dependent dilators, LNMA had no effect on cyclic GMP levels obtained in cocultures stimulated by the endothelium-independent dilator sodium nitroprusside (Figure 6). L-Arginine also was without effect on nitroprusside-stimulated cyclic GMP accumulation in cocultures.

To identify the site of action of LNMA, we used superfusion of columns containing endothelial cells on microcarrier beads. The effluent from the
endothelial columns was applied to cultured smooth muscle cells, and cyclic GMP content of these cells was measured as a sensitive index of EDRF production. In such column transfer experiments, LNMMA (1 × 10⁻⁴ M) significantly inhibited bradykinin- and A23187-stimulated cyclic GMP accumulation only when it was introduced before the superfusate reached the column with endothelial cells and not when it was infused beyond the endothelial cells and therefore came in contact only with smooth muscle cells (Figures 7 and 8). This indicates that the site of action of LNMMA is localized to the endothelial cells and does not involve an action on vascular smooth muscle.

Discussion

The identity of EDRF has been a subject of intense investigation for the past 10 years. It has been suggested that EDRF may be NO, based on several observations including the correlation of equivalent degrees of vascular relaxation by these two agents with equivalent degrees of guanylate cyclase activation in vascular smooth muscle. Recently, two groups have demonstrated independently that L-arginine may be the primary substrate in the pathway for the synthesis or release of EDRF in vascular endothelial cells. Endothelial cells were shown to synthesize L-arginine. Bradykinin- and A23187-stimulated release of EDRF from endothelial cells was increased by infusions of L-arginine but not...
D-arginine or other basic amino acids. Subsequently, LNMMMA, an analogue of L-arginine, was reported to be a specific inhibitor of EDRF formation. LNMMMA inhibited in a dose-dependent manner the endothelium-dependent vascular relaxations induced by acetylcholine, the calcium ionophore A23187, and substance P without affecting the endothelium-independent relaxation of rabbit aortic rings by nitroglycerin or sodium nitroprusside. LNMMMA administered alone caused a small endothelium-dependent contraction, suggesting inhibition of basal EDRF production. This inhibition of endothelium-dependent vasodilation by LNMMMA was reversed by L-arginine but not by D-arginine or by several other arginine analogues. LNMMMA may act by competitively inhibiting a proposed "EDRF synthetase" enzyme that uses L-arginine as a substrate to produce NO (EDRF) and citrulline. Such an enzyme may be activated by the rise in [Ca²⁺] in endothelial cells as a result of activation of phospholipase C and calcium mobilization.

Recently, Thomas et al have questioned the specificity of LNMMMA by demonstrating reversal of the relaxation response induced by several endothelium-independent vasodilators including hydroxylamine, sodium nitroprusside, forskolin, dibutyryl cyclic AMP, and amiloride. This inhibition of endothelium-independent dilators occurred, however, only if the endothelium was present.

To further investigate the specificity of LNMMMA as an inhibitor of putative EDRF production from L-arginine, we used cultured cells for which cyclic GMP accumulation in vascular smooth muscle was used as the assay for EDRF activity. The endothelium is capable of producing a number of factors that may influence vascular tone but do not activate guanylate cyclase. For instance, some tissues relax in response to prostacyclin produced from endothelial cells. These responses are blocked by indomethacin, but not by hemoglobin or methylene blue, both of which prevent guanylate cyclase activation. Endothelium-derived hyperpolarizing factor is another endothelium-derived vasodilator distinct from EDRF. In addition, endothelial cells can produce a number of vasoconstricting substances. The amount of tone detected in vascular segments or reflected by blood pressure, therefore, is the end result of several additive and opposing events. For these reasons, cyclic GMP accumulation is a much more specific indicator of the production of EDRF, which presently is the only known endogenous activator of soluble guanylate cyclase in vascular smooth muscle. The results of this study indicated that LNMMMA specifically inhibits the production of an EDRF that acts on guanylate cyclase. The ability to isolate the endothelium from the vascular smooth muscle by cell culture has allowed us to determine that the specific site (cell type) at which LNMMMA exerts its action is the endothelium.

The present study demonstrates that in cocultures LNMMMA inhibits cyclic GMP accumulation stimulated by the endothelium-dependent dilators bradykinin, melitin, ATP, and the calcium ionophore A23187. LNMMMA also inhibited basal EDRF release as measured by cyclic GMP accumulation in control, unstimulated cocultures. LNMMMA had no effect, however, on cyclic GMP accumulation induced by the endothelium-independent vasodilator sodium nitroprusside. The specificity of LNMMMA for L-arginine was reversed by the fact that L-arginine reversed LNMMMA inhibition of endothelium-dependent cyclic GMP accumulation, while D-arginine did not. Further, we have demonstrated conclusively that the effect of LNMMMA is specific to the endothelium and does not involve an action on vascular smooth muscle.

The current studies demonstrate the inhibition of EDRF production by LNMMMA in cultured endothelial cells. The techniques of coculture and endothelial cells column transfer provide excellent model systems to investigate the mechanisms of EDRF production. They allow for rapid screening of agonists, antagonists, and inhibitors and provide the opportunity for separate pharmacological manipulation of the endothelial cell and vascular smooth muscle. The number of endothelial cells can be changed easily to facilitate a wide array of biochemical and pharmacological manipulations. Finally, as mentioned above, cyclic GMP accumulation in vascular smooth muscle is a much more specific indicator of EDRF activity compared with measurement of vascular relaxation.

There are several possible explanations for the data of Thomas et al demonstrating a lack of specificity for LNMMMA as an inhibitor of vascular relaxation. In their study, LNMMMA reversed endothelium-independent vasodilators only if the endothelium was intact. The authors suggest that LNMMMA may be releasing a noncycoxygenase contractile substance such as endothelin or the superox-

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**Figure 8. Endothelial cell column to vascular smooth muscle endothelium-derived relaxing factor transfer experiments.** N⁷-Monomethyl L-arginine (LNMMMA, 1×10⁻⁴ M) was infused above or below the column of endothelial cells. See "Materials and Methods" for details of protocol. A23187, the calcium ionophore A23187 (1×10⁻⁴ M). Data are expressed as mean±SEM; n=3 for each data point. *Significantly different from control (p<0.05).
ide anion from the endothelium. A more likely explanation is derived from the methodology of their study in which phenylephrine was used to preconstrict all rat aortic rings studied. It is well established that phenylephrine stimulates the release of EDRF from the endothelium, which attenuates its direct contractile effect on vascular smooth muscle. The observed endothelium-dependent reversal by LNMMA of endothelium-independent vasodilation could thus be explained by LNMMA inhibition of EDRF production by phenylephrine. Indeed, we have observed that the addition of LNMMA (1 x 10^-4 M) to rat aortic rings precontracted with phenylephrine (EC50) causes a 30% increase in developed tone when the endothelium is intact but has no effect when the endothelium has been removed (R.A. Johns, unpublished observation).

The results of our study also support the suggestion that endothelial cells release some EDRF even under basal conditions. Adding endothelial cells to smooth muscle cells increases basal cyclic GMP production. This effect is not attributable to cyclic GMP in endothelial cells and thus most likely is due to the basal release of EDRF. Consistent with this idea is the observation that LNMMA inhibits basal cyclic GMP accumulation in cocultures.

The scientific community is in agreement that the activation of soluble guanylate cyclase probably is mediated via a free radical (NO or nitrosothiol) that interacts with the heme moiety of the cyclase. The complexing of heme results in activation of guanylate cyclase. At issue really is the source of the NO; is the NO generated within the smooth muscle target cells in response to an endothelium-derived external signal, or is NO released by the endothelium in quantities that are sufficient to ensure diffusion and accumulation by smooth muscle?

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