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Alteration of Nitric Oxide Gas on Gene Expression of Endothelin-1 and Endothelial Nitric Oxide Synthase by a Time- and Dose-Dependent Manner in Human Endothelial Cells

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Abstract

Our purpose was to investigate the gene expression of endothelin-1 (ET-1) and endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cells (HUVECs) under various concentrations and periods of exogenous nitric oxide (NO) gas exposure. Cultured HUVECs were exposed to 0, 20, and 80 ppm NO for 0, 24, and 48 h. With NO exposure for 24 h, ET-1 peptide levels decreased in both the 20 and 80 ppm groups. Thereafter, at 48 h, ET-1 peptide levels persistently decreased in the 20 ppm group, but significantly increased in the 80 ppm group. Furthermore, there was a significant decrease in the expression levels of eNOS protein and mRNA in the 80 ppm 48 h group. The data suggest a time- and dose-dependent effect of NO gas exposure on the gene expression of ET-1 and eNOS in HUVECs. A high concentration and long period of NO exposure induces an inhibition of eNOS and enhancement of ET-1, which could lead to vasoconstriction. These findings may have clinical implications in NO therapy regarding the optimal dose and period.

Key Words: endothelial nitric oxide synthase, endothelin-1, nitric oxide

Introduction

Nitric oxide (NO) is a ubiquitous gas produced by oxidation of atmospheric nitrogen (10). It is also endogenously secreted and plays a variety of roles as vasodilator, neurotransmitter, and cytotoxin. As an intercellular signaling molecule, NO is synthesized from the oxidation of L-arginine by NO synthase (NOS). Endothelial cells generate NO by the activation of endothelial NOS (eNOS) and release NO into the smooth muscle cells *via* diffusion (15). NO activates soluble guanylate cyclase and initiates a cascade leading to smooth muscle relaxation. Endothelial cells also release endothelin-1 (ET-1), the most potent vasoconstrictor. ET-1 binds to the endothelin-A receptor on the membrane of smooth muscle cells and then increases the intracellular calcium concentration, which causes the smooth muscle constriction.

The endothelium-derived mediators of vascular tone, such as eNOS and ET-1, are regulated by exogenous NO (16, 19, 20). Numerous data have demonstrated that NO serves as a negative-feedback

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regulator of eNOS expression (4, 8, 9, 22, 25). In contrast, NO seems to upregulate eNOS expression in the pulmonary system (5, 27). In addition to the complex regulation of eNOS expression, previous studies with primary cultures of endothelial cells have illustrated that NO-generating agents induce an inhibition of ET-1 expression (2, 3, 11, 13, 18, 24). In the animal and clinical studies, however, there are increasingly conflicting findings to depict that inhaled NO enhances ET-1 expression (6, 17, 19, 21, 23). These contradictory results may attribute to the differences in cell types and NO sources (5, 16, 18).

In the current study, we examined the effects of exogenous NO on the gene expression of ET-1 and eNOS. Our study was performed with isolated cultured human umbilical vein endothelial cells (HUVECs) to avoid the potential effects of *in vivo* constituents that may affect the gene expression. Furthermore, we used NO gas as the source of exogenous NO to eliminate the chemical effects of NO-creating compounds. Our data showed a time- and dose-dependent effect of NO gas exposure on the gene expression of ET-1 and eNOS in HUVECs.

Materials and Methods

Cell Culture

Endothelial cells obtained from human umbilical veins were cultured on gelatin-coated plates in the presence of heparin and endothelial cell growth factor (Roche Molecular Biochemicals, Indianapolis, IN, USA). They were incubated in M199 media (Life Technologies, Rockville, MD, USA) containing 20% Fetal Bovine Serum (Life Technologies) in 21% O_2-5% CO_2 at 37°C and sub-cultured by 0.25% (w/v) Trypsin-0.03% (w/v) EDTA (Life Technologies) every 3–4 d. At confluence, 10^6 cells/ml of endothelial cells were exposed to 0, 20, and 80 ppm NO mixtures pre-analyzed by a NO sensor (Micro Medical MicroGas, Gillingham, Kent, UK) for 48 h. Cells were then collected at 0, 24, and 48 h of NO gas exposure and stored at -70°C until assay.

Measurement of NO Concentrations in Culture Media

A chemiluminescence analyzer NOA 280 (Sievers Instrument, Inc, Boulder, CO, USA) was used to measure NO concentrations in the culture media. Two milliliter of 0.1 M Vanadium (III) chloride in 1 N hydrochloric acid was added in 10 μl aliquots of culture media. The reaction mixture was heated to 90°C in the purge vessel of the analyzer to reduce all NO metabolites (nitrites, nitrates, nitrosothiols, and peroxynitrite) to NO, which was then driven to the chamber by bubbling the mixture with argon. The calibration curves of NO concentrations were generated by measuring the production of NO with a range (10–100 pM) of sodium nitrate solutions.

Determination of Cell Viability

Cellular toxicity of NO gas was accessed after each exposure, using trypan blue exclusion. Briefly, HUVECs were re-suspended and added with equal volume of trypan blue (0.4%) in PBS. The suspensions were then mixed thoroughly and allowed to stand at room temperature for 5 min. Viable cells were counted by a hemocytometer. Results are expressed as a percentage of cells excluding trypan blue dye.

ELISA for ET-1 Peptide

Homogenized cells in PBS were centrifuged at 2,500 rpm at 4°C. The protein contents were quantified in the supernatants with the Bradford reagent (Sigma, St. Louis, MO, USA) and read in a spectrophotometer at an absorbance of 595 nm. One hundred-microliter aliquots of each supernatant were analyzed for ET-1 peptide with a commercial enzyme immunoassay kit (R & D Systems Inc, Minneapolis, MN, USA). The concentration of ET-1 in HUVECs was compared with a standard curve of known concentration of ET-1. The antibody used in this assay cross-reacts with ET-2 (27%) and ET-3 (8%). The coefficients of variation of intra-assay and inter-assay were 4.4% and 5.7%, respectively.

Western Analysis for eNOS Proteins

Twenty micrograms of cell homogenates were separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes using semi-dry electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA). These membranes were incubated with eNOS antibody (Transduction Laboratories, Lexington, KY, USA) as previously described (26).

Determination of mRNA for preproET-1 and eNOS

Pure RNA from HUVECs was isolated using Total TRIzol reagent (Life Technologies), according to the manufacturer's instructions. RT-PCR was performed using ThermoScript Reverse Transcriptase (Life Technologies) and *Tag* DNA Polymerase (Protech Technology Enterprise Co., Taipei, Taiwan). The primers for human preproET-1 (5'-TCG TCC CTG ATG GAT AAA GAG TAT GTC-3' and 5'-GGT CAC ATA ACG CTC TCT GGA GGG CTT-3'), eNOS (5'-CCC CAA GAC CTA CGT GCA -3' and 5'-TCG TGG TAG CGT TGC TGA T -3'), and β-actin (5'-ATC TGG CAC CAC ACC TTC TAC AAT

GAG CTG CG-3' and 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3') were synthesized commercially (MWG-Biotech AG, Heidelberg, Germany). The template was initially denatured for 4 min at 94°C. The amplification proceeded for 35 cycles at a denaturation temperature of 94°C for 40 sec, an annealing temperature of 62°C for 1 min (preproET-1) or 57°C for 45 sec (eNOS), and an elongation temperature of 72°C for 1 min (preproET-1) or for 45 sec (eNOS). PCR products were analyzed by FLA-2000 (Fuji Photo Film Co. Ltd, Minato-ku, Tokyo, Japan) as the ratio of preproET-1/β-actin and eNOS/β-actin by agarose gel electrophoresis after being incubated with ethidium bromide.

Statistical Analysis

For comparison between treatment groups, the null hypothesis that there was no difference between treatment means was tested by analysis of variance for multiple groups or unpaired *t*-test for two groups (SPSS 10.0, SPSS Inc., Chicago, IL, USA). Statistical significance ($P < 0.05$) between and within groups was determined by means of the Fischer method of multiple comparison.

Results

NO Concentration in Culture Media

The metabolites of NO were determined to evaluate the actual NO concentrations during the time course (Fig. 1). Before NO exposure, NO concentrations were hardly measurable in the culture media incubated with HUVECs (10 ± 1 μM). With 20 ppm exposure, NO concentrations significantly increased at 24 h (70 ± 2 μM). Further increase of NO concentrations was observed at 48 h (313 ± 10 μM). Similar results were noted at 80 ppm NO exposure for 24 h (189 ± 7 μM) and 48 h (441 ± 8 μM). Moreover, NO concentrations at 80 ppm were significantly higher than 20 ppm with similar exposure periods. These results demonstrate a dose- and time-dependent increase of NO concentrations in the culture media under NO gas exposure.

Cytotoxicity of NO Exposure

The number of viable HUVECs was measured to determine whether exogenous NO could cause cell death (Fig. 2). After NO exposure, the number of viable cells significantly decreased. However, the viability carried no significant difference among the groups exposed to NO gas. Furthermore, we did not observe any statistically significant difference in the pH value of the culture media between each group (data not shown). These findings suggest NO gas led to cell death, which was not proportional to the concentration and time of exogenous NO exposure.

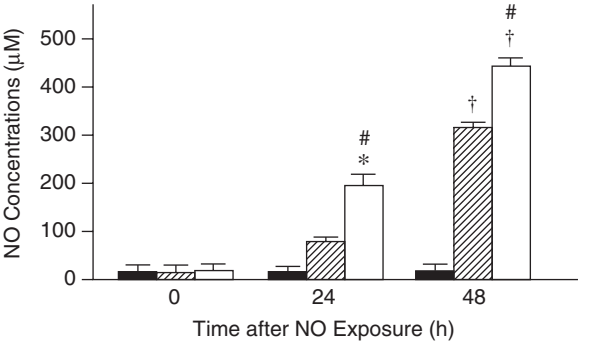

Fig. 1. NO concentrations in the culture media incubated with endothelial cells at different levels of NO exposure. The metabolites of NO were measured as NO concentrations by chemiluminescence. Solid bars: 0 ppm; hatched bars: 20 ppm; empty bars: 80 ppm. Values are mean NO concentrations ± SD of five separate experiments. $^{*}P < 0.05$ *vs*. 0 h NO exposure. $^{\dagger}P < 0.05$ *vs*. 24 h NO exposure. $^{\#}P < 0.05$ *vs*. 20 ppm NO with similar exposure periods.

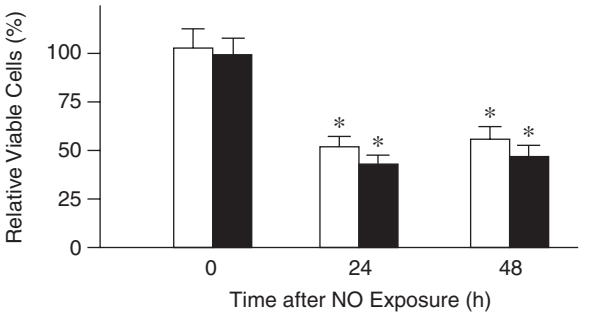

Fig. 2. Viability of human endothelial cells at different levels of NO exposure. The viable cell were measured by trypan blue exclusion. Empty bars: 20 ppm; solid bars: 80 ppm. Values are expressed as a mean percentage of viable cells to those exposed to 0 ppm NO at similar exposure periods ± SD from five separate experiments. $^{*}P < 0.05$ *vs.* 0 h NO with similar exposure concentrations.

Time- and Dose-Dependent Regulation of ET-1 by Exogenous NO

To determine the effect of exogenous NO on the expression of ET-1, the ET-1 peptide levels and preproET-1 mRNA contents were detected by ELISA and RT-PCR, respectively (Fig. 3). With NO exposure for 24 h, the ET-1 peptide levels in the 20 and 80 ppm groups were significantly decreased compared to those of the 0 ppm NO group (Fig. 3A). At 48 h NO exposure, the ET-1 peptide levels in the 20 ppm group were still lower than those of the 0 ppm group. However, the ET-1 peptide levels at 80 ppm were

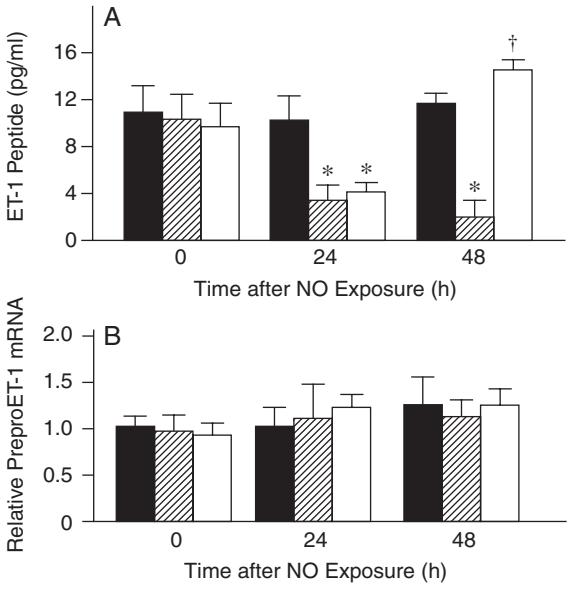

Fig. 3. The ET-1 peptide values and preproET-1 mRNA contents at different levels of NO exposure. Solid bars: 0 ppm; hatched bars: 20 ppm; empty bars: 80 ppm. (A) ET-1 peptide values detected by ELISA. Values are mean ET-1 peptide values ± SD of five separate experiments. $^{*}P < 0.05$ *vs*. 0 ppm NO with similar exposure periods. $^{\dagger}P < 0.05$ *vs*. the rest groups. (B) Quantitation of relative preproET-1 mRNA contents analyzed by RT-PCR. Densitometric values obtained for the prepro ET-1 mRNA transcripts were normalized to values for β-actin mRNA obtained from the same samples. Values are expressed as mean preproET-1/β-actin ratio, compared to 0 ppm 0 h NO exposure ± SD from five separate experiments.

significantly higher than those of the 0 ppm after NO exposure for 48 h. Furthermore, there was no significant change in the expression of preproET-1 mRNA contents between each group (Fig. 3B). These data indicate that exogenous NO gas modulated ET-1 expression in a dose- and time-dependent pattern at the post-transcriptional level.

Down-Regulation of eNOS by Exogenous NO

Western analysis was performed to measure the eNOS protein contents (Fig. 4A). A significant reduction of eNOS protein contents was detected only in the 80 ppm 48 h group. Furthermore, RT-PCR was carried out to measure the eNOS mRNA contents (Fig. 4B). Similarly, there was a significant decrease of eNOS mRNA contents in the 80 ppm 48 h group. These findings imply a transcriptional regulation of eNOS expression under a high concentration and long period of NO gas exposure.

Discussion

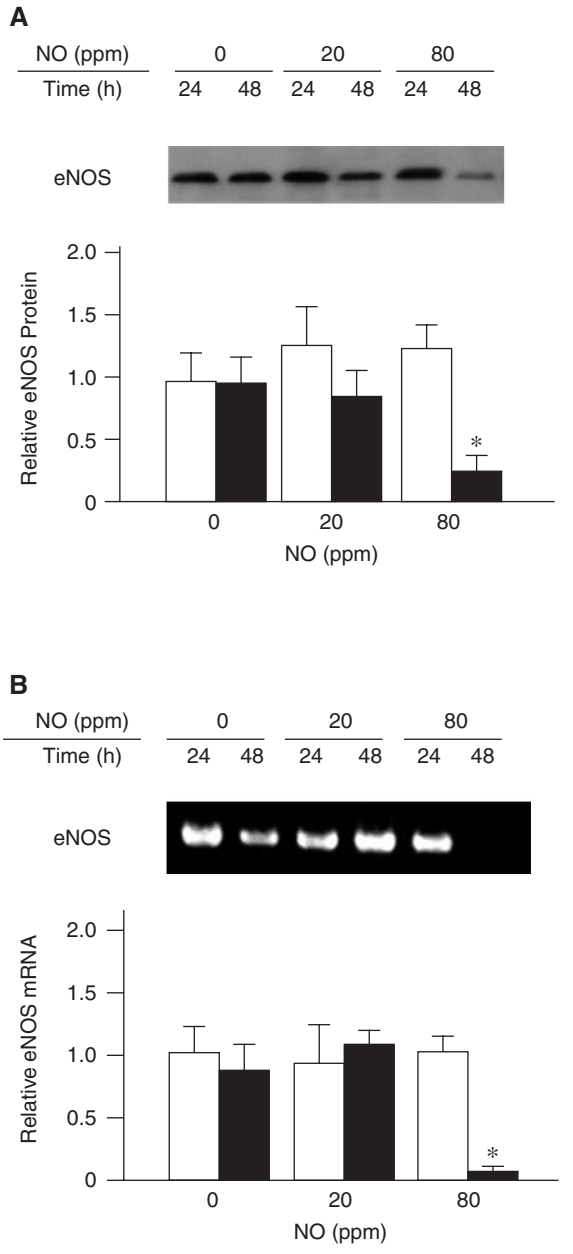

Fig. 4. The expression of eNOS protein and mRNA at different levels of NO exposure. Empty bars: 24 h; solid bars: 48 h. (A) The expression of eNOS protein determined by Western analysis. Equal loading was verified by Coomassie blue staining. Top: representative image of eNOS protein. Bottom: quantitation of relative eNOS protein levels. Values are means ± SD of relative density from 4 separate experiments. $^{*}P < 0.05$ *vs*. the rest groups. (B) The expression of eNOS mRNA determined by RT-PCR. Top: representative image of eNOS mRNA. Bottom: quantitation of relative eNOS mRNA contents. Densitometric values obtained for the eNOS mRNA transcripts were normalized to values for β-actin mRNA obtained from the same samples. Values are expressed as mean eNOS/β-actin ratio, compared to 0 ppm 0 h NO exposure ± SD from five separate experiments. $^{*}P < 0.05$ *vs*. the rest groups.

In the present study, we evaluated the effects of exogenous NO gas on the gene expression of ET-1 and eNOS in HUVECs. We used NO gas as the source of exogenous NO to ignore the chemical effects of NO-generating agents. In using a cell-culture model, we also have eliminated *in vivo* constituents that may be important determinants of gene expression. Our results showed the NO concentrations in the culture media were proportional to the dose and duration of NO exposure, indicating that our system was capable of providing a continuous and predictable source of exogenous NO during the experimental course. The steady release of NO gas allowed us to investigate the effects of NO gas in various concentrations and periods. Our data demonstrated the expression of ET-1 and eNOS was time- and dose-dependently regulated by NO gas.

In this study, we did not find any changes of eNOS expression in HUVECs exposed to either 20 ppm or 24 h of NO gas. Similar study from Smith and his colleagues showed that exposure to both 10 and 100 ppm of NO gas for 4 h did not alter the eNOS mRNA expression in bovine pulmonary artery endothelial cells (24). Our survey has further extended their inquiry by demonstrating a reduction of eNOS gene expression at 80 ppm 48 h of NO exposure. There was growing evidence to show that NO inhibited eNOS expression as a negative feedback mechanism to regulate endothelial cell function (8, 9, 22, 25). Buga *et al*. reported NO reduced eNOS expression by a dose-dependent manner in bovine aortic endothelial cells (4). The data obtained from their and our studies, although evaluated with different cells types and NO sources, highlight the fact that the gene expression of eNOS is modulated by exogenous NO in a time- and dose-dependent manner.

Although the effects of NO on ET-1 expression have been surveyed in a variety of systems, how NO regulates ET-1 remains incompletely clear. Our data illustrated that exposure to either 20 ppm or 24 h of NO gas resulted in a significant fall in ET-1 peptides. Additionally, we found the ET-1 peptides increased at 80 ppm 48 h of NO exposure. In cultured porcine aortic endothelial cells, the expression of ET-1 levels was dose-dependently reduced by exogenous NO (3, 18). In contrast to the inhibitory effect of NO-creating agents, inhaled NO was shown to induce an enhancement of ET-1 levels by a time-dependent regulation *in vivo* (17). Therefore, we suggest that, despite cell types and NO resources, different exposure periods and concentrations of exogenous NO could lead to the discrepant effects of NO gas resulting in inhibition or induction of ET-1 expression. Moreover, our study did not discover any significant alternations in the expression of preproET-1 mRNA. McMullan *et al*. showed that the ET-1 expression is regulated by exogenous NO through post-transcriptional levels, which may be involved in the clearance and release of ET-1 peptides (17). Further studies are needed to determine the underlying mechanisms.

In the current study, we found exogenous NO gas induced toxicity to HUVECs. Excess NO can disrupt cell membranes and cause cell death (7, 14). One would question whether the cytotoxic effects of NO gas may have impact on the expression of eNOS and ET-1. It has been shown that the alternation of gene expression caused by NO exposure is not related to cell death (18). In our study, the expression of eNOS and ET-1 was time- and dose-dependently regulated; we did not observe any time- and dose-dependent patterns of NO toxicity. Thus, the gene expression of eNOS and ET-1 were not simply modulated by cell death, although the toxic effects of NO gas can not be completely ruled out.

Overall, our study demonstrated that exposure to either 20 ppm or 24 h of NO gas inhibited ET-1 levels. The down regulation in ET-1 levels without an effect on eNOS expression at a lower concentration or shorter period of NO exposure could be considered beneficial in NO therapy for vasodilatation. In clinical applications, a high-dose and prolonged NO utilization was associated with rebound hypertension (1, 12). Our results support their findings by revealing that exposure to a higher concentration and longer period of NO gas led to an inhibition of eNOS expression and enhancement of ET-1 levels, which could worsen vasoconstriction. These data may provide clinical implications in the optimal dose and time period of NO therapy regarding to the greatest effectiveness and safety.

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