cGMP-Mediated Negative-Feedback Regulation of Endothelial Nitric Oxide Synthase Expression by Nitric Oxide

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Abstract—Earlier studies have demonstrated that nitric oxide (NO) exerts a fast-acting inhibitory influence on endothelial NO synthase (eNOS) enzymatic activity in isolated vascular tissue preparations. The present study was designed to examine the possible effect of NO on eNOS protein expression in cultured endothelial cells and intact animals. Human coronary endothelial cells were incubated with \( S\)-nitroso-\( N\)-acetyl-penicillamine (SNAP, an NO donor), oxyhemoglobin (HGB, an NO trapping agent), SNAP plus HGB, or inactive vehicle (control). In other experiments, cells were treated with 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor), \( 1H\)-[1,2,4]oxadiazolo-[4,3–2]quinazolin-1-one (ODQ, a guanylate cyclase inhibitor), SNAP plus ODQ, 8-bromo-cGMP (8-Br-cGMP, a cell-permeable cGMP compound), 8-Br-cGMP plus HGB, or inactive vehicle in order to discern the effect of cGMP. The incubations were conducted for 24 hours, and total nitrate plus nitrite production and eNOS protein abundance (Western analysis) were measured. To determine the effect of NO on eNOS expression in vivo, rats were treated with either the NO donor isosorbide dinitrate or placebo by gastric gavage for 48 hours, and aortic eNOS protein expression was examined. The NO donor SNAP markedly depressed, whereas the NO scavenger HGB significantly raised, eNOS protein expression. The downregulatory action of SNAP was completely abrogated by HGB. Phosphodiesterase inhibitor and 8-Br-cGMP downregulated, whereas the guanylate cyclase inhibitor ODQ upregulated eNOS protein expression. The downregulatory action of SNAP was completely overcome by the guanylate cyclase inhibitor ODQ, and the upregulatory action of the NO scavenger HGB was abrogated by 8-Br-cGMP. Administration of NO donor resulted in a marked downregulation of aortic eNOS protein expression in intact animals, thus confirming the in vitro findings. NO serves as a negative-feedback regulator of eNOS expression via a cGMP-mediated process. (Hypertension. 1999;34:1237-1241.)

Key Words: nitric oxide ■ cyclic GMP ■ nitric oxide synthase ■ endothelium ■ arteries ■ hemoglobin

Nitric oxide (NO) is produced from the conversion of \( L\)-arginine to \( L\)-citrulline by NO synthase (NOS). To date, 3 isotypes of NOS have been identified. These include neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III) isotypes. Constitutively produced endothelium-derived NO plays an important role in the regulation of renal and systemic vascular resistance and arterial blood pressure. Enzymatic activity and production of eNOS are regulated by a number of receptor agonists and by flow-induced shear stress.

In an earlier study, Buga et al. showed that addition of either NO or the NO donor \( S\)-nitroso-\( N\)-acetyl-penicillamine (SNAP) to the reaction mixture results in a dose-dependent inhibition of eNOS activity of the cell-free plasma membrane preparation of bovine aortic endothelial cells. Likewise, they showed that a brief 15-minute pretreatment with the NO donor SNAP leads to a dose-dependent reduction of both flow/shear-induced and bradykinin-stimulated NO release by isolated bovine aortic endothelial cells. The inhibitory action of NO/NO donor on eNOS activity was overcome by oxyhemoglobin (HGB), a well-known NO trapping agent. On the basis of these observations, the authors concluded that NO serves as a noncompetitive negative-feedback regulator of eNOS activity. They speculated that interaction of NO with the heme prosthetic component of NOS may lead to inhibition of NOS activity by limiting electron transport and oxygenation of \( L\)-arginine. These observations were subsequently confirmed by Ravichandran et al., who showed reversible inhibition of semipurified eNOS enzymatic activity by NO and NO donors.

Clearly, the rapid fall in eNOS activity after incubation with NO and SNAP of the plasma membrane preparation of cultured endothelial cells and its prompt reversal by the NO scavenger HGB shown by Buga et al. reflected a functional modification as opposed to a change in de novo production of the enzyme. However, an earlier study has demonstrated that
pretreatment with NO or NO donor can mitigate the endotoxin/cytokine-mediated induction of iNOS in human ramified microglial cells.\(^9\)\(^10\) In addition, sodium nitroprusside, a well-known NO donor, has been shown to suppress lipopolysaccharide-induced iNOS expression in rat neutrophils.\(^11\) The present study was designed to explore whether NO modulates expression of eNOS by human coronary endothelial cells and, if so, whether this regulatory action is mediated by cGMP, the second messenger of NO, in vascular smooth muscle.

**Methods**

**Cell Culture**

Human coronary artery endothelial cells were obtained from Bio Whittaker Inc. The cells were cultured in a specific culture medium (Endothelial Cell Growth System, Bio Whittaker Inc) in 75-cm\(^2\) flasks and were incubated in a humidified incubator at 37°C and 5% CO\(_2\). After 48 hours, 10 mL of fresh medium was added to each flask, and the incubation was continued for an additional 2 days. Thereafter, the medium was changed every 48 hours. Once a monolayer was formed, the cells were subcultured. The cells were identified by staining with a specific antibody to von Willebrand factor and fluorescently labeled LDL as described previously.\(^12\)

**Study Design**

The cells obtained on passages 3 to 4 were subcultured in 6-well plates and incubated in the medium containing 5% fetal calf serum for 48 hours, at which point 80% to 90% confluence was reached. The cells were then treated with the NO donor SNAP (0.1 mmol/L, Alexis Inc) for 24 hours. The SNAP concentration used in the present study was based on the reported use of this agent in cultured endothelial cells\(^13\) and in cell-free eNOS preparations.\(^4\) In a series of parallel experiments, cells were treated with either the NO trapping agent HGB (50 \(\mu\)mol/L, Sigma Chemical Co) alone or HGB plus SNAP. The above experiments were intended to discern the effects of addition and removal of NO on eNOS expression by endothelial cells.

The second set of experiments was designed to explore whether the effect of NO on endothelial cell eNOS expression is cGMP-mediated. To this end, cells were treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 50 \(\mu\)mol/L, Sigma) for 24 hours. This was intended to raise cGMP by inhibiting its degradation by phosphodiesterase. In a set of parallel experiments, the cells were treated with the guanylate cyclase inhibitor 1H-[1,2,4]oxadiiazolo-[4,3-2]quinoxalin-1-one (ODQ, 1 \(\mu\)mol/L, Sigma) or ODQ plus SNAP to inhibit basal and SNAP-stimulated cGMP generation. The final set of experiments was undertaken to determine the effect of cGMP directly. To this end, cells were treated with a cell-permeable cGMP compound, 8-bromo-cGMP (8-Br-cGMP, 1.0 \(\mu\)mol/L, Sigma) for 24 hours. In an attempt to dissect the effect of NO from that of cGMP, in a parallel set of experiments, cells were treated with the NO trapping agent HGB plus 8-Br-cGMP. All incubations were performed for 24 hours, after which the cells and the supernatants were harvested and stored at −70°C until processed. Trypan blue exclusion was used to discern cell viability that was >90% in all experiments.

**In Vivo Studies**

In an attempt to examine the effect of exogenous NO on eNOS expression in vivo, we treated 6 male Sprague-Dawley rats (body weight 261±4 g) with the NO donor isosorbide dinitrate (Alexis Corp) at 30 mg/kg every 8 hours by gastric gavage for 2 days. A group of 6 placebo-treated rats (body weight 268±4 g) served as controls. The dosage used was based on an earlier study.\(^14\) At the conclusion of the study period, tail blood pressure was measured by plethysmography (Harvard Apparatus) as previously described.\(^15\) The animals were then anesthetized with injections of pentobarbital (Nembutal; 50 mg/kg IP) and killed by exsanguination using cardiac puncture. The thoracic aorta was removed and processed for the measurement of eNOS protein expression by Western analysis in a manner precisely identical to that described in our earlier studies.\(^16\)

**Western Blot Analysis**

These measurements were carried out to determine eNOS protein mass by use of an anti-eNOS monoclonal antibody (Transduction Laboratories) as previously described.\(^17\) Briefly, the treated cells were washed with PBS and then extracted directly into the sample buffer (1% SDS and 10 mmol/L Tris HCl, pH 7.4), and the total protein was determined by a Bio-Rad kit. Cell lysate protein (50 \(\mu\)g) was size-fractionated on 4%–12% Tris-glycine gel at 120 V for 3 hours. In preliminary experiments, we had found that the given protein concentrations were within the linear range of detection for our Western blot technique. After electrophoresis, proteins were transferred onto a Hybond-enhanced chemiluminescence (ECL) membrane at 400 mA for 120 minutes using the Novex transfer system. The membrane was prehybridized in 10 mL of buffer A (10 mmol/L Tris HCl [pH 7.5], 100 mmol/L NaCl, 0.1% Tween 20, and 10% nonfat milk powder) for 1 hour and then hybridized for an additional 1-hour period in the same buffer containing 10 \(\mu\)L of the given anti-NOS monoclonal antibody (1:1000). Thereafter, the membrane was washed for 30 minutes in a shaking bath; the wash buffer (buffer A without nonfat milk) was changed every 5 minutes before 1 hour of incubation in buffer A plus goat anti-mouse IgG–horseradish peroxidase at a final titer of 1:1000. Experiments were performed at room temperature. The washes were repeated before the membrane was developed by a light-emitting nonradioactive method using ECL reagent (Amersham Inc). The membrane was then subjected to autoradiography for 1 to 5 minutes. The autoradiographs were scanned with a laser densitometer (model PDI211, Molecular Dynamics) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain before prehybridization. This step verified the uniformity of protein load and transfer efficiency across the test samples.

**Measurements of NOx**

The concentration of total nitrate and nitrite (NOx) in the culture medium was determined by the purge system of a NO analyzer (NOA, model 270B, Sievers Instruments Inc).\(^18\)

**Data Presentation and Analysis**

ANOVA and the Student \(t\) test were used in statistical evaluation of the data, which are given as mean±SEM. A value of \(P<0.05\) was considered significant.

**Results**

**In Vitro Results**

Incubation with the NO donor SNAP for 24 hours resulted in a significant downregulation of eNOS protein expression by cultured human coronary endothelial cells. In contrast, incubation with the NO trapping agent HGB caused a significant increase in eNOS protein expression in endothelial cells. The upregulatory action of hemoglobin on eNOS expression was abrogated by cotreatment with SNAP (Figure 1). Upregulation of eNOS expression by hemoglobin was accompanied by a significant increase in the amount of NOx recovered in the culture medium (Figure 2). Taken together, these findings suggest that NO exerts a negative-feedback regulatory role on eNOS expression in human coronary artery endothelial cells.

Treatment of cells with the phosphodiesterase inhibitor IBMX resulted in a significant downregulation of eNOS protein expression and NOx production by cultured endothelial cells, mimicking the effect of the NO donor SNAP.
(Figures 2 and 3). In contrast, incubation with the guanylate cyclase inhibitor ODQ led to a significant increase in endothelial cell NOx production and eNOS abundance (Figures 2 and 3). Moreover, cotreatment of cells with ODQ abrogated the downregulatory action of SNAP on eNOS expression in this system (Figure 4). Together, these findings provide indirect but compelling evidence that the regulatory action of NO on eNOS expression is mediated by cGMP.

Incubation with 8-Br-cGMP, a cell-permeable cGMP analogue, resulted in a significant downregulation of NOx production and eNOS protein expression in cultured human endothelial cells (Figures 2 and 3). Thus, exogenous cGMP simulated the effects of NO donor SNAP and phosphodiesterase inhibitor IBMX, which both raise cellular cGMP contents. Moreover, cotreatment of cells with 8-Br-cGMP abrogated the upregulatory action of NO scavenger HGB (Figures 2 and 5). These observations provide direct evidence for the role of cGMP as the mediator of NO action on endothelial cell expression of eNOS protein.

In Vivo Results
Administration of the NO donor isosorbide dinitrate for 48 hours resulted in a modest reduction in blood pressure (100±1.1 versus 125±1.2 mm Hg, *P<0.05) and a marked downregulation of aortic eNOS protein abundance in normal rats. Accordingly, the results of the in vivo studies paralleled those of the in vitro experiments (Figure 6).

Discussion
Incubation with NO donor SNAP for 24 hours resulted in a significant fall in eNOS protein expression by cultured human coronary endothelial cells. In contrast, incubation with HGB, a potent NO trapping agent, resulted in a significant increase in eNOS protein expression in this system. These observations indicate that addition of exogenous NO downregulates and removal of endogenous NO (by hemoglobin) upregulates eNOS expression. Thus, on the basis of these data, NO serves as a negative-feedback regulator of eNOS protein expression. We then sought to discern whether the effect of NO on the regulation of eNOS is mediated by cGMP, its second messenger in vascular smooth muscle cells. To this end, we incubated the endothelial cells with either guanylate cyclase inhibitor or phosphodiesterase inhibitor to reduce or increase cellular cGMP levels respectively. We assumed that if downregulation of NOS by NO is mediated by cGMP then inhibition of phosphodiesterase, which catalyzes cGMP degradation, should depress eNOS expression by raising cellular cGMP content. In fact, the results confirmed this supposition. Further support for the role of cGMP as the mediator of NO-induced downregulation of eNOS came from the experiments in which blockade of cGMP generation by a guanylate cyclase inhibitor resulted in a significant increase in eNOS protein expression by cultured human endothelial cells. Moreover, the downregulatory action of the NO donor SNAP on eNOS expression was overcome by blocking cGMP production with ODQ. This observation indicates that
inhibition of eNOS expression is not due to a direct action of NO, per se, but that it is a consequence of NO-stimulated cGMP generation. These experiments provided indirect but compelling evidence for the role of cGMP as the mediator of negative-feedback regulation of eNOS protein expression by NO in endothelial cells.

In an attempt to directly demonstrate the role of cGMP, we repeated the experiments using 8-Br-cGMP, a cell-permeable cGMP compound. The results showed a significant down-

regulation of eNOS expression by exogenous cGMP. Moreover, the upregulatory action of the NO trapping agent HGB was fully overcome by simultaneous treatment of cells with 8-Br-cGMP. These experiments provided direct evidence for the inhibitory action of cGMP on eNOS protein expression in the endothelial cells.

The downregulatory action of NO on eNOS expression seen in cultured endothelial cells was also evident in vascular tissues of rats treated for 2 days with the NO donor isosorbide dinitrate. The clinical implications of downregulation of vascular eNOS expression by nitrovasodilators shown in the present study is not certain. However, the reduction in the amount of NOS and, hence, depressed endogenous NO production capacity may theoretically result in transient NO deficiency, leading to episodic vasospasm and hypertension with abrupt discontinuation of irregular consumption of the drug in patients maintained on chronic nitrovasodilator therapy. This phenomenon may play a partial role in the pathogenesis of tolerance to these agents. In an earlier study, Buga et al.7 showed that NO (and NO donors) can rapidly lower eNOS enzymatic activity, thus providing a fast-acting negative-feedback regulatory influence. The present study has extended the inquiry by demonstrating the negative-
feedback regulation of eNOS protein expression by NO. The effect of NO on eNOS protein expression in human coronary endothelial cells shown here is consistent with the reported effect of NO on endotoxin/cytokine-mediated induction of iNOS in human microglial cells reported by Colasanti and colleagues and in rat neutrophils reported by Mariotto et al.  

In conclusion, the present study showed that expression of eNOS protein in endothelial cells is influenced by a negative-feedback regulatory action of NO. The study has further revealed that downregulation of eNOS protein expression by NO is mediated by its second messenger, cGMP.

References
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