J. Biochem. **133**, 501–505 (2003)
DOI: 10.1093/jb/mvg066

Nitric Oxide Synthase Induction, cGMP Elevation, and Biopterin Synthesis in Vascular Smooth Muscle Cells Stimulated with Interleukin-1β in Hypoxia

Kaori Nagao[1], Shigeo Takenaka[2], Ryoichi Yamaji[1], Hiroshi Inui[*,1] and Yoshihisa Nakano[1]

[1]Department of Applied Biological Chemistry, and [2]Department of Veterinary Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531

Received January 21, 2003; accepted January 27, 2003

In cultured rat vascular smooth muscle cells (VSMC), inducible nitric oxide synthase (iNOS) expression evoked by interleukin-1β (IL-1β) or tumor necrosis factor–α was greatly enhanced in hypoxia (2% O_2), compared to in normoxia. In contrast, iNOS induction by interferon-γ, lipopolysaccharide or their combination was barely influenced by hypoxia. These results indicate that iNOS induction is regulated by hypoxia in different manners, depending on the stimuli in VSMC. Nitric oxide (NO) production in response to stimulation with interferon-γ plus lipopolysaccharide was significantly decreased in hypoxia, due to a decrease in the concentration of O_2 as a substrate. In contrast, the level of NO production in hypoxia was almost the same as that in normoxia when the cells were stimulated by IL-1β. In addition, cGMP increased in response to IL-1β in hypoxia to a level comparable to that in normoxia. Thus, it seems that the IL-1β–induced expression of iNOS is up-regulated in hypoxia to compensate for a decrease in the enzyme activity due to the lower availability of O_2 as a substrate, and consequently a sufficient amount of NO is produced to elevate cGMP to an adequate level. In addition, the IL-1β–induced synthesis of tetrahydrobiopterin, a cofactor for iNOS, was also greatly stimulated by hypoxia in VSMC.

Key words: biopterin, cGMP, inducible nitric oxide synthase, hypoxia, vascular smooth muscle cells.

Abbreviations: HIF-1, hypoxia inducible factor-1; HRE, hypoxia responsible element; IFN-γ, interferon-γ; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor–κB; NO, nitric oxide; TNF-α, tumor necrosis factor–α; VSMC, vascular smooth muscle cells.

The formation of NO, which leads to the activation of soluble guanylate cyclase with an increased tissue concentration of cGMP, is an endogenous regulatory mechanism of cardiovascular homeostasis (*1*, *2*). Under physiological conditions, the endothelial lining of vessels contributes to the regulation of vascular tone through NO production by Ca^{2+}-calmodulin–dependent NO synthase (*3*, *4*). In contrast, VSMC do not produce NO in the resting state. However, following stimulation with LPS and proinflammatory cytokines, such as IL-1β, TNF-α, and IFN-γ, the inducible form of NO synthase (iNOS) is expressed in VSMC, as well as other types of cells such as macrophages (*4–7*). NO produced by iNOS, as well as endothelial-derived NO, activates soluble guanylate cyclase, increases the level of cGMP, and thereby results in vasorelaxation (*4*, *6*, *8*). In addition to the induction of iNOS, GTP cyclohydrolase I, which participates in biopterin synthesis, is induced in response to LPS and proinflammatory cytokines in VSMC, as well as other cell types, since tetrahydrobiopterin is required as a cofactor for iNOS (*9–11*).

Hypoxia is a condition of decreased O_2 availability, and mammals exhibit many adaptive responses to low O_2 conditions (*12*). Under hypoxic conditions, transcription of the genes encoding erythropoietin, vascular endothelial growth factor and glycolytic enzymes is triggered to increase O_2 delivery to tissues and to stimulate an anaerobic metabolic pathway for ATP production. The transcriptional activation is mediated by HIF-1, and during hypoxia HIF-1 binds to HRE in these genes (*12–14*).

HRE is present in the 5′-flanking region of the iNOS gene (*14–17*), suggesting that iNOS expression is regulated by hypoxia through the action of HIF-1. However, data on the effect of hypoxia on the expression level of iNOS are contradictory. Melillo *et al.* (*15*, *18*) have reported that the induction of iNOS by IFN-γ is up-regulated by hypoxia in murine macrophages, and that the iNOS HRE is essential for the up-regulation. In contrast, Hong *et al.* (*19*) have observed that the induction of iNOS by IFN-γ plus LPS is not influenced by hypoxia in rat VSMC. Furthermore, in rat hepatocytes, it has been reported that the IL-1β–induced expression of iNOS is inhibited by hypoxia (*20*). In addition to these contradictions, it has not yet been clarified whether or not the synthesis of tetrahydrobiopterin, a cofactor for iNOS, is regulated by hypoxia.

In the present study, we have examined the iNOS induction by cytokines and LPS in hypoxia in cultured rat VSMC. We have found that IL-1β– or TNF-α–evoked expression of iNOS is greatly enhanced by hypoxia in rat

[*]To whom correspondence should be addressed. Tel/Fax: +81-72-254-9454, Email: inui@server.biochem.osakafu-u.ac.jp

VSMC, although hypoxia does not have any effects on the iNOS induction by IFN-γ plus LPS. The enhanced expression of iNOS in cells stimulated with IL-1β is thought to be physiologically important for elevating cGMP in hypoxia to a level comparable to that in normoxia, since NO production induced by iNOS is affected by a decrease in the concentration of its substrate, O_2, in hypoxic cells. In addition, we also report that the IL-1β–induced synthesis of tetrahydrobiopterin is greatly stimulated by hypoxia in VSMC.

MATERIALS AND METHODS

Materials—Recombinant rat IL-1β and IFN-γ were obtained from Pepro Tech (Rocky Hill, NJ, USA), recombinant TNF-α from Genzyme-Techne (Boston, Mass., USA), LPS (*Escherichia coli* 055:B5) from Difco Laboratories (Detroit, Mich., USA), and rabbit anti-iNOS antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture—VSMC isolated from rat thoracic aortas were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), were fed every other day (*21*). For experiments, cells between passage level of 7 and 12 were seeded into a 35- or 100-mm dish at a density of 10^4 cells/cm^2, and then cultured at 37°C in normoxia (20% O_2/5% CO_2/75% N_2). After reaching confluence, cytokine and/or LPS were added to the medium, and immediately the cells were transferred to hypoxia (2% O_2/5% CO_2/93% N_2).

Western Blotting—Cells in a 100-mm dish were washed twice with ice-cold phosphate-buffered saline, and then lysed with 100 mM HEPES-KOH buffer, pH 7.6, comprising 0.5% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 10 μM leupeptin. After centrifugation at 20,000 ×*g* for 20 min, the supernatant obtained was used as a sample. The sample (10 μg of protein) was subjected to SDS–PAGE (7.5% gel) according to Laemmli (*22*), and proteins in the gel were transferred to a polyvinylidene difluoride membrane by electroblotting. The membrane was treated with anti-iNOS antibodies, and immunoreactive proteins were detected by a chemiluminescent method using a commercial kit (Super Signal CL-HRP, Pierce, Rockford, IL, USA). The protein content was measured according to Bradford (*23*) with bovine serum albumin as a standard.

Northern Blotting—Cells in a 100-mm dish were washed twice with ice-cold phosphate-buffered saline, and then total RNA was extracted with a commercial kit (Sepasol-RNA I; Nacalai Tesque, Kyoto). The total RNA (20 μg) was separated by electrophoresis through a 1% agarose/17.5% formaldehyde gel, and then transferred to a nylon membrane. A fragment (nucleotide positions: 1290–1943) of rat iNOS cDNA (*24*), donated by Dr. Kamitani (Shionogi Pharmacy, Osaka), was labeled with [^{32}P]dCTP and used as a probe. Hybridization was performed with the probe at 42°C for 18 h, and the hybridized probe on the membrane was detected by autoradiography. For control hybridization, a fragment of the rat 18S rRNA gene (*25*), donated by Dr. Iritani (Tezukayama Gakuin College, Sakai), was used as a probe, and the hybridized probe was detected with a commercial kit (AlkPhos Direct, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Determination of NO Production—NO production was determined by a fluorescent method using DAF-2 (Daiichi Kagaku Yakuhin, Tokyo) (*26*, *27*). Cells in a 35-mm dish were exposed to IL-1β or IFN-γ plus LPS in normoxia or hypoxia for 12 h. Then, the medium was changed to 1.5 ml of Hank's solution, supplemented with 20 mM glucose, 0.5 mM arginine and 10 μM DAF-2, which had been equilibrated in 20% O_2/5% CO_2/75% N_2 (for incubation in normoxia) or 2% O_2/5% CO_2/93% N_2 (for incubation in hypoxia), and then the cells were incubated in normoxia or hypoxia for an additional 2 h. After the incubation, the fluorescence intensity of the medium was determined to estimate the amount of NO released from these cells during the 2-h incubation.

Measurement of cGMP—Cells were disrupted with 5% trichloroacetic acid and then centrifuged to obtain a cell extract. After removal of trichloroacetic acid with diethyl ether, the cell extract was evaporated to dryness, dissolved in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, and then used as a sample. cGMP in the sample was measured by means of a radioimmunoassay method using a commercial kit (Cyclic GMP [^{3}H] Assay System, Amersham Pharmacia Biotech).

Measurement of Tetrahydrobiopterin—Cells were disrupted with 0.1 N H_3PO_4, and then centrifuged to obtain a supernatant. Tetrahydrobiopterin in the supernatant was determined by HPLC according to Fukushima and Nixon (*28*).

Statistical Analyses—Statistical analyses were performed with GB-Stat 5.4 (Dynamic Microsystems, Silver Spring, MD, USA). Data were analyzed by two-way ANOVA, and post-hoc analyses were performed with the Scheffé test. All data are presented as means ± SEM, and statistical significance is defined as $p < 0.05$.

RESULTS

iNOS Induction by Cytokines and LPS in Hypoxia in VSMC—VSMC were incubated with various doses of IL-1β in normoxia (20% O_2) for 12 h, and the expression level of iNOS protein was examined by SDS–PAGE followed by immunoblotting with anti-iNOS antibodies. As shown in Fig. 1A, the iNOS protein was induced in response to IL-1β in a dose-dependent manner in the range examined (0–5 ng/ml). In the cells incubated without any cytokines or LPS, even on incubation in hypoxia (2% O_2) for 12 h, induction of the iNOS protein was not observed. However, the IL-1β–induced expression of the iNOS protein was greatly enhanced in hypoxia, compared to in normoxia, with any dose examined. Similar results were also obtained when TNF-α was substituted for IL-1β (Fig. 1B). In addition, the level of iNOS mRNA, determined by Northern blotting, was also substantially higher in hypoxia than in normoxia when the cells were stimulated with either IL-1β or TNF-α (Fig. 2). These results suggest that the induction of iNOS by IL-1β or TNF-α is up-regulated by hypoxia to a transcriptional level in rat VSMC.

As shown in Fig. 1C, the iNOS protein was also induced when VSMC were stimulated with IFN-γ (10 ng/ml) or LPS (10 μg/ml) in normoxia for 12 h, but the level of the induction was obviously lower than that with IL-1β

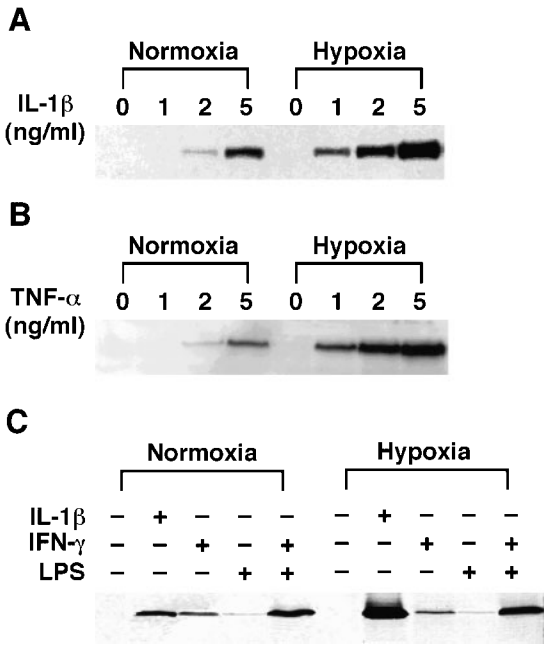

Fig. 1. **Induction of iNOS by cytokines and LPS in VSMC in hypoxia.** Panels A and B: VSMC were incubated with IL-1β (panel A) or TNF-α (panel B) at the concentration indicated under 20% (normoxia) or 2% (hypoxia) O_2 for 12 h. Panel C: VSMC were incubated with IL-1β (5 ng/ml), IFN-γ (10 ng/ml), and/or LPS (10 μg/ml) in normoxia or hypoxia for 12 h. After the incubation, the expression level of the iNOS protein was examined by SDS–PAGE, followed by immunoblotting with anti-iNOS antibodies.

(5 ng/ml). In addition, the iNOS induction by IFN-γ or LPS, in contrast to that by IL-1β, was not up-regulated by hypoxia. As has been reported previously (*29*), a synergistic increase in iNOS expression was observed when IFN-γ and LPS were combined, and the level of the iNOS protein induced by IFN-γ plus LPS was almost the same as that by IL-1β in normoxia. However, the level of the iNOS protein was not enhanced by hypoxia even if IFN-γ and LPS were combined.

Effects of Hypoxia on NO Production and cGMP Elevation—When VSMC were treated with IL-1β in hypoxia, despite the great enhancement of iNOS expression, the level of NO production was almost the same as that in normoxia (Fig. 3). In contrast, when cells treated with IL-

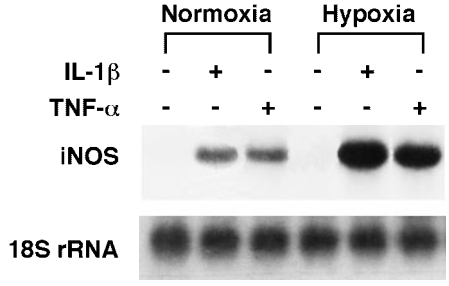

Fig. 2. **iNOS mRNA level in VSMC stimulated by IL-1β or TNF-α in hypoxia.** VSMC were incubated with IL-1β or TNF-α at 5 ng/ml under 20% (normoxia) or 2% (hypoxia) O_2 for 12 h, and then the levels of iNOS mRNA and 18S rRNA were determined by Northern blotting.

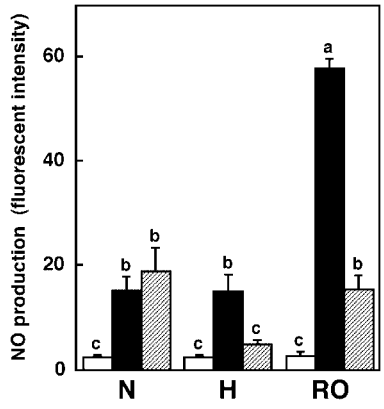

Fig. 3. **NO production induced by cytokines and LPS in hypoxia.** VSMC were stimulated without (open bars) or with IL-1β (5 ng/ml) (solid bars) or IFN-γ (10 ng/ml) plus LPS (10 μg/ml) (shaded bars) under 20% (N) or 2% (H, RO) O_2 conditions for 12 h, and then incubated in a medium containing DAF-2 under 20% (N, RO) or 2% (H) O_2 for 2 h. The amount of NO produced during the 2-h incubation was determined by measuring the fluorescence intensity of the medium. Data are presented as means ± SEM (n = 4). Values with different letters are significantly different, $p < 0.05$. N, normoxia; H, hypoxia; RO, reoxygenation.

1β in hypoxia for 12 h were transferred to normoxic conditions (reoxygenation), a marked increase (about 4-fold; $p < 0.05$) in NO production was observed. NO production was also induced in cells treated with IFN-γ plus LPS in normoxia to a level similar to that in the cells with IL-1β. However, in hypoxia, NO was barely produced in response to stimulation with IFN-γ plus LPS.

The cellular cGMP level was determined after the cells had been incubated with IL-1β under normoxic or hypoxic conditions for 12 h. As shown in Fig. 4, the cGMP level increased in response to IL-1β in hypoxia to a level comparable to that in normoxia.

IL-1β–Stimulated Synthesis of Tetrahydrobiopterin in Hypoxia—To explore whether or not hypoxia affects the IL-1β–stimulated synthesis of tetrahydrobiopterin, the

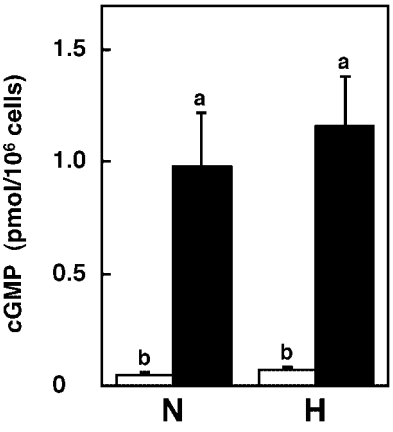

Fig. 4. **Elevation of the cGMP level in response to IL-1β in hypoxia.** VSMC were incubated with (solid bars) or without (open bars) IL-1β (5 ng/ml) under 20% (N) or 2% (H) O_2 for 12 h, and then the cGMP levels in the cells were determined. Data are presented as means ± SEM ($n = 4$). Values with different letters are significantly different, $p < 0.05$. N, normoxia; H, hypoxia.

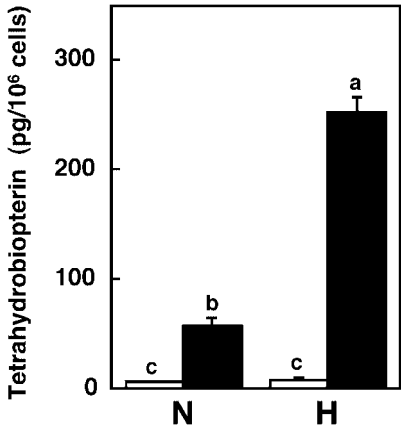

Fig. 5. **Tetrahydrobiopterin content in VSMC stimulated by IL-1β in hypoxia.** VSMC were incubated with (solid bars) or without (open bars) IL-1β (5 ng/ml) under 20% (N) or 2% (H) O_2 for 12 h, and then the contents of tetrahydrobiopterin in the cells were determined. Data are presented as means ± SEM (n = 4). Values with different letters are significantly different, $p < 0.05$. N, normoxia; H, hypoxia.

tetrahydrobiopterin content was determined in cells incubated with the cytokine in normoxia or hypoxia for 12 h. As shown in Fig. 5, a significant increase in the tetrahydrobiopterin content was induced by IL-1β in hypoxia as well as in normoxia. Furthermore, the extent of the increase in hypoxia was significantly greater than that in normoxia.

DISCUSSION

It is well known that LPS and cytokines, such as IL-1β, TNF-α, and IFN-γ, induce iNOS in certain kinds of cells, including macrophages and VSMC, and that the induction is synergistically augmented by their combinations (*6*, *7*, *16*). The promoter of the iNOS gene contains numerous potential sites for the binding of a number of different kinds of transcriptional factors (*7*, *30*–*32*). The consensus sequence for the binding of interferon regulatory factor-1 is required for the transcriptional activation of iNOS by IFN-γ. In contrast, the activation of NF-κB is crucial to the iNOS induction on treatment with IL-1β, TNF-α or LPS.

Melillo *et al.* (*18*) have reported that the IFN-γ-induced expression of iNOS is enhanced in hypoxia, compared to in normoxia, in murine macrophages. They have also found a HRE in the murine iNOS promoter, and suggested that the binding of HIF-1 to the iNOS HRE is essential for the enhancement of IFN-γ–induced expression of iNOS in hypoxia in these cells (*15*, *18*). In rat VSMC, the IL-1β– or TNF-α–evoked expression of iNOS was greatly enhanced by hypoxia (Figs. 1 and 2). It is thus reasonable to postulate that the transcription of the iNOS gene induced by IL-1β or TNF-α through the activation of NF-κB is up-regulated in VSMC when HIF-1 binds to its promoter region. The rat iNOS gene, as well as the murine one, has been reported to have a HRE in its 5′-flanking region (*17*). However, the induction of iNOS by IFN-γ, LPS or their combination was not influenced by hypoxia in rat VSMC (Fig. 1), as had been observed previously (*19*). Thus, it seems likely that in VSMC, in contrast to in macrophages, the binding of HIF-1 to the iNOS promoter does not have any effects on the transcriptional activation of iNOS through interferon regulatory factor-1. In addition, it appears that the mechanism by which NF-κB activates the iNOS promoter in response to LPS is different from that operative for IL-1β or TNF-α in VSMC. This notion is supported by previous reports that there are two NF-κB binding sites in the iNOS gene, and that the downstream site is important in mediating the effect of LPS in macrophages, whereas the upstream one is crucial for the transcriptional activation by IL-1β or TNF-α in VSMC (*7*, *17*, *30*, *32*).

Therefore, these results obtained in the present study show that the induction of iNOS is regulated by hypoxia in different manners, depending on the stimuli in VSMC. In addition, taking our present data and results reported previously for other types of cells together (*15*, *18*, *20*), it is also suggested that iNOS induction is differently regulated by hypoxia in various kinds of cells even if the same cytokine is used as an inducer. However, the detailed mechanisms by which these differences occur remain unclear.

Since iNOS requires molecular O_2, in addition to arginine and NADPH, as a substrate (*6*, *16*), the amount of NO produced through the action of iNOS decreases in cells under low O_2 conditions (*18*). Indeed, when rat VSMC were stimulated by IFN-γ plus LPS, the NO production was significantly lower in hypoxia than in normoxia (Fig. 3), although the level of expression of the iNOS protein was barely different between the cells in normoxia and hypoxia (Fig. 1). In contrast, the iNOS induction by IL-1β was up-regulated by hypoxia, and NO was produced in response to IL-1β in hypoxia to a level comparable to that in normoxia. Thus, it seems that in rat VSMC stimulated with IL-1β in hypoxia the decrease in the iNOS activity due to lowering of the concentration of its substrate, O_2, is compensated for by an increase in its protein content. When cells stimulated with IL-1β in hypoxia were exposed to normoxic conditions (reoxygenation), the amount of NO produced was greatly increased, confirming that the iNOS protein induced by IL-1β in hypoxia is fully active if a sufficient level of O_2 is provided as a substrate.

NO produced through the action of iNOS activates soluble guanylate cyclase, and the activation results in elevation of the cellular cGMP level in VSMC (*4*, *6*, *8*). Reflecting the level of NO production, the elevated level of cGMP in response to IL-1β in hypoxia was comparable to that in normoxia (Fig. 4). Thus, it seems likely that the IL-1β-induced expression of iNOS is enhanced in hypoxia, compared to that in normoxia, to elevate cGMP to an adequate level through the action of NO in VSMC, since the NO production induced by iNOS is limited by the decrease in the concentration of its substrate, O_2, in the hypoxic cells.

Tetrahydrobiopterin is an essential cofactor for iNOS as well as other types of NO synthase (*4*). Together with the induction of iNOS, GTP cyclohydrolase I, which is the first and rate-limiting enzyme in the biosynthesis of biopterin, is induced by LPS and cytokines in certain types of cells including VSMC, and consequently the content of tetrahydrobiopterin in these cells increases (*9*–

11). The amount of tetrahydrobiopterin synthesized in response to IL-1β was significantly greater in hypoxia than in normoxia in rat VSMC (Fig. 5). These results suggest that the IL-1β–induced synthesis of tetrahydrobiopterin is stimulated by hypoxia to support the enhanced expression of the iNOS protein. Thus, it appears that the IL-1β–induced expression of GTP cyclohydrolase I, as well as iNOS, is up-regulated by hypoxia in VSMC; however, the details remain obscure. As far as we know, this is the first report showing that the cytokine-induced synthesis of biopterin is regulated by hypoxia.

We are grateful to Dr. Kamitani (Shionogi Pharmacy, Osaka) for the gift of rat iNOS cDNA, Dr. Iritani (Tezukayama Gakuin College, Sakai) for the gift of a fragment of the rat 18S rRNA gene, and Dr. Ichinose (Fujita Health University, Toyoake) for his advice regarding the measurement of tetrahydrobiopterin.

REFERENCES

1. Murad, F. (1986) Cyclic guanosine monophosphate as a mediator of vasodilatation. *J. Clin. Invest.* **78**, 1–5
2. Lee, Y.-C., Martin, E., and Murad, F. (2000) Human recombinant soluble guanylate cyclase: expression, purification, and regulation. *Proc. Natl Acad. Sci. USA* **97**, 10763–10768
3. Palmer, R.M.J., Ashton, D.S., and Moncada, S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333**, 664–666
4. Moncada, S., Palmer, R.M.J., and Higgs, E.A. (1991) Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**, 109–142
5. Busse, R. and Mulsch, A. (1990) Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.* **275**, 87–90
6. Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6**, 3051–3064
7. Spink, J., Cohen, J., and Evans, T.J. (1995) The cytokine responsive vascular smooth muscle cell enhancer of inducible nitric oxide synthase. *J. Biol. Chem.* **270**, 29541–29547
8. Beasley, D. (1990) Interleukin 1 and endotoxin activate soluble guanylate cyclase in vascular smooth muscle cells. *Am. J. Physiol.* **259**, R38–R44
9. Hattori, Y. and Gross, S.S. (1993) GTP cyclohydrolase I mRNA is induced by LPS in vascular smooth muscle; characterization, sequence and relationship to nitric oxide synthase. *Biochem. Biophys. Res. Commun.* **195**, 435–441
10. Togari, A., Arai, M., Mogi, M., Kondo, A., and Nagatsu, T. (1998) Coexpression of GTP cyclohydrolase I and inducible nitric oxide synthase mRNAs in mouse osteoblastic cells activated by proinflammatory cytokines. *FEBS Lett.* **428**, 212–216
11. Vann, L.R., Twitty, S., Spiegel, S., and Milstein, S. (2000) Divergence in regulation of nitric-oxide synthase and its cofactor tetrahydrobiopterin by tumor necrosis factor-α. Ceramide potentiates nitric oxide synthesis without affecting GTP cyclohydrolase I activity. *J. Biol. Chem.* **275**, 13275–13281
12. Bunn, H.F. and Poyton, R.O. (1996) Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76**, 839–885
13. Wang, G.L. and Semenza, G.L. (1993) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc. Natl Acad. Sci. USA* **90**, 4304–4308
14. Wenger, R.H. and Gassmann, M. (1997) Oxygen(es) and the hypoxia-inducible factor-1. *Biol. Chem.* **378**, 609–616
15. Melillo, G., Musso, T., Sica, A., Taylor, L.S., Cox, G.W., and Varesio, L. (1995) A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *J. Exp. Med.* **182**, 1683–1693
16. MacMicking, J., Xie, Q.W., and Nathan, C. (1997) Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15**, 323–350
17. Keinanen, R., Vartiainen, N., and Koistinaho, J. (1999) Molecular cloning and characterization of the rat inducible nitric oxide synthase (iNOS) gene. *Gene* **234**, 297–305
18. Melillo, G., Taylor, L.S., Brooks, A., Cox, G.W., and Varesio, L. (1996) Regulation of inducible nitric oxide synthase expression in IFN-γ-treated macrophages cultured under hypoxic conditions. *J. Immunol.* **157**, 2638–2644
19. Hong, Y., Suzuki, S., Yatoh, S., Mizutani, M., Nakajima, T., Bannai, S., Sata, H., Soma, M., Okuda, Y., and Yamada, N. (2000) Effect of hypoxia on nitric oxide production and its synthase gene expression in rat smooth muscle cells. *Biochem. Biophys. Res. Commun.* **268**, 329–332
20. Inoue, T., Kwon, A.H., Oda, M., Kaibori, M., Kamiyama, Y., Nishizawa, M., Ito, S., and Okumura, T. (2000) Hypoxia and heat inhibit inducible nitric oxide synthase gene expression by different mechanisms in rat hepatocytes. *Hepatology* **32**, 1037–1044
21. Inui, H., Kitami, Y., Kondo, T., and Inagami, T. (1993) Transduction of mitogenic activity of platelet-derived growth factor (PDGF) AB by PDGF-β receptor without participation of PDGF-α receptor in vascular smooth muscle cells. *J. Biol. Chem.* **268**, 17045–17050
22. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
23. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein dye-binding. *Anal. Biochem.* **72**, 248–254
24. Nunokawa, Y., Ishida, N., and Tanaka, S. (1993) Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **191**, 89–94
25. Iritani, N., Fukuda, H., and Tada, K. (1996) Nutritional regulation of lipogenic enzyme expression in rat epididymal adipose tissue. *J. Biochem.* **120**, 242–248
26. Kojima, H., Nakatsubo, N., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H., Hirata, Y., and Nagano, T. (1998) Detection and imagining of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal. Chem.* **70**, 2446–2453
27. Nakatsubo, N., Kojima, H., Kikuchi, K., Nagoshi, H., Hirata, Y., Maeda, D., Imai, Y., Irimura, T., and Nagano, T. (1998) Direct evidence of nitric oxide production from bovine aortic endothelial cells using new fluorescence indicators: diaminofluoresceins. *FEBS Lett.* **427**, 263–266
28. Fukushima, T. and Nixon, J. (1980) Analysis of reduced forms of biopterin in biological tissues and fluids. *Anal. Biochem.* **102**, 176–188
29. Sirsjö, A., Söderkvist, P., Sundqvist, T., Carlsson, M., Öst, M., and Gidlöf, A. (1994) Different induction mechanisms of mRNA for inducible nitric oxide synthase in rat smooth muscle cells in culture and in aortic strips. *FEBS Lett.* **338**, 191–196
30. Xie, Q.W., Whisnant, R., and Nathan, C. (1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J. Exp. Med.* **177**, 1779–1784
31. Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W., and Murphy, W.J. (1993) Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon γ and lipopolysaccharide. *Proc. Natl Acad. Sci. USA* **90**, 9730–9734
32. Xie, Q.W., Kashiwabara, Y., and Nathan, C. (1994) Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269**, 4705–4708