Release of Nitrogen Oxides From Cultured Bovine Aortic Endothelial Cells Is Not Impaired by Calcium Channel Antagonists

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**Background.** The endothelium-derived relaxing factor has been shown to be nitric oxide or a related nitroso compound, synthesized by the enzyme nitric oxide synthetase, which oxidizes the guanidino nitrogens of arginine. This enzyme is activated by increases in cytosolic calcium. The effect of the clinically used calcium channel antagonists on this process is controversial. The present study was performed to determine whether calcium channel blockade with these pharmacological agents would alter the activity of nitric acid synthetase in intact endothelial cells.

**Methods and Results.** A specific and sensitive chemiluminescence assay was used to measure the release of nitrogen oxides (nitric oxide and one-electron oxidation products of nitric oxide) from bovine aortic endothelial cells grown in culture. Under basal conditions, the release of nitrogen oxides was about 0.2 nmol/100 μg protein/hr. Bradykinin doubled this response. Removal of extracellular calcium abolished basal and bradykinin-stimulated release of nitrogen oxides. Neither diltiazem, verapamil, nor nifedipine in concentrations that are encountered clinically altered the release of nitrogen oxides.

**Conclusions.** These experiments show that although the production of nitrogen oxides is dependent on extracellular calcium, the clinically used calcium channel antagonists do not inhibit the release of the endothelium-derived relaxing factor. *(Circulation 1991;83:1404–1409)*

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urchott and Zawadzki showed that the vasodilator action of acetylcholine on isolated rabbit aorta was mediated by the release of a nonprostanoid substance from intact endothelial cells subsequently termed “endothelium-derived relaxing factor” (EDRF). Further studies demonstrated that a variety of stimuli including increased vascular flow and pharmacological agents such as bradykinin, substance P, ATP, and histamine produce vascular relaxation by the release of EDRF.4–7 Recently, EDRF has been identified as nitric oxide (NO) or as a related NO-containing compound.8,9

The synthesis or release of EDRF(NO) in response to various agonists depends strongly on the presence of extracellular calcium.1 The calcium ionophore A23187, which facilitates the entry of extra-

cellular calcium, evokes strong endothelium-dependent relaxations in a variety of arteries isolated from different animal species.5,6 Removal of extracellular calcium depressed or abolished endothelium-dependent responses in isolated blood vessels and reduced or inhibited the release of EDRF(NO) under bioassay conditions or in cultured bovine aortic endothelial cells (BAECs).14–15 The mechanism by which Ca2+ enters vascular endothelial cells is not fully understood. Calcium channels in mammalian cells have been classified as being receptor or voltage operated. The latter are modulated by membrane voltage and are subject to stimulation by calcium channel agonists and to inhibition by the clinically used calcium channel antagonists. Selective calcium channel agonists have evoked relaxations of isolated rings of canine femoral artery16 and have triggered the release of EDRF(NO) from these vessels as measured by bioassay.17 These findings suggest that endothelial cells possess voltage-sensitive calcium channels and that production or release of EDRF is linked to these channels.17,18

There is conflicting evidence, however, as to whether calcium channel antagonists may alter the synthesis or release of EDRF. Pretreatment with verapamil and nifedipine has inhibited the maximal endothelium-dependent response to methacholine in
isolated rabbit aorta. With a bioassay system, d-cis-diltiazem partially reversed the relaxation induced by acetylcholine in canine femoral arteries. In contrast, flunarizine and verapamil failed to inhibit endothelium-dependent relaxation in isolated rat aorta; furthermore, nicardipine did not alter the acetylcholine-induced release of EDRF in rabbit aorta under bioassay conditions. One limitation of these studies is that the release of EDRF was measured indirectly by comparing vascular smooth muscle relaxations. This approach is problematic because the calcium channel antagonists likely have unavoidable direct effects on the vascular smooth muscle of either intact rings or the bioassay tissue. This is important because the calcium channel antagonists may reduce the bioactivity of released NO compounds from the endothelium by reducing the activity of the enzyme responsible for EDRF(NO) production or by either directly or indirectly inactivating EDRF(NO).

An alternative approach to determine the effect of an intervention on the release of NO and related compounds is to use chemical detection of these compounds. In the present study, we measured the release of EDRF(NO) in the presence of verapamil, diltiazem, and nifedipine by cultured BAECs with a chemiluminescence technique. This technique allows the direct quantification of nitrogen oxides (NO and one-electron oxidation products, including R-NO and NO$_3^-$) with an extremely high sensitivity. Moreover, this technique measures nitrogen oxides without interference by the agonists or calcium antagonists. Our results show that the release of nitrogen oxides by cultured endothelial cells is not affected by short-term application of organic calcium channel antagonists.

**Methods**

**Cell Culture**

BAECs were obtained as previously described. Cells from passages 5 to 9 were plated at a density of about 200,000 cells/well in six multiwell plates and were grown to confluence in 4–5 days in medium 199 with Earle’s salts and 20% fetal calf serum. At the time of study, the BAECs were washed two times and incubated with 1 ml modified Krebs’ buffer at 37°C for 60 minutes unless otherwise noted. The buffer had the following composition (mM): NaCl 118.3, KCl 4.7, CaCl$_2$ 2.5, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, NaHCO$_3$ 25, EDTA 0.026, glucose 11.1, at pH 7.40; this solution was continuously aerated with 95% O$_2$–5% CO$_2$ before use. All drugs were dissolved directly in this incubation buffer. After incubation, an aliquot of 500 μl was used to measure nitrogen oxide release by chemiluminescence. The BAECs were solubilized with 1 ml 1N NaOH overnight, and protein content was determined according to Bradford.

**Chemiluminescence**

The release of nitrogen oxides from the cultured BAECs was measured with a commercially produced NO analyzer (model 2108, Dasibi, Glendale, Calif.). The injected samples were carried by a continuous stream of deoxygenated water into a reflux chamber containing 1% NaI and glacial acetic acid. The samples were exposed to this reducing environment to degrade NO-containing compounds and to reduce NO$_2^-$ to release NO gas. Any released NO was then carried into the NO analyzer by a stream of nitrogen gas under vacuum. Within the analyzer, the gas was heated to 45°C and mixed with ozone in a reaction chamber. Ozone and NO spontaneously react to release light at a wavelength of 6,500–8,000 Å. The amount of light generated by this system is dependent on the concentration of NO present and is measured by a photomultiplier tube. Equimolar amounts of authentic NO (dissolved in deoxygenated water as described previously) and NaNO$_2$ yielded identical signals by this technique; NaNO$_3$ (up to 100 nmol tested) was undetectable (Figure 1).

**Drugs**

Bradykinin, diltiazem HCl, (±)verapamil HCl, nifedipine, and N-nitro-l-arginine were obtained from Sigma Chemical Co. (St. Louis, Mo.). Stock solutions of verapamil were made in 0.02N HCl and were further diluted in distilled water. Nifedipine was dissolved in dimethyl sulfoxide; the final concentration of dimethyl sulfoxide did not exceed 0.1%. Vehicles were used in the control experiments. Incubations with nifedipine and verapamil were protected from light. All drugs revealed no chemiluminescence signal by themselves.

**Data Analysis**

Data are presented as mean±SEM. The NO analyzer was calibrated before every experiment with known concentrations of authentic NO or NaNO$_2$. The amount of nitrogen oxide release from the cultured BAECs was determined with the respective standard curve. The amount of nitrogen oxide was expressed as nanomoles per well per 100 micrograms of protein. Statistical significance was tested with a t test; a probability value less than 0.05 was considered significant.

**Results**

The basal and bradykinin-stimulated release of nitrogen oxides from BAECs increased with the incubation time (Figure 2). After 1 hour of incubation, basal release of nitrogen oxides was about 0.2 nmol/100 μg protein; this release of nitrogen oxides increased twofold in response to 0.1 μM bradykinin. The basal or bradykinin-stimulated release of nitrogen oxides was inhibited in the presence of the l-arginine analogue N-nitro-l-arginine (1 and 10 μM, Figure 3).

Removal of calcium from the incubation buffer abolished, whereas an increase of the extracellular Ca$^{2+}$ concentration from 2.5 to 5.0 mM augmented, basal and bradykinin-stimulated release of nitrogen oxides (Figure 4).
Incubations with verapamil (0.1–10 μM) (Figure 5), diltiazem (0.1–10 μM) (Figure 6), and nifedipine (0.001–0.1 μM) (Figure 7) for 60 minutes did not influence the basal and bradykinin-stimulated release of nitrogen oxides by BAECs. Only the highest concentration of nifedipine (1 μM) attenuated the release of nitrogen oxides.

Discussion

Previous studies demonstrated that EDRF(NO) is formed in endothelial cells from the terminal guanidino nitrogen atom(s) of the amino acid L-arginine.26,27 In the present experiments, we directly measured the release of nitrogen oxides by the vascular endothelium with chemiluminescence. That the release of these compounds was dependent on the NO synthase enzyme is supported by the observation that release of nitrogen oxides was completely inhibited by an analogue of L-arginine, N-nitro-L-arginine. Biochemical characterization of NO synthetase revealed a strong requirement for calmodulin and Ca2+.28 The calcium ions that activate the NO synthetase may originate from intracellular stores or from the extracellular space. Several groups have shown that removal of extracellular Ca2+ can markedly inhibit the release of EDRF by boas-say.12,13 This concept is supported by our findings that removal of extracellular calcium abolished and that an increase in extracellular calcium concentration augmented the basal and bradykinin-stimulated release of nitrogen oxides from BAECs. The lack of a role of intracellular Ca2+ mobilization was suggested by a previous study in which TMB-8, a compound that
Figure 4. Bar graph showing release of nitrogen oxides by bovine aortic endothelial cells under basal conditions and in the presence of 0.1 μM bradykinin. Removal of calcium from the incubation buffer (in addition to 10 mM EDTA) abolished, whereas an increase in the extracellular calcium concentration (5 mM) augmented, basal and bradykinin-stimulated release of nitrogen oxides. *p<0.05 vs. control (2.5 mM Ca2+).

Figure 5. Bar graph showing effect of verapamil (0.1–10 μM) on the release of nitrogen oxides by bovine aortic endothelial cells under basal conditions (top) and in the presence of 0.1 μM bradykinin (bottom). Number of experiments are given in parentheses.

Figure 6. Bar graph showing effect of diltiazem (0.1–10 μM) on the release of nitrogen oxides by bovine aortic endothelial cells under basal conditions (top) and in the presence of 0.1 μM bradykinin (bottom). Number of experiments are given in parentheses.

Figure 7. Bar graph showing effect of nifedipine (0.001–1 μM) on the release of nitrogen oxides by bovine aortic endothelial cells under basal conditions (top) and in the presence of 0.1 μM bradykinin (bottom). *p<0.05 vs. control. Number of experiments are given in parentheses.
inhibits mobilization of Ca$^{2+}$ from intracellular stores, had no effect on bradykinin-stimulated EDRF(NO) release from BAECs.\textsuperscript{[15]}

Indirect measurements of EDRF release by bioassay systems, in which a vascular ring without endothelium is superfused by a solution passing through a segment of artery with intact endothelium, have yielded conflicting results concerning the effect of calcium channel antagonists. d-cis-Diltiazem has been reported to partially reverse the acetylcholine-induced release of EDRF in canine femoral arteries,\textsuperscript{[16]} whereas the dihydropyridine derivative nifedipine failed to inhibit EDRF release in rabbit aorta.\textsuperscript{[22]} The reason for this discrepancy is unclear. Direct measurements of the release of nitrogen oxides by BAECs in the present study clearly indicate that the transmembrane influx of Ca$^{2+}$ necessary for EDRF(NO) production does not involve channels that are sensitive to verapamil, diltiazem, and nifedipine. Only nifedipine at the highest concentration used (1 $\mu$M) somewhat attenuated the release of nitrogen oxides. This high concentration, however, may have nonspecific effects on endothelial cells and is considerably higher than the therapeutic plasma concentration in humans (0.07–0.2 $\mu$M).\textsuperscript{[20]} Thus, the attenuation of the release of nitrogen oxides by 1 $\mu$M nifedipine seems to have minor, if any, therapeutic relevance.

The failure of organic calcium channel antagonists in our study to inhibit the release of nitrogen oxides is consistent with the view that BAECs likely do not possess voltage-sensitive calcium channels. Previously, investigators used Ca$^{2+}$ channel agonists that presumably activate voltage-operated Ca$^{2+}$ channels. Those studies revealed conflicting results concerning the release of EDRF. The 1,4-dihydropyridine derivative compound Bay K 8644 did not evoke relaxations in rat aortic rings with intact endothelium.\textsuperscript{[30]} However, the presence of endothelium attenuated the maximal contractile responses to the optically pure dihydropyridine Ca$^{2+}$ agonists (+)S202,791 and (-)Bay K 8644 in porcine coronary arteries.\textsuperscript{[31]} Furthermore, the contraction in response to (+)S202,791 could be augmented by the calcium channel antagonist (-)R202,791 in the presence of intact endothelium.\textsuperscript{[31]} The investigators concluded that optically pure Ca$^{2+}$ agonists may stimulate the release of EDRF in porcine coronary arteries. Experiments performed under bioassay conditions demonstrated that at concentrations less than those that evoke smooth muscle contraction, Bay K 8644 and (+)202,791 caused release of EDRF in canine femoral arteries.\textsuperscript{[17]} Under these conditions, the EDRF release could be prevented by pretreatment with the calcium channel antagonist nitrindipine.\textsuperscript{[17]} Based on these data, a close association between voltage-operated calcium channels and the production of EDRF has been assumed.\textsuperscript{[17]} All of these studies used indirect assessments of EDRF release by bioassay or studies of intact vascular rings. A direct effect of the Ca$^{2+}$ antagonist or agonist on vascular smooth muscle cannot be excluded and may explain some of the discrepancies between these studies and the present experiments.

Direct measurement of voltage-sensitive Ca$^{2+}$ current by patch clamp techniques, however, revealed no evidence for voltage-operated Ca$^{2+}$ channels in cultured pulmonary\textsuperscript{[32]} or aortic\textsuperscript{[33]} bovine endothelial cells. The observation that dichlorobenzamil, an amiloride analogue, inhibited the vasodilator response to acetylcholine and A23187 in isolated aortic rings of rat and rabbit indicates that the Ca$^{2+}$ influx may be associated with the release of EDRF by sodium–calcium exchange.\textsuperscript{[11]}

The present observations likely have important clinical implications. The calcium channel antagonists studied in these experiments are commonly used for the treatment of hypertension and angina pectoris. These clinical entities have been associated with impaired production or release of EDRF (for review, see References 6 and 7). This defect of endothelial function is not augmented by the administration of the calcium channel antagonists.

References

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