Biochemistry (Moscow), Vol. 65, No. 12, 2000, pp. 1376-1379. Translated from Biokhimiya, Vol. 65, No. 12, 2000, pp. 1634-1638.

Comparative Study of Induction of iNOS mRNA Expression in Vascular Cells of Different Species

J.-K. Wen* and M. Han

Department of Biochemistry and Molecular Biology, Hebei Medical University, Shijiazhuang 050017, People's Republic of China; fax: +86-311-6048177; E-mail: wjk@hebmu.edu.cn

Received March 13, 2000
Revision received May 26, 2000

Abstract—To determine the difference in induction of inducible nitric oxide synthase (iNOS) mRNA expression in cultured vascular cells of different species, the expression of iNOS genes and their regulatory mechanisms in rat, human, bovine, and rabbit vascular endothelial cells and smooth muscle cells (SMC) were studied by Northern blotting, chloramphenicol acetyltransferase (CAT) assay, and electrophoretic mobility shift assay (EMSA). Qualitative estimation of iNOS mRNA by Northern-blot analysis demonstrated that the combination of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and lipopolysaccharides (LPS) drastically induces iNOS expression in rat and human SMC, and a more moderate effect was observed for endothelial cells; the effect of IL-1β alone was much weaker than that of the three factors. IL-1β alone or a mixture of IL-1β, TNF-α, and LPS both showed negligible effect on iNOS expression in bovine and rabbit vascular endothelial cells and SMC. Results of CAT assay corresponded well with Northern analysis indicating 7-fold increase in CAT activity by the mixture of IL-1β, TNF-α, and LPS in SMC and more moderate, 2-fold increase, in endothelial cells. IL-1β alone produced an intermediate effect (less than 2-fold) on vascular SMC of rats and humans. The results of EMSA showed that two shifted bands appeared when the nuclear protein from rat and human vascular endothelial cells bound to the region from −1037 to −787 of the rat iNOS gene, while vascular SMC nuclear protein only produced a single shifted band under the same conditions. These results suggest that cell- and species-specific mechanisms exist in the induction of iNOS expression.

Key words: iNOS gene, expression regulation, species, vascular cell, cytokine, endotoxin

Nitric oxide (NO) is a potent vasodilator. It can be generated by at least two types of nitric oxide synthase (NOS) in vascular cells (see [1-3] for reviews). A constitutively expressed enzyme is present predominantly in healthy endothelial cells. The release of NO by this type of enzyme is necessary to maintain normal vascular tone. In addition, an isoform of NOS can be induced by certain inflammatory mediators (interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and endotoxin) in most types of vascular cells. However, it is not clear whether iNOS gene expression in response to specific cytokines is regulated identically in vascular endothelial cells and vascular smooth muscle cells (SMC) of the same species and in the same cell types in different species [4, 5]. In the present study, the expression of iNOS genes and their regulatory mechanisms by inflammatory mediators in human, rat, bovine, and rabbit vascular endothelial cells and vascular SMC were investigated by determining iNOS mRNA level, the activity of the specific transcriptional factor, and the ability of iNOS gene promoter to direct reporter expression in each cell type.

Abbreviations: NOS) nitric oxide synthase; iNOS) inducible NOS; IL-1β) interleukin-1β; TNF-α) tumor necrosis factor-α; IFN) interferon; LPS) lipopolysaccharides; EC) endothelial cells; SMC) smooth muscle cell; CAT) chloramphenicol acetyltransferase; EMSA) electrophoretic mobility shift assay.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials. All cell culture reagents were obtained from GIBCO-BRL. Chloramphenicol, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), lipopolysaccharides (LPS, *E. coli* 055:B_5), and type IV collagenase were purchased from Sigma (USA). [α-^{32}P]dCTP and [α-^{32}P]dATP (3000 Ci/mmol) were supplied by DuPont NEN (USA). [^{3}H]Acetyl CoA and nylon nucleic acid transfer membrane (Hybond) were purchased from Amersham Corp. (England). TNF-α and IL-1β were from Boehringer Mannheim (Germany), and restriction endonucleases, phage T_4 DNA polymerase, and Klenow fragment were from Promega (USA). The rat iNOS cDNA fragment in pUC19 was generously provided by Dr. Takashi Osumi (Himeji Institute of Technology, Hyogo, Japan).

0006-2979/00/6512-1376$25.00

Cell culture. Vascular SMC and endothelial cells were derived by enzymatic digestion of segments of artery or vein from a variety of species—human, rat, bovine, and rabbit—by standard procedures [6, 7]. Human endothelial cells were harvested from umbilical vein, and the other endothelial cells were from aorta by digestion with collagenase and dispase. Human, rat, bovine, and rabbit vascular SMC were isolated and cultured as described previously [8]. Endothelial cells were cultured in endothelial cell growth media (EGM) supplemented with 20% fetal bovine serum at 37°C in 5% CO_2. Vascular SMC were cultured in M199 with 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. For all experiments, cells of less than six passages were used.

Northern-blot analysis of iNOS mRNA. The two types of cells were grown to 80% confluence and washed with Hanks' balanced salt solution before incubation for 12 h in fresh medium 199 or EGM containing 10 ng/ml IL-1β, 25 ng/ml TNF-α, and 30 μg/ml LPS. Total cellular RNA was isolated using guanidine isothiocyanate [9]. Equal amounts of RNA (25 μg) from each preparation, including the control of each cell type, were subjected to electrophoresis in 1% agarose gels and transferred overnight onto Hybond nylon membranes by capillary action. The labeled iNOS probe, with [α-^{32}P]dCTP by random oligonucleotide-primed synthesis, was incubated with the nylon membranes at 42°C in 5× SSPE, 5× Denhardt's solution, 50% formamide, 0.1% SDS, and 100 μg/ml salmon sperm DNA for 24 h. The membranes were rinsed twice with 5× SSPE, 0.5% SDS at room temperature, following by two washes with 0.1× SSPE, 1% SDS for 10 min each at 65°C prior to autoradiography.

Transfection of vascular cells and determination of CAT activity in transfected cells. The rat iNOS promoter (nucleotides −1037 to −438) was liberated as a HindIII/PstI fragment from the rat iNOS gene 5′-flanking region (J. K. Wen and M. Han, unpublished data) and ligated into pCAT-basic (Promega) to create pNOS-CAT.

Cells of the different species were grown to 90% confluence in 60-mm tissue culture Petri dishes and transfected with 10 μg of pNOS-CAT by the calcium phosphate coprecipitation method as described by Wigler et al. [10]. IL-1β, TNF-α, and LPS were added, as described above, to the medium 36 h after transfection, plates were incubated at 37°C for another 12 h, and then the cells were harvested. In each experiment, an expression vector containing the β-galactosidase gene, pCMVβ, was cotransfected as an internal control. β-Galactosidase was assayed as described by Sambrook et al. [11].

The CAT content of cell lysates was assayed by a liquid scintillation counting-based assay (Promega) as described in the manufacturer's protocol. Values from control reactions were subtracted from all results. Determinations were standardized by protein content and β-galactosidase activity of each sample. Results are expressed as the *n*-fold increase in CAT activity following the specified cytokine treatment.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA). Nuclear extracts of various cells were prepared 3 h after exposure to IL-1β, TNF-α, and LPS, as described above, according to Spink et al. [12], in the presence of leupeptin (10 μg/ml), antipain (5 μg/ml), pepstatin (5 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). Protein concentrations of nuclear extracts were determined according to Bradford [13]. The 5′-flanking region of the rat iNOS gene (nucleotides −1037 to −787), containing TNF-α-responsive elements, IFN-γ-responsive elements, NF-κB DNA-binding consensus sequences, and IFN-α-stimulated responsive elements was liberated as a EcoRI/Sau3AI fragment. The resultant cohesive ends were in-filled with Klenow fragment using [α-^{32}P]dATP as the label. The probe was resuspended in TE buffer.

The binding reactions were performed at room temperature in a volume of 20 μl containing 10% glycerol, 25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1 M NaCl, 2 mg/ml poly(dI-dC), 1 μg/ml pepstatin, antipain, and aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg nuclear extract. A preincubation of 5 min was allowed prior to adding 0.5 μl of the labeled DNA probe, and the incubation was continued for a further 20 min. DNA–protein complexes were separated on native 5% polyacrylamide gels in 0.5× Tris borate buffer containing EDTA.

RESULTS

The iNOS gene in vascular cells of different species exhibits markedly distinct expression activity. Under normal circumstances, cultured rat, human, bovine, and rabbit vascular endothelial cells and vascular SMC possessed no detectable iNOS mRNA. As shown in Fig. 1, stimulation with IL-1β alone as well as combined administration with IL-1β, TNF-α, and LPS could markedly induce iNOS expression in rat and human vascular endothelial cells and vascular SMC. The effect of a single factor on iNOS expression, however, was weaker than that of the three inflammatory mediators combined. Furthermore, under the same conditions, the induction of iNOS expression in rat and human vascular SMC was much higher than in the corresponding vascular endothelial cells. Whether IL-1β alone or a mixture of IL-1β, TNF-α, and LPS hardly stimulated iNOS expression in bovine and rabbit vascular endothelial cells and vascular SMC (results not shown).

Significant differences in transcriptional regulation of iNOS gene exist in vascular cells of different species. As shown in Fig. 2, after rat and human vascular SMC transfected with the pNOS-CAT were exposed to IL-1β alone, CAT activity increased 4-fold, and it reached 7-fold in

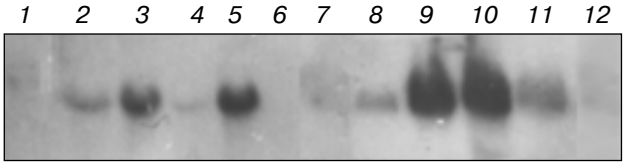

Fig. 1. Northern-blot analysis of iNOS mRNA in vascular cells of different species following stimulation with inflammatory mediators. EC, vascular endothelial cells; com., combination of IL-1β + TNF-α + LPS. *1*) Rat EC; *2*) rat EC + IL-1β; *3*) rat EC + com.; *4*) human EC + IL-1β; *5*) human EC + com.; *6*) human EC; *7*) rat SMC; *8*) rat SMC + IL-1β; 9) rat SMC + com.; *10*) human SMC + com.; *11*) human SMC + IL-1β; *12*) human SMC.

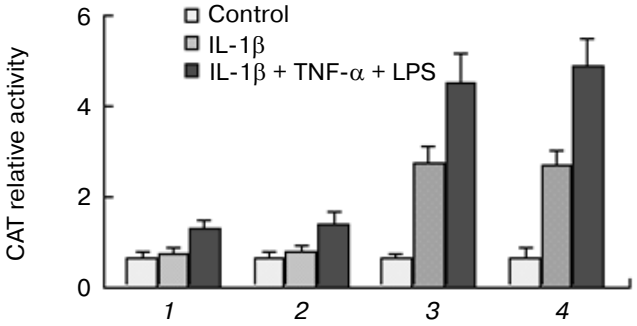

Fig. 2. Induction of CAT activity by IL-1β and IL-1β + TNF-α + LPS in vascular cells transfected with pNOS-CAT: *1*) rat EC; *2*) human EC; *3*) rat SMC; *4*) human SMC.

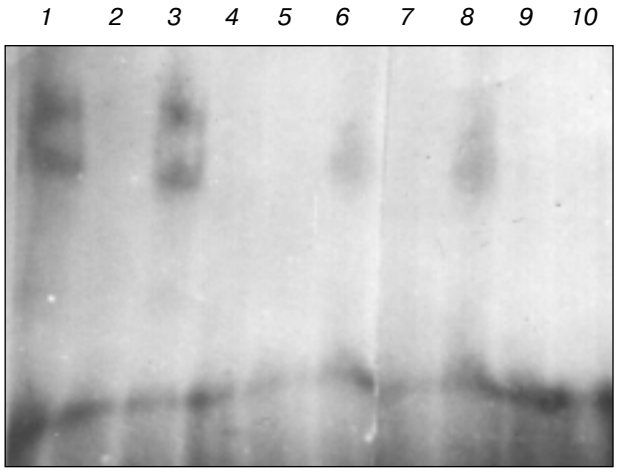

Fig. 3. Effect of IL-1β + TNF-α + LPS on DNA-binding activity of nuclear factors in vascular cells of different species: *1*) rat EC+ IL-1β + TNF-α + LPS; *2*) rat EC (control); *3*) human EC + IL-1β + TNF-α + LPS; *4*) human EC (control); *5*) rat SMC (control); *6*) rat SMC+ IL-1β + TNF-α + LPS; *7*) human SMC (control); *8*) human SMC+ IL-1β + TNF-α + LPS; *9*) same sample as for lane *8* incubated with 50-fold excess of specific competitive polynucleotide; *10*) 100-fold excess of specific competitive polynucleotide.

these two types of SMC stimulated by the combination of IL-1β, TNF-α, and LPS. The three inflammatory mediators could induce CAT to 2-fold over basal levels, but IL-1β alone was ineffective in rat and human vascular endothelial cells. However, a combination of IL-1β, TNF-α, and LPS had no effect on CAT activity in bovine and rabbit vascular cells (data not shown).

EMSA demonstrated that the nuclear extracts from human and rat vascular cells treated with a mixture of IL-1β, TNF-α, and LPS could bind to the 5′-flanking region of the rat iNOS gene (nucleotides −1037 to −787). As shown in Fig. 3, two shifted bands appeared when human and rat vascular endothelial cell nuclear extracts bound to the probe, whereas SMC nuclear proteins only produced one shifted band under the same conditions. These shifted bands could be abolished in the presence of 50-fold molar excess of unlabeled specific competitor, suggesting that both human and rat vascular SMC and endothelial cell nuclear extracts contain some protein component(s) that could specifically bind to a 250-base pair region extending from position −1037 to −787 of the rat iNOS gene.

DISCUSSION

Previous work has demonstrated that certain inflammatory mediators can stimulate the expression of the iNOS gene in most types of cell present in the blood vessel wall [1, 2]. Until now, however, iNOS induction in vascular endothelial cells and vascular SMC has mostly been studied in rats [14]. Little is known about whether there are differences in the responses of vascular cells of different species to inflammatory mediators [4, 15]. In the present study, we simultaneously compared iNOS induction in human, rat, bovine, and rabbit vascular endothelial cells and vascular SMC and provided evidence that iNOS expression is regulated differently in rat and human vascular SMCs stimulated with IL-1β, TNF-α, and LPS compared to their endothelial cells stimulated with the same factors (summarized in table).

Under our experimental conditions, bovine and rabbit vascular cells barely generated significant amounts of iNOS mRNA in response to the mixture of the three inflammatory mediators. Thus, we speculate that cell- and species-specific mechanisms exist in the regulation of iNOS expression.

We performed transfection studies with a cloned 5′ region of the rat iNOS gene (pNOS-CAT), which contains multiple consensus sites for transcription factors, including the NF-κB site, TNF-α responsive element, IFN-γ response element, and IFN-α-stimulated responsive element. The results indicate that induction of CAT enzyme by the inflammatory mediators in transfected vascular cells also reveal cell- and species-specific differences. To explore the molecular mechanisms contributing

Effect of IL-1β, TNF-α, and LPS on iNOS mRNA level, CAT activity, and nuclear factors

Method and conditions	Rat SMC	Human SMC	Rat EC	Human EC
RNA				
IL-1β	+	+	+	+
Combination	++++	++++	+++	+++
EMSA				
Combination	1 band	1 band	2 bands	2 bands
CAT				
IL-1β	++	++	—	—
Combination	+++	+++	+	+

Note: EC, vascular endothelial cells; combination, a combination of IL-1β, TNF-α, and LPS.

to these differences, we observed the induction of binding activities of nuclear factors in EMSA with the upstream sequence of the rat iNOS gene (nucleotides −1037 to −787) when the cells were stimulated with a mixture of IL-1β, TNF-α, and LPS. The results of EMSA revealed that these mediators could induce novel binding activities in human and rat but not bovine and rabbit vascular endothelial cells and SMC. According to the binding pattern of nuclear factors with the probe in EMSA, nuclear factors activated by the inflammatory mediators and/or the interaction of nuclear factors with *cis*-acting elements might be different between vascular endothelial cells and vascular SMC. The degree of expression of the iNOS gene is dependent on the interactions of its regulatory regions with nuclear factors. Therefore, heterogeneity of nuclear factors is probably the molecular basis for the difference in induction of iNOS mRNA expression in vascular cells of different species. In summary, regulation of iNOS expression involves complex interactions of nuclear factors with *cis*-acting elements. Understanding the precise mechanisms of vascular iNOS induction may be helpful in the development of new therapeutic strategies for some vascular diseases.

This project was supported by the National Natural Science Foundation of China.

REFERENCES

1. Schini-kerth, V. B., and Vanhoutte, P. M. (1995) *Exp. Physiol.*, **80**, 885-905.
2. Bredt, D. S., and Snyder, S. H. (1994) *Annu. Rev. Biochem.*, **63**, 175-195.
3. Forstermann, U., Gath, I., Schwarz, P., Gloss, E. I., and Kleinert, H. (1995) *Biochem. Pharmacol.*, **50**, 1321-1332.
4. Yan, Z. Q., and Hansson, G. K. (1998) *Circ. Res.*, **82**, 21-29.
5. Brahmajothi, M. V., and Campbell, D. L. (1999) *Circ. Res.*, **85**, 575-587.
6. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) *J. Clin. Invest.*, **52**, 2745-2756.
7. Lynch, C. M., Clowes, M. M., Osborne, W. R., Clowes, A. W., and Miller, A. D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 1138-1142.
8. Gunther, S., Alexander, R. W., Atkinson, W. J., and Gimbrone, M. A. (1982) *J. Cell. Biol.*, **92**, 289-298.
9. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.*, **162**, 156-159.
10. Wigler, M., Silverstein, S., Lee, L. S., Pellicer, A., Cheng, Y. C., and Axel, R. (1977) *Cell*, **11**, 223-232.
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 1666-1667.
12. Spink, J., Cohen, J., and Evans, T. J. (1995) *J. Biol. Chem.*, **270**, 29541-29547.
13. Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248-254.
14. Kinugawa, K., Shimizu, T., Yao, A., Kohmoto, O., Serizawa, T., and Takahashi, T. (1997) *Circ. Res.*, **81**, 911-921.
15. Cernadas, M. R., Miguel, L. S., García-Durán, M., González-Fernández, F., Millás, I., Montón, M., Rodrigo, J., Rico, L., Fernández, P., Frutos, T., Rodríguez-Feo, J. A., Guerra, J., Caramelo, C., Casado, S., and López-Farré, A. (1998) *Circ. Res.*, **83**, 279-286.