Negative Feedback Regulation of Endothelial Cell Function by Nitric Oxide

Georgette M. Buga, Jeanette M. Griscavage, Norma E. Rogers, Louis J. Ignarro

The objective of this study was to determine whether nitric oxide (NO) could function as a negative feedback modulator of endothelial cell function by inhibiting NO synthase in vascular endothelial cells. The rationale for this approach was a previous study from this laboratory, which revealed that NO inhibits neuronal NO synthase from rat cerebellum. In the present study, NO and NO-donor agents noncompetitively inhibited NO synthase derived from bovine aortic endothelial cells. Oxyhemoglobin blocked the inhibitory action of NO and by itself increased NO synthase activity. This finding suggests that NO acts as a negative feedback modulator of NO synthase. In intact aortic endothelial cells grown on microcarrier beads and perfused in a bioassay cascade system, pretreatment of cells with NO-donor agents caused a marked inhibition of endothelial NO biosynthesis in response to bradykinin and increased fluid shear or flow. When isolated bovine pulmonary arterial rings precontracted by phenylephrine were used, pretreatment of arterial rings with NO-donor agents diminished endothelium-dependent arterial relaxation involving the l-arginine–NO pathway without altering endothelium-independent relaxation to NO itself. On the basis of these studies, NO is suggested to play an important negative feedback regulatory role on endothelial NO synthase and, therefore, vascular endothelial cell function. (Circ Res. 1993;73:808-812.)

KEY WORDS • nitric oxide • nitric oxide synthase • endothelium-dependent vasorelaxation • negative feedback regulation

Nitric oxide (NO) synthase is a heme-containing monoxygenase that catalyzes the conversion of L-arginine to NO plus L-citrulline.1-4 This conversion appears to involve a five-electron oxidation of one of the basic guanidino nitrogen atoms of L-arginine to NO. The membrane-bound constitutive isoform of NO synthase from vascular endothelial cells is one of three principal isoforms of mammalian NO synthase.5 The other two enzyme isoforms are the cytosolic constitutive NO synthase present in neuronal tissue6 and the cytosolic inducible NO synthase present in activated rodent macrophages.7 Substantial information is now available on cofactor requirements and the inhibitory effects of N\(^6\)-substituted L-arginine analogues.8 What is lacking is information on the regulation of NO synthase activity by endogenous substances. We reported recently that NO, both exogenously added and enzymatically generated by NO synthase, causes reversible inhibition of the cytosolic constitutive isoform of NO synthase from rat cerebellum.9 The physiological significance of this observation, however, remains unknown.

The objective of the present study was to determine whether NO could inhibit endothelium-derived NO synthase activity and, thereby, interfere with endothelial cell function. In addition to examining the influence of NO and NO-donor agents on endothelium-derived NO synthase activity, advantage was taken of the ability to study endothelial cell function. Thus, the influence of pretreatment of isolated vascular endothelial cells and isolated arterial rings with NO-donor agents on endothelium-dependent relaxation responses to chemical agents, electrical stimulation, and shear or mechanical forces was studied.

Materials and Methods

Harvesting and Growth of Endothelial Cells

Bovine aortic endothelial cells were harvested and grown exactly as described previously,10 and cells obtained from passages 4 to 6 were used. Endothelial cells that were used as the source of NO synthase were removed from culture flasks, washed in phosphate-buffered saline, and homogenized as described below. Endothelial cells were also transferred to Cytodex microcarrier beads and perfused in a bioassay cascade system as described previously.10 Briefly, beads containing cells (0.5 to 0.7 mL containing approximately 2×10\(^7\) cells) were packed into a small chromatography column and perfused with Krebs’ bicarbonate solution gassed with a mixture of 10% O\(_2\)-5% CO\(_2\)-85% N\(_2\) at 37°C. The perfusion rate through the column was regulated from 0.3 to 3 mL/min by a perfusion pump, and the perfusate was allowed to superfuse three precontracted helical strips of endothelium-denuded bovine intrapulmonary artery arranged in a cascade. Relaxant responses of only the first strip in the cascade are illustrated in Fig 2. In each experiment, the lower two strips relaxed in a typical decremental manner characteristic of NO.10 Increments in the perfusion rate caused increments in the generation and release of NO. This flow-dependent increase in NO generation has been attrib-
uted to an increase in fluid shear stress, mechanical forces, or a combination of both.10 In some experiments, bradykinin was added to stimulate the generation and release of NO.

**NO Synthase Assay**

Bovine aortic endothelial cells that were harvested, grown, and washed as described above were homogenized (10% [vol/vol]) in 50 mmol/L Tris HCl, pH 7.4, containing 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.5 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L pepstatin A, and 2 μmol/L leupeptin at 0° to 4°C. In some experiments, homogenates were centrifuged at 100 000g for 60 minutes at 4°C to obtain the sediment or membrane fraction. Enzymatic reactions were conducted exactly as described previously.9,11 Briefly, NO synthase reactions were conducted at 37°C for 15 minutes in 50 mmol/L Tris HCl, pH 7.4, containing 50 μmol/L L-arginine (200 000 dpm of L-[2,3,4,5-3H]arginine HCl, 77 Ci/mmol, Amersham Corp, Arlington Heights, Ill), 100 μmol/L NADPH, 10 μmol/L tetrahydrobiopterin, 1 μg calmodulin, 2 mmol/L CaCl2, and homogenate fraction (0.82 mg protein) or washed sediment fraction (0.58 mg protein) in a final assay volume of 100 μL. Enzymatic reactions were terminated, and [3H]l-citrulline was separated from [3H]l-arginine by cationic exchange chromatography.11 NO, S-nitroso-N-acetylpenicillamine (SNAP), and oxyhemoglobin were prepared as described previously.9 The chemicals and reagents used in this study were purchased from Sigma Chemical Co, St Louis, Mo, and Fisher, Pittsburgh, Pa.

**Preparation of Arterial Rings and Electrical Stimulation**

Rings of bovine intrapulmonary artery were prepared and mounted under optimal tension for recording of changes in isometric force.12 Briefly, arterial rings were bathed in Krebs' bicarbonate solution gassed with a mixture of 95% O2–5% CO2 at 37°C and precontracted to 70% of maximal tone with 1 to 10 μmol/L phenylephrine just before electrical field stimulation (EFS). EFS was conducted with the aid of two parallel platinum electrodes as described previously13 and was applied at 25 V and a frequency of 4 Hz in the form of square wave pulses of 0.2-millisecond duration and delivered as 20-second trains. EFS of segments of pulmonary artery from bovine and other species has been shown to cause endothelium-dependent vascular smooth muscle relaxation13,14 attributed to NO. In some experiments, EFS was not performed, but acetylcholine and bradykinin were used as endothelium-dependent relaxants, whereas SNAP was used as an endothelium-independent relaxant.

**Results**

Initial experiments with endothelial cell homogenates and washed sediment fractions as sources of membrane-bound NO synthase revealed consistently that the rate of conversion of L-arginine to l-citrulline was nonlinear through 15 minutes of incubation, although excess substrate and cofactors were present. Addition of 30 μmol/L oxyhemoglobin to reaction mixtures increased product formation by 30% to 40% (12 determinations), whereas superoxide dismutase (100 to 500 U) decreased product formation by 20% to 30% (10 determinations). This was consistent with the hypothesis that the NO generated during enzyme catalysis feeds back to inhibit NO synthase.9 Fig 1 illustrates the concentration-dependent inhibitory actions of NO and SNAP on NO synthase activity from the homogenate fraction of bovine aortic endothelial cells. Quantitatively similar observations were made when the washed sediment fraction was used as the source of NO synthase (not shown). Oxyhemoglobin (30 μmol/L) blocked the inhibitory effect of SNAP and, when added alone, increased NO.
synthase activity. The effect of 30 μmol/L oxyhemoglobin was nearly abolished when excess SNAP (1 mmol/L) was added to enzyme reaction mixtures, producing 75% inhibition of NO synthase activity when compared with the control value (not shown). Methemoglobin (30 μmol/L) was completely without effect. Kinetic experiments with crude enzyme preparations revealed that the $K_m$ for L-arginine was 8 μmol/L, which is in the range of the 3 μmol/L value reported for purified preparations of endothelium-derived NO synthase. SNAP, tested at a concentration of 100 μmol/L, was a noncompetitive inhibitor of NO synthase with respect to L-arginine, as assessed by analysis of double-reciprocal plots. The $V_{max}$ values for NO synthase in the absence (control) and presence of 100 μmol/L SNAP, respectively, were 41 and 20 pmol L-citrulline per minute per milligram protein.

Experiments were next designed to ascertain whether NO interferes with endothelial cell function that is dependent on the L-arginine–NO pathway. Increasing the flow rate of Krebs' bicarbonate solution through a column of endothelial cells attached to microcarrier beads caused a flow-dependent increase in the release of a relaxing factor (Fig 2) that has been previously characterized as NO. Pretreatment of endothelial cells with 1 μmol/L SNAP for 15 minutes caused a marked inhibition of flow-dependent NO generation, as monitored by relaxation of target arterial strips. NO generation by endothelial cells recovered after 30 to 40 minutes of perfusion with Krebs' bicarbonate solution (not shown). Bradykinin caused a concentration-dependent generation of NO from perfused endothelial cells, and this was suppressed in endothelial cells pretreated with SNAP (Fig 2). Endothelial cells retained their viability and remained attached to the beads, as assessed by microscopic examination at the end of each experiment.

The next experiment was designed to determine whether NO interferes with endothelium-dependent relaxation of isolated arterial rings. Precontracted rings of bovine intrapulmonary artery relaxed in response to EFS (Fig 3) and to both endothelium-dependent (acetylcholine and bradykinin) and endothelium-independent (SNAP) relaxing agents (Fig 4). Arterial rings that had been pretreated with 1 μmol/L SNAP for 15 minutes showed a marked decrease in relaxation responses to both EFS (Fig 3) and acetylcholine or bradykinin without a significant change in the relaxation response to SNAP itself (Fig 4). The inhibitory effects of SNAP against EFS-induced relaxation (Fig 3) and agonist-elicited relaxation (Fig 4) were mimicked by 1 μmol/L N-methyl-$N'$-nitro-$N'$-nitrosoguanidine and by 0.1 μmol/L glyceryl trinitrate, both of which generate NO in tissues, and caused selective inhibition of endothelium-dependent relaxation. Arterial rings recovered their responsiveness to EFS and endothelium-dependent relaxants within 30 to 40 minutes of withdrawing the NO-donor agents (not shown).

**Discussion**

The data indicate that NO and NO-donor agents markedly inhibit endothelium-dependent relaxation of arterial rings and NO generation from intact endothelial cells without diminishing the sensitivity of the vascular smooth muscle cells to the direct relaxant effect of NO. Appropriate control experiments indicated that the effects of NO and NO-donor drugs cannot be attributed to any time-dependent attenuation of tissue responsiveness or tissue desensitization. The cellular mechanism of this inhibitory action of NO appears to be the inhibition of intracellular NO synthase activity, since NO and the NO-donor agent, SNAP, inhibited endothelium-derived NO synthase activity. The inhibition of NO synthase activity was noncompetitive with respect to L-arginine. Since NO is one of the two principal end products of L-arginine oxygenation catalyzed by NO synthase, NO could act as a negative feedback modulator of its own biosynthesis. The evidence for this hypothesis is that oxyhemoglobin, which chemically inactivates NO, increased NO synthase activity, whereas superoxide dismutase, which prolongs the biologic half-life of NO by destroying superoxide anion, decreased NO synthase activity. Moreover, the effect of oxyhemoglobin was overcome by the addition of a large excess of NO in the form of SNAP. In
Fig. 3. Bar graph shows inhibition of electrically induced endothelium-dependent relaxation of isolated arterial rings by nitric oxide–donor agents. S-Nitroso-N-acetylpenicillamine (SNAP), N-methyl-N’-nitro-N-nitrosoquani- dine (MNNG), and glyceryl trinitrate (GTN) were used as nitric oxide–donor agents. Control relaxant responses to electrical field stimulation were obtained, and 15 minutes later the rings were washed extensively over a 40-minute period. A second set of control responses to electrical field stimulation were obtained, and then the rings were bathed in 1 μmol/L concentrations of SNAP, MNNG, or GTN for 15 minutes as indicated. After extensive washing of arterial rings over a 40-minute period, responses to electrical field stimulation were obtained. Data points represent mean±SEM of 12 determinations from four separate experiments.

In addition, the rate of L-citrulline formation was nonlinear for short incubation periods unless oxyhemoglobin was added to reaction mixtures.

Although low concentrations (1 μmol/L) of NO-donor agents were sufficient to impair NO generation in intact endothelial cells, higher and seemingly unphysiological concentrations were required to inhibit NO synthase activity in broken cell preparations. There are many possible reasons for this apparent discrepancy, including the possibility that certain cellular components that may augment the inhibitory action of NO on NO synthase in intact cells are absent or present at diluted concentrations in broken cell homogenate fractions containing NO synthase.

The molecular mechanism by which NO inhibits NO synthase is not known. NO may inhibit NO synthase activity by either indirect or direct mechanisms. One possible direct mechanism is that NO interacts directly with the heme prosthetic group of NO synthase and thereby interferes with electron transport and substrate oxygenation. The constitutive and inducible isofoms of NO synthase from brain and activated rodent macrophages, respectively, have been characterized as hemoproteins.1-4 It is likely that the constitutive isofom of NO synthase in vascular endothelial cells is also a hemoprotein. The precise function of enzyme-bound heme is unknown but may play a role in catalysis by serving as the terminal electron acceptor in the oxygenation of L-arginine to the intermediate N^G-hydroxy-L-arginine. Cytochrome P-450 reductase is a closely related monoxygenase that interacts with cytochrome P-450 hemoproteins,18 and a recent report indicated that 50 to 150 μmol/L NO and NO-donor agents interfere with substrate oxygenation by binding to the heme moiety of cytochrome P-450 and thereby cause inhibition of substrate oxygenation.19

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References

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