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CLONING OF INDUCIBLE NITRIC OXIDE SYNTHASE IN RAT VASCULAR SMOOTH MUSCLE CELLS+

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SUMMARY: We previously showed that interferon(IFN)-γ inhibited the proliferation of rat vascular smooth muscle cells(VSMC) by generation of nitric oxide(NO) through the induction of an NO synthase(NOS). To identify the NOS in the VSMC at molecular level, we analyzed messenger RNA(mRNA) levels and primary structure of the novel NOS by cDNA cloning with application of polymerase chain reaction(PCR). mRNA of the NOS was induced and the level of induction was significantly increased by IFN-γ in VSMC within a few hours. The amino acid sequence deduced from the cloned NOS cDNA was distinct from that of the previously reported constitutive types of NOSs, while highly similar to that of macrophage NOS. Cofactor binding regions were highly conserved among these NOSs. These findings show that the NOS is inducible and could regulate the proliferation of VSMC. Besides, this is the first report of cloning of the NOS in VSMC.

Nitric oxide (NO) is a recently identified messenger molecule with diverse functions throughout the body (1). In the brain and nervous system, NO displays many properties of a neurotransmitter (2). NO is responsible for endothelial derived relaxing factor activity regulating blood pressure (3). In macrophage cells, NO mediates tumoricidal and bactericidal actions (4). NO is synthesized from L-arginine by NO synthase (NOS). NOSs exist as constitutive enzymes in brain and endothelial cells (5-7). The constitutive NOSs known so far are Ca^{2+}/calmodulin dependent and the rapid increase in the enzyme activity in response to activation of specific cell receptors, such as N-methyl-D-aspartate receptor, is not dependent on new protein synthesis (8). On the other hand, NOS in macrophage cells (mac-NOS) is induced by endotoxin and/or cytokines such as interferon (IFN)-γ, and the activation of NOS occurs over several hours (9, 10).

+Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under Accession No. D14051.

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Abbreviations: NO, nitric oxide; NOS, NO synthase; IFN, interferon; VSMC, vascular smooth muscle cells; RT, reverse transcription; PCR, polymerase chain reaction; mRNA, messenger RNA; M-MLV, Moloney murine leukemia virus; RACE, rapid amplification of cDNA ends; FMN, flavin mononucleotide; FAD-PPi, flavin adenine dinucleotide pyrophosphate; FAD-ISO, flavin adenine dinucleotide isoalloxazine; NADPH-ribose, nicotinamide adenine dinucleotide phosphate ribose; NADPH-Ade, nicotinamide adenine dinucleotide phosphate adenine; bp, base pairs.

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Recently NOS was shown to be induced in vascular smooth muscle cells (VSMC) also, in a manner similar to that in macrophage cells (11). Previously, we showed that NOS induced by IFN-γ was involved in the inhibition of the proliferation of VSMC (12). However, the molecular characterization of the NOS in the VSMC (VSM-NOS) had not been done. In this communication, we characterized the VSM-NOS not only by reverse transcription (RT)-polymerase chain reaction (PCR) and RNA blot analysis of its messenger RNA (mRNA) levels but also by molecular cloning of the cDNA.

MATERIALS AND METHODS

Analysis of Messenger RNA Levels for VSM-NOS by RT-PCR: RNA was prepared by ISOGEN (Nippon Gene, Tokyo, Japan) (13) from rat VSMC cultured in Dulbecco's modified Eagle's medium (Life Technologies Inc. MD, U.S.A.) supplemented with 10 % fetal bovine serum and 1000 U/ml of IFN-γ (Holland Biotechnology, Netherlands) for the indicated periods(see Figure 1). RT-PCR was performed as described before (14). Briefly, the first strand cDNA was synthesized using an oligo$(dT)_{12-18}$ primer and M-MLV reverse transcriptase (Life Technologies Inc. MD, U.S.A.) followed by PCR with gene specific primers whose sequence corresponded to the amino acid residues 1-8 (forward primer, YN017) and 265-273 (reverse primer, YN056) of the murine mac-NOS 5'-region (9, 10). PCR amplification was performed using GeneAmp DNA amplification reagent kit (Takara, Kyoto, Japan) according to the following schedule: Denaturation, annealing and elongation at 94, 55, and 72 °C for 1, 1, and 1.5 minutes, respectively, for 25 cycles. To confirm the change in the VSM-NOS mRNA level, the β-actin mRNA level using Amprimer set for RT-PCR (Clontech laboratories, Inc. CA, U.S.A.) was used as a control. The PCR products were electrophoresed on a 0.8 % agarose gel containing ethidium bromide.

RNA Blot Hybridization Analysis: Total RNA was isolated from rat VSMC cultured for 12 hours with or without IFN-γ. RNA blot hybridization analysis was performed as described (15). The cDNA probe used was RT-PCR product amplified with YN017 and YN056 primers.

3'-RACE Procedure to Prepare 3'VSM-NOS cDNA Probe: To obtain the initial information of the 3'-coding region of VSM-NOS, RNA was prepared from VSMC cultured with 1000 U/ml of IFN-γ for 12 hours and applied to 3'-RACE procedure(16). First, RNA was applied to single strand cDNA synthesis by using reverse transcriptase and oligo$(dT)_{17}$ adaptor primer (5'-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3', Life Technologies Inc. MD, U.S.A.). Next, the cDNA was amplified by the gene specific primer, which was designed from murine mac-NOS (9, 10) region corresponded to amino acid residues 855-864 (forward primer, YN057), and universal adapter primer (reverse primer, YN058). PCR amplification was performed as described before except for the schedule: Denaturation, annealing and elongation at 94, 55, and 72 °C for 1, 2, and 3 minutes, respectively, for 30 cycles.

Isolation and Sequencing of cDNA Clone Encoding VSM-NOS: The PCR products obtained above were sequenced directly with an Applied Biosystems model 373A DNA sequencer and Dye Deoxy Terminator kit (Applied Biosystems, U.S.A.). The 3'-terminal sequence primer (YN086) obtained from 3'-RACE was applied to construction of cDNA from mRNA prepared from VSMC cultured with 1000 U/ml of IFN-γ for 12 hours. YN086-primed cDNA was ligated with an EcoRI/Not I adaptor and introduced into the EcoRI site of a pCRII (Invitrogen, SD, U.S.A.) plasmid. Positive clones were selected with PCR using gene specific primers (YN017 and YN056) and sequenced the inserted cDNA fragments as described above.

Design of Primers: Sequences utilized for the experiments were as follows:
YN017: 5'-ATGGCTTGCCCCTGGAAGTTTCTC-3' YN086: 5'-AGATGCTGTAACTCTTCTGGGTGTCA-3'
YN058: 5'-GGCCACGCGTCGACTAGTAC-3' YN056: 5'-CCTCTGATGGTGCCATCGGGCATCTG-3'
YN057: 5'-CCTTGTGTCAGCCCTCAGAGTACAATGA-3'

RESULTS AND DISCUSSION

In previous study, we observed induction of NOS by IFN-γ in VSMC (12). To analyze the induction at the mRNA level, we carried out RT-PCR analysis on the NOS. For VSM-NOS has inducible nature, we assumed that the VSM-NOS would have significantly high similarity to murine mac-NOS (9, 10), which is only inducible NOS isozyme so far cloned to our knowledge. We designed RT-PCR primers YN017 and YN056 based on the sequence of murine mac-NOS. These primers were located in the 5'-terminal in murine mac-NOS (corresponded to the 5'-terminal of the VSM-NOS). The DNA fragment of about 830 bp was amplified by the RT-PCR (Figure 1A). The nucleotide sequence of the DNA fragment partially sequenced revealed that it had a unique sequence but was highly similar to murine mac-NOS. The results indicate that the efficiency of RT-PCR using YN017 and YN056 primers works as an indicator for mRNA levels of VSM-NOS. As shown in Figure 1A, mRNA level of the VSM-NOS was maximal 6 hour after IFN-γ treatment and then decreased. In this experiment, no VSM-NOS mRNA was detected in the VSMC at basal level. Even though mRNA level of the VSM-NOS was decreased after 6 hour, the enzyme activity is thought to be maximal 24 hour after IFN-γ treatment(12).

To investigate primary structure of VSM-NOS, we first, performed 3'-RACE procedure. The first strand cDNA template was synthesized with an oligo$(dT)_{17}$ adaptor primer (5'-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3'). The gene specific primer (YN057), which was located 900

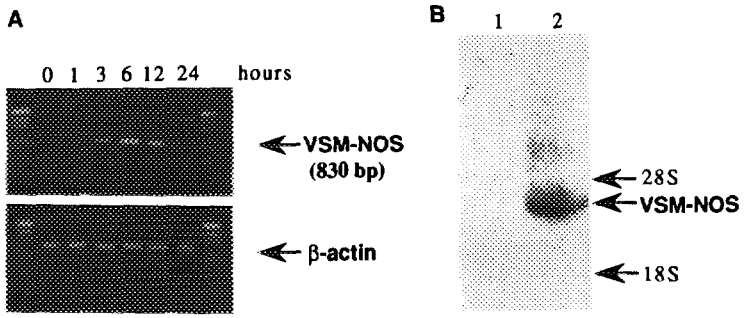

Figure 1. (A) Time course of induction of VSM-NOS mRNA. At time 0, IFN-γ was added to each dish in which rat VSMC were grown at a final concentration of 1000 U/ml, and incubation was continued at 37 °C. At each time point indicated, the cells were lysed in ISOGEN and total RNA was extracted as described in MATERIALS AND METHODS. Each total RNA preparation (1.5 μg) was reverse transcribed and half of the cDNA product was PCR-amplified using either YN017/YN056 (upper panel) or β-actin primers (lower panel), for 25 cycles each. A portion of the PCR reaction product was electrophoresed on a 0.8 % agarose gel containing ethidium bromide.
(B) RNA blot hybridization analysis of VSM-NOS. Total RNAs analyzed (20 μg each) were as follows: lane 1, rat VSMC (no-IFN-γ treatment); lane 2, rat VSMC(IFN-γ treatment for 12 hours). Migration of ribosomal RNA is indicated (28 S and 18 S).

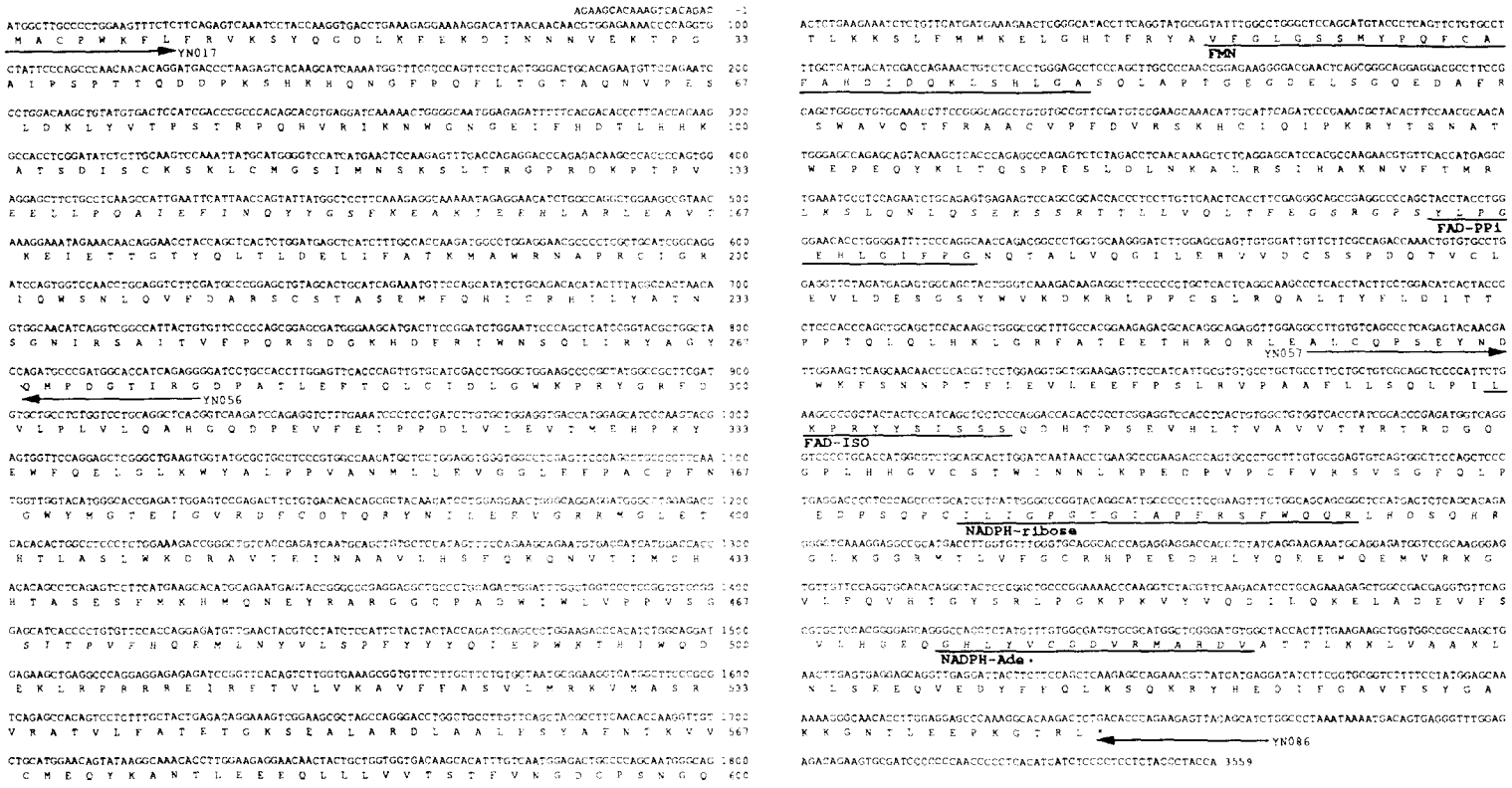

Figure 2. Nucleotide and deduced amino acid sequences of VSM-NOS. Nucleotides are numbered beginning with the first potential initiating methionine codon. The deduced amino acid sequence is shown using the single letter code. Consensus sequences of cofactor binding regions for FMN, FAD-PPi, FAD-ISO, NADPH-ribose, and NADPH-Ade are underlined according to Ref. 5 and 10. A position for amino acid substitution obserbed between VSM-NOS and mac-NOS in the consensus sequence is indicated by •(Val 1078).

bp upstream from the 3'-terminal of murine mac-NOS, and universal adaptor primer (YN058) were applied to PCR. The products from 3'-RACE were sequenced directly. The nucleotide sequence of 3'-RACE products was also quite similar to 3'-terminal sequence of mac-NOS (Figure 2) as described in the RT-PCR study. As the result, we could speculate the termination codon (TGA) of the VSM-NOS and obtain the gene specific sequence primer (YN086).

YN086 primer, we newly obtained, was applied to construct the gene specific 3'-primed cDNA library. The cDNA library thus constructed was convenient for screening the clone encoding VSM-NOS. In fact, by simple PCR screening as described in MATERIALS AND METHODS, we obtained 2 positive clones from a total of 35 clones in the plasmids of which had been inserted some cDNAs. One of the two clones was sequenced with an auto-sequencer and Dye-Deoxy Terminator kit.

In this way, we could obtain rat VSM-NOS clone containing complete coding region. The cDNA insert of the clone contains a 3441 bp open reading frame encoding a polypeptide of 1147 amino acids with a calculated molecular mass of 131 kilo-dalton. The translational start site contains a Kozak consensus sequence (CAGACATGG) (17). The similarity of amino acid sequence to NOS in rat brain (5), endothelial cells (6, 7) and murine macrophage (9, 10) is 46, 50 and 93%, respectively (Figure 2). NADPH, FMN, and FAD binding regions predicted as the characteristics for NOSs in previous reports (5, 10), were highly conserved, and these regions, except for one amino acid residue(Val 1078), were completely identical to those of murine mac-NOS (Figure 2). The results suggest that VSM-NOS and mac-NOS would be identical isozymes. It is, however, also possible that they would be closely related genes such as cytochromes P450 CMF1a (18) (P450IID1 (19)) and P450 CMF1b (18, 20) (P450IID5 (19)). These P450s are as high as 96 % similar, while they are coded on separated genes each other in a genome. Cloning of rat mac-NOS or murine VSM-NOS will clarify these points.

RNA blot hybridization analysis was performed to confirm the expression and to examine the size of the NOS mRNA. RNA blot hybridization gave rise to a hybridization band with an estimated mRNA size of above 4 kilo-nucleotides under high-stringency conditions (Figure 1B). In the case of RNA prepared from VSMC cultured without IFN-γ, no signal was detected under the same conditions. These findings were consistent with those obtained by the RT-PCR experiment.

As for VSM-NOS, no report has been made on its protein purification, cloning nor detail-characterization, although its existence has been confirmed (11, 12). We now show the cDNA for

rat inducible VSM-NOS, which could be one of the key enzymes for VSMC proliferation and is identical to the NOSs cloned so far, by the application of PCR. The time-course analysis of VSM-NOS mRNA levels suggests that IFN-γ will act as an effective antiproliferative agent to VSMC, at least, by 6-hour treatment. Hansson et al. reported that IFN-γ inhibited the proliferation of VSMC *in vivo* (21). Therefore our findings are interesting in the administration of IFN-γ to the patients of cardiovascular diseases such as atherosclerosis and restenosis. Further studies such as analysis of the promoter regions of the gene are going on now, and will elucidate the mechanisms of the induction of NOS in VSMC.

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