Regulation of Endothelial Nitric-oxide Synthase during Hypoxia*

(Received for publication, October 27, 1995, and in revised form, January 5, 1996)

Urs A. Arnet‡, Audrey McMillant, Jay L. Dimerant, Barbara Ballermann§, and Charles J. Lowenstein¶

From the Divisions of ‡Cardiology and §Nephrology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The mechanism by which nitric-oxide (NO) production increases during hypoxia is unknown. To explore the effect of hypoxia upon endothelial nitric-oxide synthase (ecNOS) activity and expression, we exposed bovine aortic endothelial cells (BAEC) to hypoxia (1% O2) for 0–24 h and measured levels of ecNOS mRNA, protein, and activity. The amount of ecNOS mRNA increases to more than twice the basal level after 6 h of hypoxia. Incubation of BAEC with actinomycin D during hypoxia prevents this increase, demonstrating that higher levels of mRNA observed during hypoxia are due to increased transcription, not to increased stability of ecNOS mRNA. Levels of ecNOS protein increase throughout 24 h of hypoxia to more than twice normoxic levels. Although ecNOS expression increases within 2 h of hypoxia, total activity remains unchanged. To explore the transcriptional regulation of ecNOS, we constructed a reporter plasmid containing the ecNOS promoter region upstream of the luc gene and transfected this reporter plasmid into BAEC. In this system, hypoxia induces a linear increase over time in the expression of luciferase driven by the ecNOS promoter. It is concluded that hypoxia induces an increase in transcription of ecNOS in endothelial cells, activating the regulatory region of ecNOS by undefined transcription factors.

Systemic arteries vasodilate in response to hypoxia, delivering more blood to peripheral tissues. This dilation occurs within seconds after hypoxia, and is maintained for hours. The molecular basis of hypoxic vasodilation is not completely understood. Possible mechanisms include direct relaxation of smooth muscle cells induced by changes in pH, ion channel conductance changes, and decreases in ATP levels. Endothelial cells may also release lower amounts of vasoconstrictors, such as endothelin or thromboxane, or may release increased amounts of vasodilators such as adenosine, prostacyclin, endothelial hyperpolarizing factor, and nitric oxide (NO)1 (1–3).

NO is made in endothelial cells by endothelial nitric-oxide synthase (ecNOS), which converts arginine and oxygen into citrulline and NO (4–12). Although ecNOS is expressed constitutively in resting endothelial cells, the level of ecNOS expression can be altered by various stimuli. Shear-stress and estrogen can increase ecNOS expression both in vitro and in vivo (13–23). Tumor necrosis factor-α decreases expression of ecNOS (17, 24–27). Thus, although ecNOS is called a "constitutive" isoform of NOS, its expression can be altered.

Several studies have shown that hypoxia increases the synthesis of NO from systemic arteries both in vivo (28–30) and in vitro (31, 32). The effect of hypoxia on NO synthesis from endothelial cells in vitro is less clear; some studies report an increase, decrease, or no change (33–35). These studies exposed cells isolated from systemic vessels to hypoxia for different periods of time, which may explain the different effects of hypoxia. An increase in NO synthesis during hypoxia could be due to increased levels of calcium prolonging the activation of ecNOS, post-translational modification increasing the activity of ecNOS, or an increase in ecNOS synthesis. One study has shown that hypoxia for 1 day decreases ecNOS levels in vitro (33), and another study has shown that hypoxia for 7 days increases ecNOS in vivo (36).

In this study we tested the hypothesis that hypoxia induces the transcription of ecNOS. We show that hypoxia increases the amount of ecNOS mRNA and protein. Furthermore, we show that hypoxia can activate the transcription of a reporter gene under control of the ecNOS 5′-flanking region. However, while ecNOS levels increase, there is no increase in NOS activity in hypoxic cells.

EXPERIMENTAL PROCEDURES

Materials—The bovine ecNOS cDNA and the human ecNOS genomic clone have been previously described (24, 37). Actinomycin D was obtained from Sigma; Opti-MEM and Lipofectin were obtained from Life Technologies, Inc. The pGL2-Basic plasmid, luciferase assay system, and β-galactosidase enzyme assay system were obtained from Promega. Donkey anti-rabbit Ig antibody conjugated to horseradish peroxidase and the chemiluminescent assay are from Amer sham Corp.

Antibody Preparation—Affinity-purified polyclonal antibody against bovine ecNOS was prepared by standard techniques (38). In brief, a peptide composed of the terminal 15 amino acids of bovine ecNOS was synthesized by the Johns Hopkins Biopolymer Laboratory. The peptide was conjugated to thyroglobulin and injected into rabbits (Hazelton Research Products). Immune sera from injected rabbits was affinity-purified on a peptide-bovine serum albumin-agarose column.

Cell Culture—Bovine aortic endothelial cells were prepared from aortas obtained from the slaughterhouse and then cloned as described elsewhere (39). Their endothelial phenotype was verified by demonstrating acetylated LDL uptake and the expression of von Willebrand's factor by immunofluorescence. Cells were grown on 10-cm dishes coated with bovine gelatin in RPMI 1640 medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were used up to passage 19. When cells were approximately 90% confluent, they were re-fed with media and exposed to normoxia or hypoxia.

Hypoxia—BAEC grown in tissue culture plates were placed in an incubator that was continuously flushed with 1% O2, 5% CO2, 94% N2 (Puritan Bennett, Linthicum Heights, MD). Prior measurements with an oxygen electrode showed that the partial pressure of oxygen was 8
tor. Cells were harvested 0–24 h after being placed in this chamber.

Construction of Reporter Plasmid—A fragment of DNA containing the 5′-flanking region of the human ecNOS gene was amplified by the PCR. The template DNA for the PCR was a BlueScript plasmid (Stratagene) containing 6 kilobase pairs of genomic human DNA, including 1.6 kilobase pairs upstream from the human ecNOS gene (37). The primers for the PCR, designed to amplify DNA from 1.6 kilobase pairs upstream of the ecNOS AUG to the transcriptional start site of ecNOS, were: CGCGGTACCATCTGATGCTGCCTGTCACC (upstream) and GCGCGCAAGCTTGTTACTGTGCGTCCACTCTGC (downstream). The 1.6-kilobase pair PCR product (not shown) was inserted into KpnI and HindIII sites in the pGL2-Basic plasmid (Promega) upstream of the luc gene, and the nucleotide sequence was determined to confirm that no errors had been introduced by PCR amplification.

Transfection of BAEC—The luciferase reporter plasmid and the pSVβ-galactosidase plasmid (Promega) as an internal control were introduced into BAEC by liposomal-mediated transfection according to the manufacturer’s instructions (Life Technologies, Inc.). In brief, BAEC were incubated for 6 h with a mixture of 0.5 μg of pSVβ-galactosidase plasmid, 0.5 μg of luciferase reporter plasmid, and 10 μl of Lipofectin. BAEC were then fed with RPMI and grown for an additional 16 h. Transfected BAEC were then exposed to hypoxia as above for various times and harvested with lysis buffer within 30 s of removal from hypoxia. Cell lysates were assayed for luciferase activity using a luciferase assay kit (Promega) and a luminometer (Turner Designs 20). Lysates were also assayed for β-galactosidase activity with a kit (Promega) and assayed for protein concentration using a kit (Bio-Rad). The luminometer reported relative light units, which were normalized by dividing by galactosidase activity.

NOS Catalytic Assay—After exposure to hypoxia, BAEC were immediately harvested in sample buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA), sonicated, and centrifuged at 1000 × g for 5 min, and the supernatant was used to measure NOS activity by the conversion of [14C]arginine to [14C]citrulline (40). In brief, 75 μM of total protein was incubated in 50 mM HEPES, pH 7.4, 10 μM CaCl₂, 3 μM tetrahydrobiopterin, 1 μM FAD, 1 μM FMN, 0.1 μM calmodulin, and 1 mM NADPH at 37°C for 10 min. The reaction was stopped by adding 3 ml of 20 mM HEPES, pH 5.5, 2 mM EDTA, and 2 mM EGTA. The resulting samples were applied to 1 ml columns of Dowex AG 50WX-8 (Na⁺ form), and the radioactivity in the flow-through was determined by liquid scintillation counting. Specific ecNOS activity is calculated as citrulline production from the above reaction mixture with 10 μM CaCl₂

RESULTS

Hypoxia Increases ecNOS Expression—BAEC were exposed to hypoxia for increasing periods of time, harvested, and assayed for ecNOS mRNA and protein. During hypoxia, the amount of ecNOS mRNA rises from a basal level in normoxic cells. The increase is slight during 2–4 h of hypoxia and peaks after 6 h; levels remain elevated after 24 h of hypoxia (Fig. 1). Re-oxygenation of BAEC for 1 h reduces the mRNA to basal levels. Densitometry shows that mRNA levels of ecNOS after 6 h hypoxia are roughly twice the basal levels (not shown). This experiment was repeated three times with similar results.

Hypoxia also increases the levels of ecNOS protein. The

2 H. Silverman, personal communication.
protein, which is expressed at a basal level in normoxic BAEC, begins to increase 2–4 h after hypoxia and continues to rise during 24 h of hypoxia (Fig. 2A). Densitometry shows that ecNOS protein increases by a factor of 2.2 after 6 h of hypoxia (Fig. 2B). Re-oxygenation for 1 h has little effect on ecNOS protein after 24 h of hypoxia, in contrast to the decline in ecNOS mRNA levels. This experiment was repeated five times with similar results.

Hypoxia Decreases ecNOS Specific Activity—Arginine to citrulline conversion assays were performed to measure the activity of ecNOS in hypoxic BAEC. During 24 h of hypoxia, total NOS activity in BAEC decreases slightly (Fig. 3A), although the amount of ecNOS increases (Fig. 2). When relative NOS specific activity is calculated by dividing NOS activity (Fig. 3A) by NOS protein levels (Fig. 2B), the relative specific activity of ecNOS gradually decreases below that of NOS in normoxic controls (Fig. 3B). This decrease is not due to any increase in general protein synthesis, since the total amount of protein in each dish remains constant during hypoxia (Fig. 3C). These results are the average of three experiments in which NOS activity was measured in triplicate. Thus hypoxia decreases ecNOS relative specific activity.

Hypoxia Does Not Change ecNOS mRNA Stability—Hypoxia could increase ecNOS mRNA levels either by increasing its rate of transcription or by decreasing its destruction. In order to distinguish between these possibilities, actinomycin D was added to BAEC. BAEC were then placed either in an incubator or in a hypoxic chamber. The BAEC were harvested at various times and their RNA analyzed by Northern blotting. Hypoxia does not affect the stability of ecNOS mRNA (Fig. 4). Thus the increase of ecNOS mRNA levels during hypoxia is not due to increased stability.

Hypoxia Increases Transcription of a Reporter Gene Controlled by the ecNOS Regulatory Region—A reporter plasmid was constructed by inserting the 5'-regulatory region of the ecNOS gene into a plasmid so that it drives the expression of the luc cDNA (see “Experimental Procedures”). This reporter plasmid was transfected into BAEC, along with a plasmid constitutively expressing β-galactosidase as an internal control. Transfected BAEC were exposed to hypoxia, harvested, and assayed for luciferase and β-galactosidase activity.

Hypoxia increases expression of luciferase in BAEC transfected with the reporter plasmid containing the ecNOS regulatory region (Fig. 5). Luciferase is expressed in normoxic BAEC, just as ecNOS is normally expressed at a basal level in BAEC. Expression of luciferase relative to β-galactosidase rises linearly from 2 to 24 h of hypoxia, increasing 2.1-fold over normoxia after 24 h. This experiment was repeated three times with similar results.

Discussion

Our study provides several lines of evidence that hypoxia regulates ecNOS and its transcription. Hypoxia increases the levels of ecNOS mRNA and protein. The increase in ecNOS mRNA is not due to an increase in its stability, since this does not change during hypoxia. Hypoxia also increases transcription of a reporter gene driven by the ecNOS 5'-flanking region. Thus increased transcription produces more ecNOS during hypoxia.
Our data suggest that the influence of NOS on vascular tone during hypoxia is complex. Others have shown that during acute hypoxia, intracellular calcium levels rise within minutes in endothelial cells (35), which can activate eNOS to synthesize NO. We show that during chronic hypoxia, more eNOS is produced within hours, although total NOS activity does not change. The amount of NO synthesized during hypoxia is complex. Others have shown that during hypoxia, the enhancer elements and transcription factors are added exogenously for the NOS assay (see “Experimental Procedures”), eNOS can be myristoylated, palmitoylated, and phosphorylated (50, 51). However, it is not known whether these modifications occur during hypoxia. None of these modifications have been shown to change the activity of eNOS.

Thus oxygen regulates the transcription of eNOS. The transcription factors that sense hypoxia and activate transcription of eNOS have not yet been identified.

Acknowledgments—We thank Dr. Thomas Michel for helpful discussions and Dr. Howard Silverman for his assistance.

REFERENCES
1. Greenberg, B., and Kishiyama, S. (1993) Am. J. Physiol. 265, H1712–H1720
2. Isozumi, K., Fukuuchi, Y., Takeda, H., and Itoh, Y. (1994) Kexio. J. Med. 43, 31–36
3. McPherson, R. W., Koehler, R. C., and Traylor, R. J. (1994) Am. J. Physiol. 266, H476–H482
4. Palmer, R. M., Ashton, D. S., and Moncada, S. (1988) Nature 333, 664–666
5. Marietta, M. A. (1994) Cell 78, 926–930
6. Marietta, M. A. (1993) J. Biol. Chem. 268, 12231–12234
7. Ignarro, L. J. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 535–560
8. Moncada, S., Palmer, R. M., and Higgs, E. A. (1991) Pharmacol. Rev. 43, 109–142
9. Loweinstein, C. J., and Snyder, S. H. (1992) Cell 70, 705–707
10. Nathan, C., and Xie, Q. W. (1994) Cell 79, 915–918
11. Luscher, T. F., and Tanner, F. C. (1993) Am. J. Hypertens. 6, 2835–2935
12. Ignarro, L. J. (1993) Thromb. Haemostasis 70, 148–153
13. Koller, A., Sun, D., Huang, A., and Kaley, G. (1994) Am. J. Physiol. H326–H332
14. Buga, G. M., Gold, M. E., Fukuto, J. M., and Ignarro, L. J. (1991) Hypertension 187–193
15. Goetz, R. M., Morano, I., Calovini, T., Studer, R., and Hoftz, J. (1995) Biochem. Biophys. Res. Commun. 205–209
16. Kuchan, M. J., and Frangos, J. A. (1994) Am. J. Physiol. 266, H628–H636
17. Nishida, K., Harrison, D. G., Navas, J. P., Fisher, A. A., Dockery, S. P., Uematsu, M., Neren, R. M., Alexander, R. W., and Murphy, T. J. (1992) J. Clin. Invest. 90, 2092–2096
18. Jannides, R., Haeefel, W. E., Linder, L., Richard, V., Bakkali, E. H., Thuilliez, C., and Luscher, T. F. (1995) Circulation 91, 1314–1319
19. Kondera, A., Ando, J., Tsuboi, H., Yang, W., Sakuma, I., Toyo-oka, T., and Kaniya, A. (1994) Biochem. Biophys. Res. Commun. 190, 213–219
20. Noris, M., Morigli, R., Donadelli, R., Aiello, S., Foppolo, M., Todeschini, M., Fabbri, G., Loi, S., Renuuzzi, G., and Renuuzzi, A. (1995) Circ. Res. 76, 536–543
21. Van Buren, G. A., Yang, D. S., and Clark, K. E. (1992) Biochim. Biophys. Acta 1105, 90–95
22. Weiner, C. P., Lizasoain, I., Baylis, S. A., Knowles, R. G., Charles, I. G., and Kilbourn, R. G., and Belloni, F. L. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 5212–5216
23. Hishikawa, K., Nakaki, T., Marumo, T., Suzuki, H., Kato, R., and Saruta, T. (1995) FEBS Lett. 360, 291–293
24. Lamas, S., Marsden, P. A., Li, C., Tempst, P., and Michel, T. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 6348–6352
25. kilbourn, R. G., and Belloni, F. L. (1990) Am. J. Physiol. H355–H364
26. Marsden, P. A., Schoppert, K. T., Chen, H. S., Flowers, M., Sundell, C. L., Wilcox, J. N., Lamas, S., and Michel, T. (1992) FEBS Lett. 307, 287–293
27. Shaul, P. W., Farrar, A. A., and Zellers, T. M. (1992) Am. J. Physiol. 262, H335–H364
28. Pohl, U., and Busse, R. (1989) Am. J. Physiol. 255, H1955–H1960
29. Brown, I. P., Thompson, C. I., and Belloni, F. L. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 5122–5126
30. Ross, R. (1993) Annu. Rev. Cardiovasc. Med. 4, 311–317
31. Sun, M. K., and Reis, D. J. (1992) Life Sci. 50, 555–565
32. McQuillan, L. P., Leung, K. G., Marsden, P. A., Kostyk, S. K., and Kourer-banas, S. (1994) Am. J. Physiol. 257(H1), H1922–H1927
33. Shaul, P. W., and Wells, L. B. (1994) Am. J. Respir. Cell Mol. Biol. 11, 432–438
34. Hampl, V., Conrinfeld, D. N., Cowan, N. J., and Archer, S. L. (1995) Eur. Respir. J. 8, 515–522
35. Shaul, P. W., North, A. J., Brannon, T. S., Ujiie, K., Wells, L. B., Nisen, P. A., Lowenstein, C. J., Snyder, S. H., and Star, R. A. (1995) Am. J. Respir. Cell Mol. Biol. 13, 167–174
36. Robinson, L. J., Waremowicz, S., Morton, C. C., and Michel, T. (1994) Genomics 19, 350–357
37. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 72–78, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
38. Uchida, K., and Ballermann, B. J. (1995) Am. J. Physiol. 263, C200–C209
39. Breit, D. S., and Snyder, S. H. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 9030–9033
40. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
41. Chomczynski, P. (1993) Bio/Technology 15, 532–537
43. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, pp. 7.3–7.84, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
44. Nguyen, T., Brunson, D., Crespi, C. L., Penman, B. W., Wishnok, J. S., and Tannenbaum, S. R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3030–3034
45. Marletta, M. A., Tayeh, M. A., and Hevel, J. M. (1990) Biofactors 2, 219–225
46. Ford, P. C., Wink, D. A., and Stanbury, D. M. (1993) FEBS Lett. 326, 1–3
47. Schwartz, S. E., and White, W. H. (1983) in Trace Atmospheric Constituents: Properties, Transformation, and Fates (Schwartz, S. E., ed) pp. 1–117, John Wiley and Sons, New York
48. Arroyo, C. M., Forray, C., el Fakahany, E. E., and Rosen, G. M. (1990) Biochem. Biophys. Res. Commun. 170, 1177–1183
49. Marsden, P. A., Heng, H. H., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X. M., Tsui, L. C., and Schappert, K. T. (1993) J. Biol. Chem. 268, 17478–17488
50. Busconi, L., and Michel, T. (1993) J. Biol. Chem. 268, 8410–8413
51. Robinson, L. J., Busconi, L., and Michel, T. (1995) J. Biol. Chem. 270, 995–998
Regulation of Endothelial Nitric-oxide Synthase during Hypoxia
Urs A. Arnet, Audrey McMillan, Jay L. Dinerman, Barbara Ballermann and Charles J. Lowenstein

J. Biol. Chem. 1996, 271:15069-15073.
doi: 10.1074/jbc.271.25.15069

Access the most updated version of this article at http://www.jbc.org/content/271/25/15069

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 13 of which can be accessed free at http://www.jbc.org/content/271/25/15069.full.html#ref-list-1