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Short communication

Interrelation between nitric oxide synthase and heme oxygenase in rat endothelial cells

Toshirou Seki [a,*], Mitsuhide Naruse [a], Kiyoko Naruse [a], Takanobu Yoshimoto [a], Akiyo Tanabe [a], Toshihiro Imaki [a], Hiromi Hagiwara [b], Shigehisa Hirose [c], Hiroshi Demura [a]

[a] *Department of Medicine, Institute of Clinical Endocrinology, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162, Japan*
[b] *Research Center for Experimental Biology, Tokyo Institute of Technology, Yokohama 226, Japan*
[c] *Department of Biological Sciences, Tokyo Institute of Technology, Yokohama 226, Japan*

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Abstract

The gene expression and interrelation of the constitutive type nitric oxide (NO) synthase-III as a NO-forming enzyme and heme oxygenase-2 as a carbon monoxide-forming enzyme were studied in cultured rat aortic endothelial cells. Both NO synthase-III and heme oxygenase-2 mRNAs were demonstrated in the endothelial cells by RNAase protection analysis. NO synthase-III mRNA was upregulated in the presence of the heme oxygenase inhibitor, zinc protoporphyrin IX, but not in the presence of the NO synthase inhibitor, N^G-nitro-L-arginine. Although heme oxygenase-2 mRNA was significantly upregulated in the presence of both NO synthase inhibitor and heme oxygenase inhibitor, the increase was greater with the NO synthase inhibitor. These results provide the first evidence for the concomitant gene expression of NO synthase-III and heme oxygenase-2, and their compensatory interrelation in endothelial cells. © 1997 Elsevier Science B.V.

Keywords: N^G-nitro-L-arginine; Zinc protoporphyrin IX; Nitric oxide (NO); Nitric oxide (NO) synthase; Carbon monoxide; Heme oxygenase

1. Introduction

Nitric oxide (NO) as one of the most representative endothelium-derived relaxing factors has been shown to be deeply involved in the regulation of vascular function and structure through the activation of soluble guanylate cyclase and production of cGMP (Moncada and Higgs, 1993; Förstermann et al., 1994; Nathan and Xie, 1994; Inagami et al., 1995). Although NO is produced from L-arginine by three kinds of NO synthase: NO synthase-I, as a neuronal type, NO synthase-II, as an inducible type, and NO synthase-III, as an endothelial type. However, NO synthase-III which is constitutively expressed in the vascular endothelial cells has a key role in cardiovascular homeostasis through local generation of NO (Moncada and Higgs, 1993; Förstermann et al., 1994; Nathan and Xie, 1994; Inagami et al., 1995).

Carbon monoxide has also been shown to have a direct cardiovascular action (Schmidt, 1992; Kharitonov et al., 1995; Suematsu et al., 1995). It is produced endogenously as a by-product of heme catabolism by two kinds of heme oxygenase: heme oxygenase-1 as an inducible type and heme oxygenase-2 as a constitutive type, respectively (Maines, 1988). Morita et al. (1995) have demonstrated that carbon monoxide is produced by hypoxia-induced heme oxygenase-1 and activates soluble guanylate cyclase/cGMP in cultured vascular smooth muscle cells.

It is therefore suggested that the vascular wall modulates its tonus and structure via production of two biologically active monoxides, NO and carbon monoxide, with guanylate cyclase/cGMP as a common signal transduction system. However, details of the gene expression of heme oxygenase and its interrelation with the NO synthase/NO system in the endothelial cells remain to be elucidated. In the present study, we investigated the gene expression of constitutive type heme oxygenase-2 and the effects of inhibiting enzyme activity of NO synthase or heme oxygenase on their mRNA expression levels in cultured endothelial cells.

* Corresponding author. Tel.: (81-3) 3359-5559; Fax: (81-3) 3397-7898.

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2. Materials and methods

2.1. Animals

Eight-week-old male Wistar rats (Japan Laboratory Animals, Tokyo, Japan) were used. The rats were handled in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, revised in 1985). Techniques for euthanasia followed current guidelines established by the American Veterinary Medical Association Panel on Euthanasia (Report of the AVMA Panel on Euthanasia, 1993).

2.2. Cell culture

Primary cultures of rat aortic endothelial cells were prepared using enzymatic and dissociation techniques and were characterized morphologically and immunocytochemically as described previously (Emori et al., 1993). Cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (Sankojunyaku, Tokyo, Japan), and the antibiotics, penicillin and streptomycin (Gibco BRL, Grand Island, NY, USA), at 37°C in a humidified 5% CO_2 incubator. Endothelial cells were passaged with trypsin-EDTA every 3–4 days and were used between passages 10 and 20.

2.3. Protocol of incubation studies

Confluent endothelial cells were washed with phosphate-buffered saline and incubated at 37°C with or without test compounds in Dulbecco's modified Eagle's medium for 6 h. This incubation time was used since both NO synthase-III and HO-1 mRNA expression have been reported to reach their peak level several hours after appropriate stimuli (Morita et al., 1995; Arnet et al., 1996; Lopez-Ongil et al., 1996). At the beginning of the 6 h incubation period, N^G-nitro-L-arginine (Sigma) as the inhibitor of NO synthase and zinc protoporphyrin IX (Sigma) as the inhibitor of heme oxygenase were added at the concentrations of 2.0 mM (Morita et al., 1995) and 10 μM (Christodoulides et al., 1995; Morita et al., 1995; Zakhary et al., 1996), respectively. The concentration of N^G-nitro-L-arginine was shown not to affect heme oxygenase activity (Morita et al., 1995). In addition, the concentration of zinc protoporphyrin was demonstrated not to inhibit NO synthase and soluble guanylate cyclase activity (Zakhary et al., 1996).

2.4. Reverse transcription (RT) of RNA and polymerase chain reaction (PCR)

RT-PCR was performed by using specific oligonucleotide primers and cDNA templates of RT product from rat testis for heme oxygenase-2. Specific primers for PCR were designed according to details previously published for rat testis heme oxygenase-2 (Rotenberg and Maines, 1990). The sequences of the primers were 5′-AGAAGTATGTGGATCGGA-3′ (sense) and 5′-TACTCAGGTCCAAGGCA-3′ (antisense), respectively.

The PCR was run for 35 cycles by repeating denaturation at 95°C for 1 min, annealing at 50°C for 2 min, and polymerization at 72°C for 3 min in the presence of *Taq* polymerase (Takara, Kyoto, Japan). The PCR product was size-fractionated by 3% agarose-gel electrophoresis and subcloned into the plasmid pBluescript II SK$^-$ (Stratagene, La Jolla, CA, USA) for RNAase protection assay. PCR products were sequenced by the dideoxy chain termination method (Sanger et al., 1977) after subcloning into pBluescript II SK$^-$.

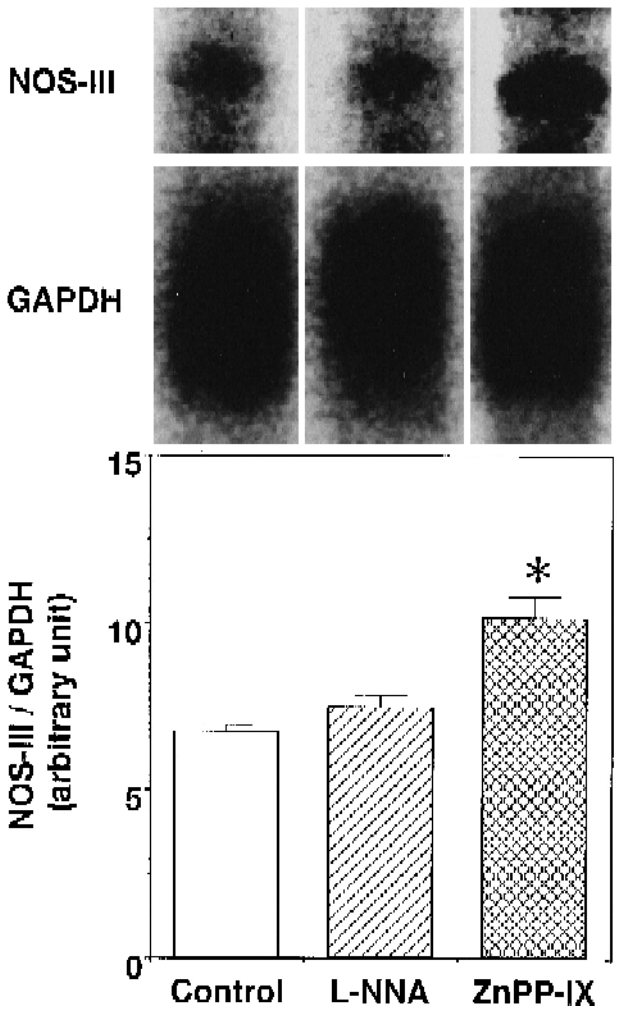

Fig. 1. Effects of NO synthase inhibitor, N^G-nitro-L-arginine (L-NNA), and heme oxygenase inhibitor, zinc protoporphyrin IX (ZnPP-IX), on mRNA expression levels of endothelial NO synthase-III (NOS-III) in cultured endothelial cells. Upper panel: Typical autoradiogram of polyacrylamide gel electrophoresis. Lower panel: The relative optical density of NO synthase-III mRNA levels corrected by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Values are the means ± SEM ($n = 5$). * $P < 0.01$ vs. control.

RT-PCR for NO synthase-III was performed with methods we have described previously (Seki et al., 1996, 1997).

2.5. RNA extraction

Total RNA was extracted from cultured rat aortic endothelial cells according to the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987).

2.6. RNAase protection analysis

Antisense cRNA probe for each enzyme was transcribed in the presence of Type 3 phage (heme oxygenase-2) and Type 7 phage (NO synthase-III) RNA polymerase (Stratagene) and [α-^{32}P]UTP (30 TBq/mmol) (DuPont NEN Research Products, Boston, MA, USA). Antisense cRNA probe for glyceraldehyde-3-phosphate dehydrogenase was generated with methods we have described previously (Yoshimoto et al., 1995). After the transcription reaction, the template DNA was removed by digestion with RNAase-free DNAase I (Boehringer-Mannheim, Mannheim, Germany). The radioactive cRNA of each enzyme (2×10^5 cpm) and that of glyceraldehyde-3-phosphate dehydrogenase (1×10^5 cpm) were hybridized with 20 μg total RNA for 12 h at 45°C (NO synthase-III) or 50°C (heme oxygenase-2) in 80% formamide, 40 mmol/l Pipes (pH 6.4), 5 mmol/l Na_2EDTA and 400 mmol/l NaCl. Non-annealing nucleic acids were digested with a combination of ribonuclease A and T1 (Boehringer-Mannheim) at final concentrations of 40 and 2 μg/ml, respectively, in 10 mmol/l Tris-HCl buffer, pH 7.4, containing 300 mmol/l NaCl and 5 mmol/l Na_2EDTA at 30°C for 1 h.

The protected fragments were analyzed by electrophoresis in a 5% polyacrylamide gel containing 7 M urea and exposed to a photostimulatable imaging plate for 12 h. The radioactivities of the protected fragments were quantified by means of a Fuji Imaging Analyzer (BAS 2000, Fuji Photo Film, Tokyo, Japan). The results for each enzyme mRNA were normalized to that of glyceraldehyde-3-phosphate dehydrogenase in each sample.

2.7. Statistical analysis

Results were expressed as the means ± SEM from multiple experiments. Statistical analysis was performed using Fisher's protected least significant difference after one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant.

3. Results

There was a significant mRNA expression of both heme oxygenase-2 and NO synthase-III in the cultured endothelial cells (Figs. 1 and 2). NO synthase-III mRNA levels showed a significant upregulation in the presence of the heme oxygenase inhibitor, zinc protoporphyrin IX, while it did not change in the presence of the NO synthase inhibitor, N^G-nitro-L-arginine (Fig. 1). Heme oxygenase-2 mRNA levels showed a significant upregulation in the presence of both N^G-nitro-L-arginine and zinc protoporphyrin IX. However, the increase was significantly greater with N^G-nitro-L-arginine than with zinc protoporphyrin IX (Fig. 2).

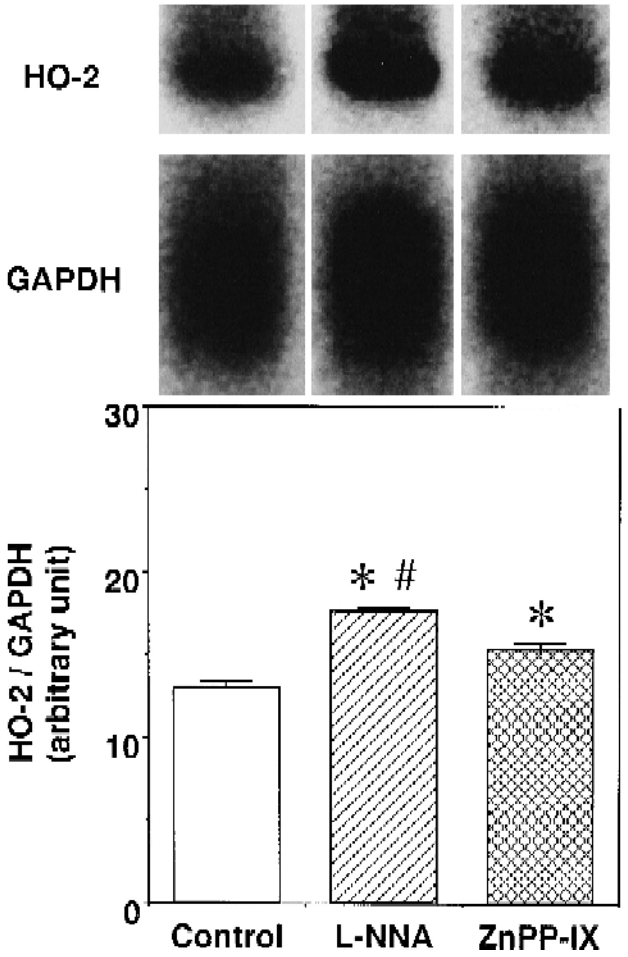

Fig. 2. Effects of NO synthase inhibitor, N^G-nitro-L-arginine (L-NNA), and heme oxygenase inhibitor, zinc protoporphyrin IX (ZnPP-IX), on mRNA expression levels of heme oxygenase-2 (HO-2) in cultured endothelial cells. Upper panel: Typical autoradiogram of polyacrylamide gel electrophoresis. Lower panel: The relative optical density of heme oxygenase-2 mRNA levels corrected by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Values are the means ± SEM ($n = 5$). * $P < 0.01$ vs. control; # $P < 0.01$ vs. ZnPP-IX.

4. Discussion

Morita et al. (1995) demonstrated that heme oxygenase-1 mRNA is expressed in vascular smooth muscle cells and that its product, carbon monoxide, may regulate vascular tone under physiological and/or pathological conditions.

Christodoulides et al. (1995) indicated that vascular smooth muscle cells have both heme oxygenase-1 and heme oxygenase-2 activity, and that they respond to specific stimuli to generate guanylate cyclase-stimulatory carbon monoxide in the same cells. Although heme oxygenase-2 was immunohistochemically localized (Zakhary et al., 1996), details of the expression of heme oxygenase-2 mRNA in endothelial cells remain unknown. In the present study, we clearly demonstrated mRNA expression of heme oxygenase-2 as well as NO synthase-III in the cultured rat aortic endothelial cells. These results suggest that endothelial cells have the heterogeneous biosynthetic pathways to produce biologically active monoxides which may play an important role in the regulation of vascular function.

Although it has been demonstrated that the endothelial cells may show phenotypic changes (Myers, 1993), the cultured endothelial cells used in the present study did not show any significant change in either morphology or immunoreactivity for factor-VIII (data not shown). It is therefore suggested that the influence of the phenotypic change of the cells, if any, on the present findings may not be significant.

Since NO and carbon monoxide share similar chemical and biological properties (Maines, 1988; Schmidt, 1992; Inagami et al., 1995; Kharitonov et al., 1995; Suematsu et al., 1995), it is hypothesized that the NO synthase/NO and heme oxygenase/carbon monoxide systems may closely interact with each other at various levels of biosynthesis. In the present study, it was shown that inhibition of carbon monoxide production by zinc protoporphyrin IX resulted in a significant upregulation of NO synthase-III mRNA, while inhibition of NO production by N^G-nitro-L-arginine resulted in a significant upregulation of heme oxygenase-2 mRNA. These results suggest a compensatory upregulation of NO synthase-III and heme oxygenase-2 gene expression by the end product of their counterpart in the endothelial cells. The reciprocal changes in NO synthase-III (McQuillan et al., 1994) and heme oxygenase-1 (Morita et al., 1995) in the vascular smooth muscle cells during hypoxia also agree with the concept. Since both NO synthase-III and heme oxygenase-2 are constitutively expressed, the compensatory interrelation between these two enzymes producing low molecular monoxides may be of physiological significance in vivo also.

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