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Original Research Communication

Effect of cAMP on Inducible Nitric Oxide Synthase Gene Expression: Its Dual and Cell-Specific Functions

AYAKO OKADO-MATSUMOTO,[1] AKIO MATSUMOTO,[1] JUNICHI FUJII,[2] and NAOYUKI TANIGUCHI[1]

ABSTRACT

The effects of some cAMP-elevating agents on the induction of nitric oxide synthase II (NOS II) were investigated for a macrophage-derived cell line, RAW264.7, stimulated with lipopolysaccharide (LPS) or interferon-γ (IFN-γ) and the results were compared for the case of vascular smooth muscle cells (VSMC) stimulated with interleukin-1β (IL-1β). Forskolin, dibutyryl cAMP, and a phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine, resulted in an elevated production of nitrite and nitrate, NOS II activities, NOS II mRNA accumulation, and the protein level in RAW264.7 cells stimulated with LPS or IFN-γ. However, the addition of combinations of these reagents decreased these levels in RAW264.7 cells, but enhanced them in VSMC that had been stimulated with IL-1β. When intracellular cAMP levels in VSMC were measured, they were elevated by about 100 times more in the forskolin-treated cells, compared to the untreated cells. Stimulated RAW264.7 cells, on the other hand, produced much lower levels of cAMP than VSMC. It is likely that cAMP functions in two opposing directions in terms of NOS II gene induction in RAW264.7 cells in a dose-dependent manner. The effects of cAMP-elevating agents on promoter activities of the 5′-flanking region of the mouse NOS II gene were then examined. The promoter activities were enhanced in RAW264.7 cells, even in the presence of all three cAMP-elevating agents. Although the binding of NF-κB to responsive elements is essential for the induction of the NOS II gene, cAMP-elevating agents had no effect on NF-κB binding to the element, thus eliminating the involvement of NF-κB in the suppression of the NOS II gene by high concentrations of cAMP. These data suggest that a putative responsive element to high levels of cAMP is present outside of the region examined in this study. The inhibitory effects of cAMP in RAW264.7 cells would be due to the presence of a negative regulatory factor that is absent in VSMC. Antiox. Redox Signal. 631–642.

INTRODUCTION

NITRIC OXIDE (NO) is produced by NO synthases (NOS), which utilize L-arginine and molecular oxygen as substrates in an NADPH-dependent manner (Moncada *et al.*, 1991). Of the three NOS isozymes that have been identified thus far, NOS II is induced in various cells such as macrophages, smooth muscle cells, and hepatocytes by stimulation with cytokines and a lipopolysaccharide (LPS) (Busse and Mulsch, 1990; Nathan 1992; Koide *et al.*, 1993a). The activity of NOS II is not dependent on Ca^{2+}, and it produces levels of NO that are sufficiently high to damage surrounding tissues as well as the cell in which it is produced (Kaneto *et al.*, 1995; Seo *et al.*, 1995).

Interferon-γ (IFN-γ) and LPS are potent inducers of NOS II gene expression in macrophages, whereas interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are more effective in vascular smooth muscle cells (VSMC). A mouse macrophage-derived line, RAW264.7, is commonly used as a model cell system for investigating the mechanism of expression of the NOS II gene. The participation of NF-κB (Xie *et al.*, 1994; Spink *et al.*, 1995; Hecker *et al.*, 1996) and interferon regulatory

[1]Department of Biochemistry, Osaka University Medical School, Osaka 565-0871, Japan.
[2]Department of Biochemistry, Yamagata University School of Medicine, Yamagata 990-9585, Japan.

factor-1 (IRF-1) (Kamijo *et al.*, 1994; Martin *et al.*, 1994) in gene induction has previously been demonstrated. The involvement of some other factors, such as STAT1α, has also been suggested for IFN-γ-dependent induction (Ohmori *et al.*, 1997).

cAMP-elevating agents, such as dibutyryl cAMP, forskolin, adenosine, and purine nucleotides, are also known to stimulate NOS II gene induction in VSMC (Koide *et al.*, 1993b; Imai *et al.*, 1994). Although cAMP alone is a weak stimulator, it synergistically enhances NOS II gene induction by IL-1β and TNF-α. cAMP, on the other hand, is a well-known immunosuppressant and is involved in the negative regulation of the expression of some immune-responsive genes such as IL-1β, IL-2, TNF, and IFN-γ (Kammer, 1988). Both the enhancing and suppressive effects of cAMP have been reported in terms of NOS II induction in macrophages and related cells (Mauel *et al.*, 1995; Pang and Hoult, 1997; Park *et al.*, 1997; Eberhardt *et al.*, 1998; Hasko *et al.*, 1998; Mullet *et al.*, 1997; Galea and Feinstein, 1999; Mustata and Olson, 1998). A well-known pathway involves the activation of cAMP. This pathway is mediated by cAMP-dependent protein kinase, which phosphorylates and thereby activates cAMP-dependent transcription factors including CREB, C/EBP, and CCAAT enhancer binding protein family members. Although no canonical cAMP-responsive element (CRE) has been found to date in the mouse NOS II gene (Xie *et al.*, 1993; Lowenstein *et al.*, 1994), a putative CRE has been reported in the 5'-flanking region of the rat NOS II gene (Eberhardt *et al.*, 1996). However, the mechanism by which cAMP suppresses the gene induction is largely unknown (Galea and Feinstein, 1999).

In this communication, we show that cAMP functions as both an inducer, at lower levels, and a suppressor, at higher levels, in terms of NOS II induction in RAW264.7 cells that have been stimulated with LPS or IFN-γ, but that it simply enhances gene expression in VSMC. This suggests the presence of a feedback, suppressive mechanism for the induction of the NOS II gene by high concentrations of cAMP in macrophages under severe inflammatory conditions.

MATERIALS AND METHODS

Materials

L-[U-^{14}C]Arginine monohydrochloride, [γ-^{32}P]ATP, and an ECL kit were obtained from Amersham Pharmacia Biotech. Pepstatin, leupeptin, mouse IFN-γ, forskolin, and poly(dI-dC) were purchased from Sigma. NADPH was obtained from Boehringer Mannheim. Tetrahydrobiopterin was obtained from Research Biochemical Inc. Soybean trypsin inhibitor, (*p*-amidinophenyl) methanesulfonylfluoride and FAD were obtained from Wako Pure Chemical Industries. Dowex 50WX-8 was obtained from Muromachi Kagaku. Fetal bovine serum (FBS) was obtained from GIBCO/BRL. A Zeta-Probe membrane was obtained from Bio-Rad. pT7 Blue-T vector was obtained from Novagen. pCAT3-Enhancer vector and pGV-Control vector were obtained from Promega. BCA kit was obtained from Pierce. [^{3}H]Acetyl coenzyme A and Enonofluor-2 were obtained from DuPont/New England Nuclear. A T4 polynucleotide kinase was obtained from Takara. cAMP assay kit was obtained from Yamasa. Rabbit anti-NOS II antibody was obtained from Transduction Laboratories. Goat anti-rabbit IgG was obtained from Organon Teknika Corp. Recombinant human IL-1β was kindly provided by Otsuka Pharmaceutical Co. LTD. Calmodulin was purified from bovine brain. The other reagents were of the highest grade available.

Culture and stimulation of RAW 264.7 cells

RAW264.7 cells, a mouse macrophage line transformed with the Abelson leukemia virus, were obtained from the American Type Culture Collection, and grown in Dulbecco's modified minimum essential medium containing 4.5 grams of glucose/liter, 100 units of penicillin, and 100 units of streptomycin supplemented with 10% heat-inactivated FBS at 37°C under an atmosphere of 95% air and 5% CO_2. A total of 6.5×10^5 cells/well, in 12-well plates, were incubated for 22 hr in a medium supplemented with 10 ng/ml LPS and varying concentrations of dibutyryl cAMP, forskolin, and IBMX.

Culture and stimulation of VSMC from rat

The VSMC used in this work were prepared using previously described methods (Seo *et al.*, 1996). VSMC were cultured in the Dulbecco's modified Eagle's medium containing 100 units of penicillin and 100 units of streptomycin supplemented with 20% heat-inactivated FBS at 37°C under an atmosphere of 95% air and 5% CO_2. A total of 6.5×10^5 cells/well in 12-well plates were incubated for 24 hr in the medium supplemented with 5 ng/ml of IL-1β in the presence of various concentrations of reagents.

Quantification of nitrite and nitrate formation

Nitrite in media was quantified using the Griess reagent on aliquots of the supernatant (Green *et al.*, 1982). The combined level of nitrite and nitrate was measured by means of the chemiluminescent reaction of NO with ozone using a NO analyzer 270B NOA (Sievers) after reduction to NO with 0.1 *M* VCl_3 in 1 *M* HCl at 95°C (Braman and Hendrix, 1989).

Assay of NOS activity

After stimulation, cells were collected by centrifugation, washed twice in phosphate-buffered saline (PBS), and sonicated. NOS activity was determined based on the rate of conversion of L-[U-^{14}C]arginine to L-citrulline using a previously described method (Bredt and Snyder, 1990) with a minor modification (Seo *et al.*, 1994). Briefly, a 10-μl aliquot sample was incubated for 10 min at 37°C in a solution of 50 m*M* HEPES, 1 m*M* dithiothreitol (DTT), 1 m*M* $CaCl_2$, 0.1 m*M* tetrahydrobiopterin, 1 m*M* NADPH, 10 μg/ml calmodulin, 10 μ*M* FAD, and 1.55 μ*M* L-[U-^{14}C]arginine, pH 7.8, in a final volume of 100 μl. The reaction was terminated by the addition of 200 μl of buffer A (100 m*M* HEPES and 10 m*M* EDTA, pH 5.2). The entire reaction mixture was then applied to a 300-μl Dowex 50 WX column (Na^+ form, 200–400 mesh) that had previously been equilibrated with buffer A. Citrulline was eluted with 0.5 ml of buffer A, and its radioactivity was subsequently determined with a liquid scintillation counter (Aloka).

RNA preparation and Northern blotting

Total RNA was prepared from cultured VSMC in 10-cm dishes essentially as described by Chomczynski and Sacchi (1987), and was then quantified by an absorbance measurement at 260 nm. Twenty micrograms of total RNA were heat-denatured at 65°C for 15 min in the presence of 50% formamide and the running gel buffer (40 m*M* MOPS, 10 m*M* sodium acetate, and 1 m*M* EDTA, pH 7.0), and then electrophoresed on a 1% agarose gel containing 2.2 *M* formaldehyde. The size-fractionated RNAs were transferred onto a Zeta-Probe membrane over a 20- to 40-hr period by capillary action, and the blotted RNAs were immobilized on the membrane by UV irradiation, followed by incubation for 2 hr at 80°C under vacuum. An 818-bp NOS II cDNA fragment, which had been synthesized from total RNA of the RAW264.7 mouse macrophage cell line and amplified by PCR, was used as described previously (Kaneto *et al.*, 1995; Seo *et al.*, 1995). After hybridization with a ^{32}P-labeled NOS II cDNA probe at 42°C in the presence of 50% formamide, the membranes were washed twice at 55°C with 2× SSC and 0.1% sodium dodecyl sulfate (SDS) for 80 min, and then twice at high stringency in 0.3× SSC and 0.1% SDS for 60 min. Kodak XAR films were exposed for 1–3 days with an intensifying screen at −80°C.

SDS-PAGE and Western blot analysis

Protein samples were subjected to 8% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to nitrocellulose membranes (Shleicher & Schuell) under semidry conditions with the use of a Transfer-blot SD Semi-dry transfer cell (Bio-Rad). After blocking by incubation with 5% skim milk in TBST (20 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, and 0.05% Tween 20) for 2 hr at room temperature, the membranes were reacted with an antibody against NOS II, diluted with 1:1,000, for 12 hr at 4°C. After washing with TBST, the membranes were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) for 1 hr. After washing, the chemiluminescence method in the form of an ECL kit was employed to detect peroxidase activity.

Cloning of rat NOS II 5′ flanking region and construction of CAT gene fusion plasmids

5′-Flanking region of the mouse NOS II gene (Lowenstein *et al.*, 1994) was cloned by the polymerase chain reaction (PCR) from the genomic DNA isolated from RAW264.7 cells. Briefly, the cultured RAW264.7 were washed twice with ice-cold PBS and removed by scraping with a rubber policeman. After precipitation by centrifugation, the cells were lysed in buffer containing 10 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 10 m*M* EDTA-NaOH, pH 8.0, and 0.1% SDS and then incubated with 100 μg/ml proteinase K for 90 min at 55°C. High-molecular-weight DNA was extracted from the lysate with phenol followed by an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). After centrifugation, the DNA pellet was dried and dissolved in TE buffer (10 m*M* Tris-HCl, pH 8.0; 1 m*M* EDTA). A 1,496-bp DNA fragment, corresponding to the 5′-flanking region of the mouse NOS II gene was amplified by PCR using a set of oligonucleotide primers according to the reported sequence (forward, 5′-GAAGACAATCTCAGCTCTTG-3′; reverse, 5′-TTCACCAAGGTGGCTGAG-3′, GenBank accession number L23806). The size-matched PCR product was subcloned into the pT7 Blue-T vector. Sequences of the PCR product were confirmed using an automated sequencer DSQ-1000L (Shimadzu). The DNA fragment was digested with restriction enzymes followed by subcloning into the pCAT3-Enhancer vector, which contains a simian virus 40 (SV40) enhancer element and SV40 late poly(A) region for chloramphenicol acetyltransferase (CAT) analysis. The original plasmid, which extended from 88 bp downstream of the transcriptional initiation site to −1496 bp upstream sequences in the NOS II gene was designated as pCAT3-Full. Similarly other deletion mutants were constructed by digestion with restriction enzymes at internal restriction enzyme sites.

DNA transfection and CAT assay

Promoter activities of the 5′-flanking region of the mouse NOS II gene was measured by transfecting the plasmid DNAs into RAW264.7 and VSMC cells by electroporation as described earlier (Saito *et al.*, 1995). Briefly, about 1×10^6 cells were suspended in 0.8 ml of HeBS buffer (20 m*M* Hepes, 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_2, and 6 m*M* glucose, pH 6.95) containing 20 μg of plasmid DNA at room temperature. Transient transfection of cells was performed by electroporation (Gene Pulser, Bio-Rad) at 300 V/0.4 cm and 300 V/0.4 cm, respectively, with the capacitance 960 μF. At 24 hr after transfection, the medium was exchanged for fresh medium. The cells were harvested 48 hr after induction, sonicated in 500 μl of 0.1 *M* Tris-HCl, pH 7.4, and then incubated for 10 min at 60°C, to inactivate acetylase. A 100-μl sample was added to the reaction mixture, which contained 140 μl of 0.1 *M* Tris-HCl, pH 7.4, 1 μl of [^{3}H]acetyl coenzyme A (200 μCi/mmol), and 9 μl of 50 μ*M* chloramphenicol in 50% ethanol in a glass vial. After slowly layering a water-immiscible scintillation cocktail, Econofluor-2, on top of the reaction mixture, it was incubated at 37°C for 2–3 hr, and the radioactivity was measured in a liquid scintillation counter as previously described. The resultant CAT activity was normalized by co-transfection of a luciferase control plasmid (pGV-Control) as an internal control.

Electrophoretic mobility shift assay

Approximately 1×10^7 cells were washed with ice-cold phosphate-buffered saline, followed by lysis with Nonidet P-40 buffer (10 m*M* HEPES, pH 7.8, containing 10 m*M* KCl, 2 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* DTT, 5% glycerol, 2 μg/ml leupeptin, 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF) and 0.2% (vol/vol) Nonidet P-40). After centrifugation at $3{,}000 \times g$ for 5 min at 4°C, nuclear protein was extracted from the pellet with 50 m*M* HEPES buffer, pH 7.8, containing 420 m*M* KCl, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* DTT, 0.5 m*M* PMSF, and 25% glycerol. Extracts were cleared by centrifugation at $14{,}000 \times g$ for 15 min at 4°C. Aliquots of the resulting supernatants were frozen at −70°C. The protein concentration of the nuclear extract was measured using a BCA kit with bovine serum albumin (BSA) as the standard. These nuclear proteins were used for the electrophoretic mobility-shift assay (EMSA). Two established NF-κB binding sequences were used as follows: upstream NF-$\kappa\beta$ probe, 5′-GGATGTGCTAGG-GGGATTTTCCCTCTC-3′, and the downstream

NF-κB probe, 5′-CACCAACTGGGGACTCTC-CCTTTG-3′. The DNA probe, which contained the putative CREB binding sequences of the upstream regulation regions of the NOS II gene was 5′-GATTGCAAGAAGTCACCCAG-3′. Complementary oligonucleotides were annealed and used as probes or competitors. The probes were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. For the gel mobility-shift assay, a labeled DNA probe (10,000 cpm) and 5 μg of nuclear proteins were preincubated for 10 min at room temperature with 400 ng of poly(dI-dC) in 20 μl of binding buffer (25 m*M* Tris-HCl, pH 7.9, 65 m*M* KCl, 6 m*M* $MgCl_2$, 0.25 m*M* EDTA, and 10% glycerol). For the competition assay, the unlabeled competitor oligonucleotides were preincubated with nuclear proteins for 10 min, prior to the addition of labeled oligonucleotide. Samples were loaded onto 5% nondenaturing polyacrylamide gels, 0.25 × TAE (1 × TAE = 40 m*M* Tris-HCl, pH 7.8, 1.1 m*M* EDTA, and 37 m*M* sodium acetate) containing 2.5% glycerol, and electrophoresis was then carried out at 4°C at 100 V for 1 hr. After electrophoresis, the gels were dried with a gel dryer and exposed to X-ray films.

cAMP assay

The cells (5×10^5 cells per well in 12-well dishes) were incubated with the indicated reagents for various periods of time. After discarding the media, the reaction was terminated by adding 1 ml of ice cold 0.1 *N* HCl. After centrifugation at 1,500 × *g* for 10 min, the supernatant was neutralized by adding 10 μl of 10 *N* NaOH. The cAMP contents of a 0.1-ml aliquot of the supernatant was determined by radioimmunoassay using a cAMP assay kit.

Statistical analysis

The paired Student's *t*-test was used to compare the significance of the differences between data. All data are expressed as means ± SD.

RESULTS

Dual functions of cAMP-elevating agents on nitrite and nitrate formation in RAW 264.7 cells and VSMC

Three reagents, dibutyryl cAMP, forskolin, and IBMX, all of which elevate intracellular

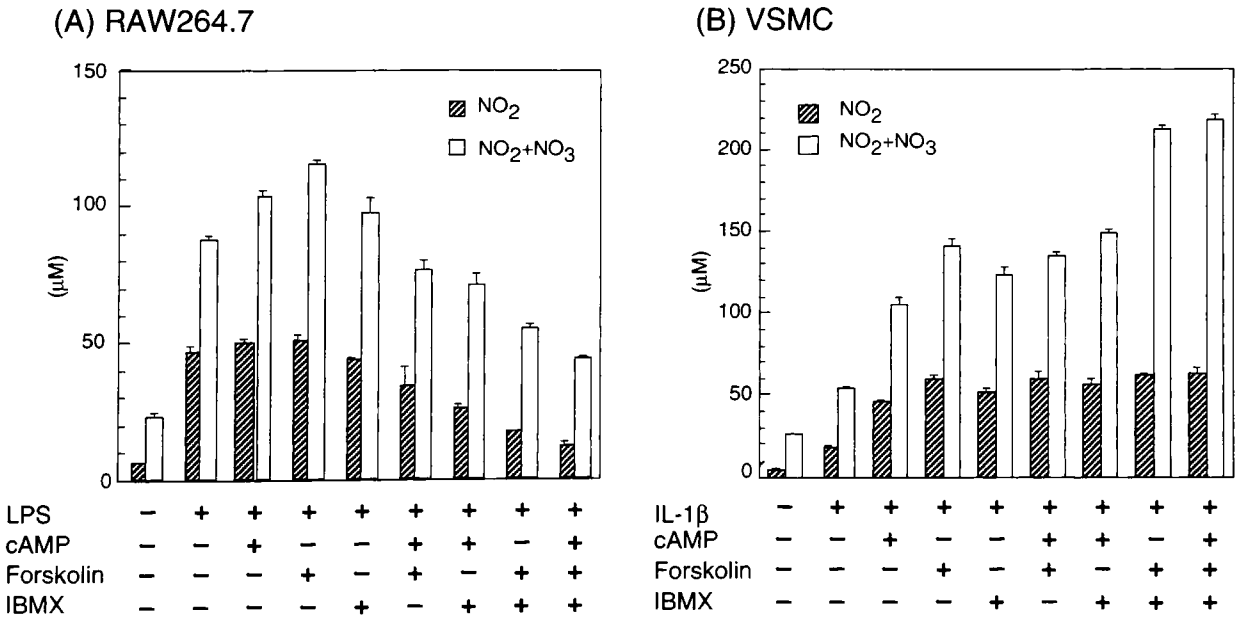

FIG. 1. Effects of cAMP-elevating agents on nitrite/nitrate production by RAW264.7 cells stimulated with LPS and by VSMC stimulated with IL-1β. (**A**) A total of 1.5×10^6 RAW264.7 cells/well in six-well plates were stimulated with 10 ng/ml LPS for 24 hr in a medium containing dibutyryl cAMP, forskolin, and 3-isobutyl-1-methyl xanthine (IBMX). (**B**) A total of 3.5×10^5 VSMC/well in six-well plates were incubated for 24 hr in a medium supplemented with 5 ng/ml IL-1β in the presence of dibutyryl cAMP, forskolin, and IBMX. Levels of nitrite alone and nitrite/nitrate were quantified by the Griess method and an NO analyzer, respectively.

cAMP by different mechanisms, were examined with respect to nitrite and nitrate formation by RAW264.7 cells stimulated with LPS. Figure 1 shows the levels of nitrite, as determined by the Griess method (Green *et al.*, 1982) and the sum of nitrate and nitrite, as determined by chemiluminescence (Braman and Hendrix, 1989) in conditioned media. Each of the three reagents increased the levels of both nitrite and nitrate, but high concentrations of these agents or combinations of them resulted in suppression, in the cells stimulated with LPS (Fig. 1A). Because cAMP-elevating agents induce NO formation in VSMC via the induction of the NOS II gene (Koide *et al.*., 1993b; Imai *et al.*, 1994), we also examined effects of these reagents on nitrite/nitrate production in VSMC, stimulated with 5 ng/ml of IL-1β (Fig. 1B). These reagents increased the production of nitrite/nitrate in VSMC at all concentrations examined, and there was essentially no suppression compared with RAW264.7 cells. Although the viability of VSMC decreased at high concentrations of these reagents, probably due to cytotoxic effect of NO (Seo *et al.*, 1996), only a slight decrease in the viability of RAW264.7 cells was observed with these reagents as judged by measuring lactate dehydrogenase activities released from the cells (data not shown). Thus, RAW264.7 cells appears to be more resistant to the cytotoxic effects of NO than VSMC.

Levels of intracellular cAMP

The effects of these reagents on the levels of intracellular cAMP in RAW264.7 cells, which had been stimulated with LPS and in VSMC stimulated with IL-1β by radioimmunoassay was also investigated (Fig. 2). Levels of cAMP were actually elevated in the case of RAW264.7 cells by the administration of forskolin and IBMX, and this effect was sustained for at least 24 hr, even in the absence of IBMX. The extent of the elevation of cAMP was much more prominent in VSMC than in RAW264.7, but, in this case, these levels began to decrease in 1 hr. This suggests that both suppressive and inducible effects of these agents are related to the intracellular levels of cAMP and that the sustained levels may be related to the suppression in RAW 264.7 cells.

Effects of cAMP-elevating agents on the levels of NOS II mRNA, protein, and activity

To determine the effect of cAMP on the NOS II transcript, the levels of the NOS II mRNA were determined by Northern blotting (Fig. 3) and the protein by Western blotting (Fig. 4). NOS II activities of the cell lysate were also assayed in the presence of 1 m*M* EGTA under the same conditions (Fig. 5). Both dibutyryl cAMP and forskolin, at a concentration of 100 μM, in-

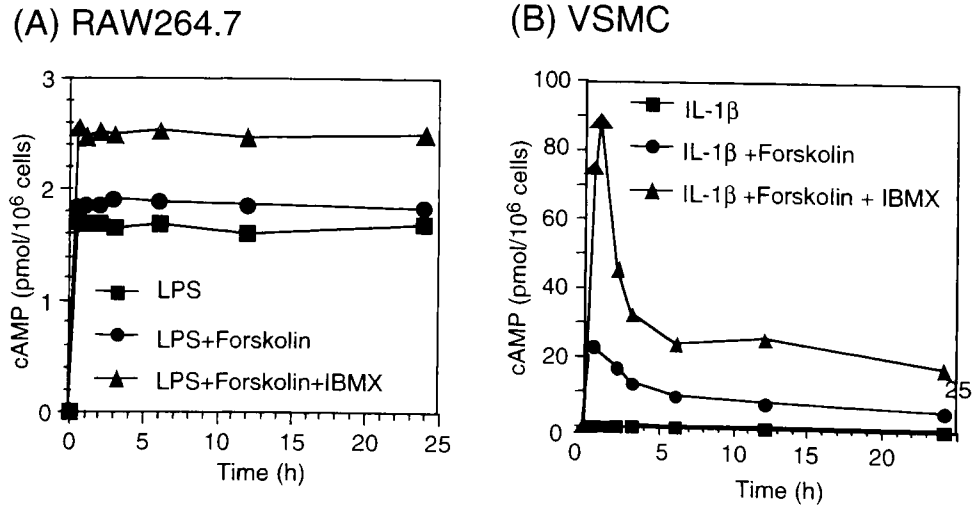

FIG. 2. Effects of cAMP-elevating agents on the levels of cAMP in RAW264.7 cells stimulated with LPS and VSMC stimulated with IL-1β. RAW264.7 cells stimulated with 10 ng/ml LPS (**A**) and VSMC stimulated with IL-1β (**B**) were incubated in the presence of forskolin alone or forskolin plus IBMX. Levels of intracellular cAMP were measured by radioimmunoassay.

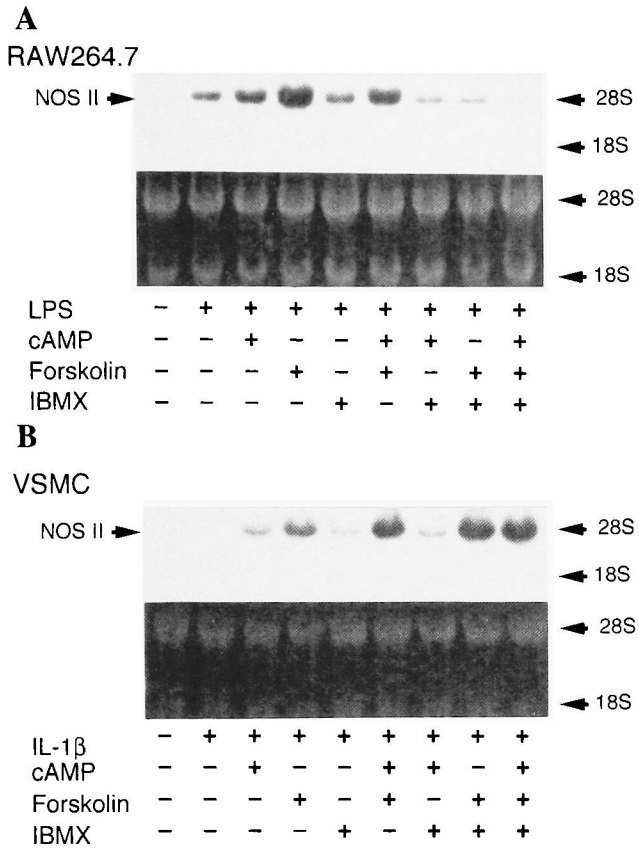

FIG. 3. Effects of cAMP-elevating agents on the levels of the NOS II mRNA in RAW264.7 cells and VSMC. Total RNAs were isolated from RAW264.7 cells stimulated with 10 ng/ml LPS (**A**) and from VSMC stimulated with IL-1β (**B**) in the media containing dibutyryl cAMP, forskolin, and /or IBMX. Northern blotting was carried out using mouse NOS II cDNA as a probe.

creased the levels of NOS II mRNA, protein, and the activity in RAW264.7 cells stimulated with LPS. The combined administration of these reagents, however, suppressed these levels in the RAW264.7 cells and enhanced them in VSMC. Thus, the opposing effects of cAMP appear to reflect transcriptional activities of the NOS II gene.

Effects of cAMP-elevating agents on promoter activities of the mouse NOS II gene

Although no canonical CRE was postulated from the sequences in the original report (Xie *et al.*, 1993; Lowenstein *et al.*, 1994), the gene was, in fact, induced by the cAMP-elevating agents. To examine effects of cAMP on the promoter activity of the NOS II gene, the 1.5-kbp 5′-flanking region of the mouse NOS II gene was cloned by PCR using isolated genomic DNA from RAW264.7 cells as a template. Nucleotide sequences of the amplified DNA matched the reported sequences (Lowenstein *et al.*, 1994). The DNA fragment was then subcloned into the upstream of the CAT gene in the pCAT3-enhancer vector. Three constructs with shorter 5′-flanking region were also prepared by digestion with restriction endonucleases (Fig. 6). After transfection of the plasmids into RAW264.7 or VSMC by electroporation, the cells were incubated with LPS and IL-1β in the presence or absence of the cAMP-elevating agents, respectively. CAT activities were assayed at 48 hr after incubation and were normalized using a luciferase control vector as an internal control. The effects of cAMP-elevating agents on the promoter activities were more

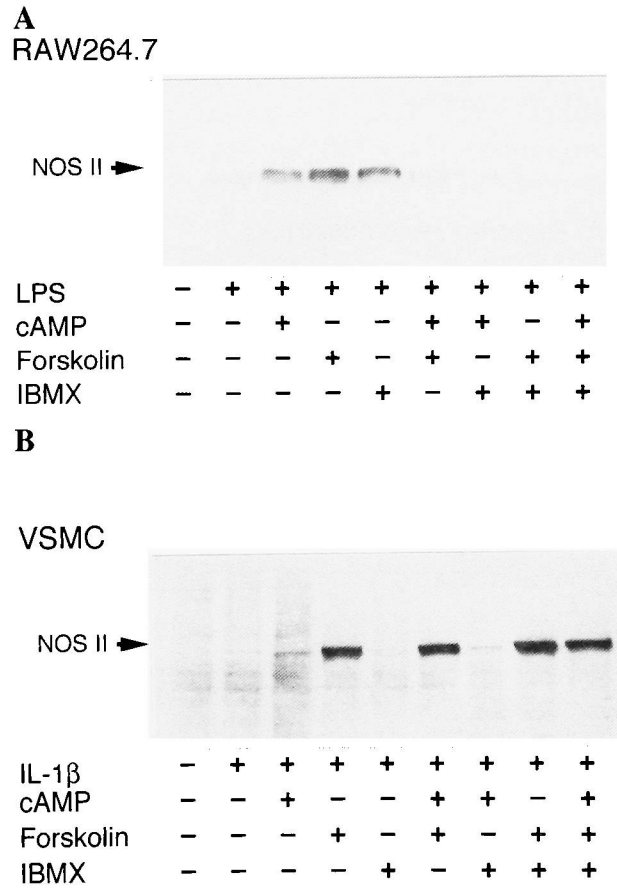

FIG. 4. Effects of cAMP-elevating agents on the levels of the NOS II protein in RAW264.7 cells and VSMC. Proteins were isolated from RAW264.7 cells stimulated with 10 ng/ml LPS (**A**) and VSMC stimulated with IL-1β (**B**) in the media containing dibutyryl cAMP, forskolin, and/or IBMX. Western blotting was carried out using anti-NOS II IgG.

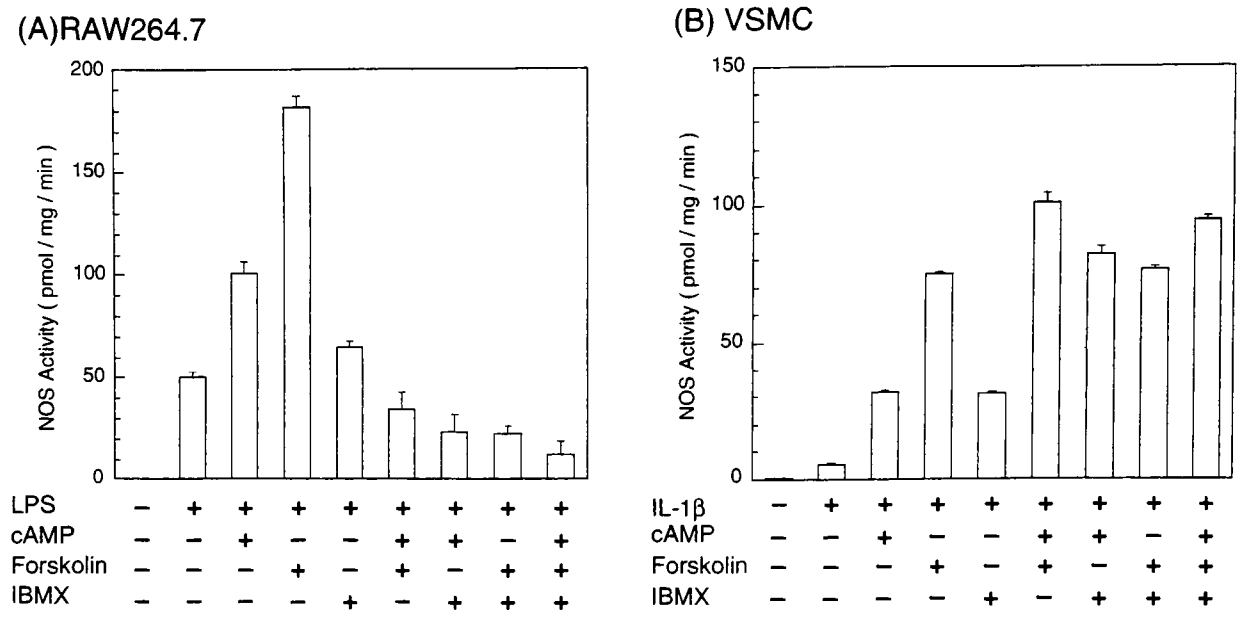

FIG. 5. Effects of cAMP-elevating agents on NOS II activity in RAW264.7 cells stimulated with LPS and in SMC stimulated within IL-1β. (**A**) A total of 5×10^6 RAW264.7 cells/dish in 10-cm plates were stimulated with 10 ng/ml LPS for 22 hr in a medium containing dibutyryl cAMP, forskolin, and/or IBMX. (**B**) A total of 1×10^6 VSMC/well in 10-cm plates were stimulated with 5 ng/ml IL-1β for 24 hr in media containing dibutyryl cAMP, forskolin, and IBMX. The NOS II activity was assayed in the presence of 1 m*M* EGTA by measuring conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline.

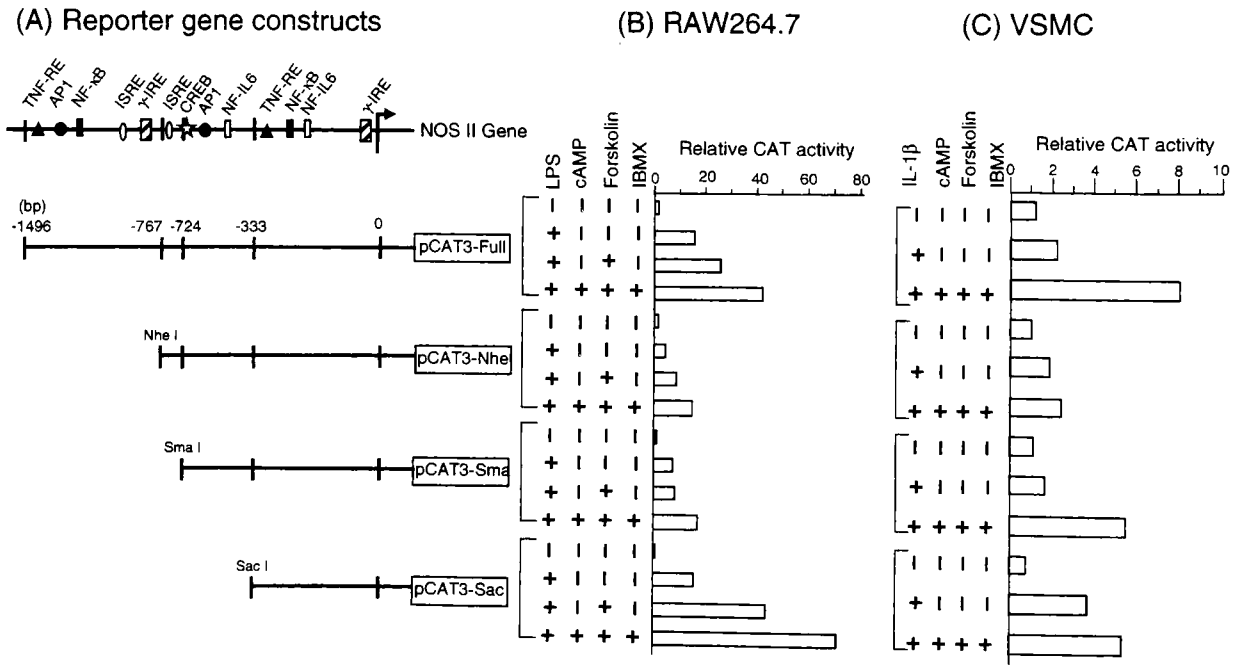

FIG. 6. Promoter assay of the mouse NOS II gene in RAW264.7 and VSMC. Reporter gene constructs containing the 5′ upstream region of the mouse NOS II gene (**A**). Four different CAT constructs were prepared by restriction enzyme digestion in the pCAT3-E vector. pCAT3-Full contained from 88 bp downstream from the transcriptional initiation site to −1,496 bp of the upstream sequences in the 5′ region of the mouse NOS II gene. Others are: pCAT3-Nhe, −767 bp; pCAT3-Sma, −724 bp, pCAT3-Sac, −133 bp. After transfection of these DNAs by electroporation, the cells were stimulated with LPS or IL-1β in the presence or absence of cAMP-elevating agents. CAT activities in RAW264.7 (**B**) and VSMC (**C**) were measured at 48 hr after stimulation using a luciferase control vector as an internal standard.

prominent in RAW264.7 cells than in VSMC, and were contrary to the data shown above. The cells, which had been transfected with all of these DNAs, showed an enhancement of the promoter activities by cAMP-elevating agents, but no suppression was observed, even by the addition of combinations of these reagents. When the effects of these cAMP-elevating agents on the binding of NF-κB to the consensus sequences in the NOS II gene were investigated by the gel-shift assay, they had no effect on the binding activity of NF-κB to the probe in nuclear extracts from the RAW264.7 and VSMC, which had been stimulated with LPS and IL-1β, respectively (data not shown). Thus, it is unlikely that cAMP regulates the expression of the NOS II gene through perturbing the binding of DNA of NF-κB to these elements. A probe to the putative CRE sequences, GAAGTCA, started at -664 and, likewise, did not show specific binding. Thus, the suppressive effect of high concentrations of cAMP on the promoter activity of the region presented here were not observed.

DISCUSSION

IFN-γ and LPS are well-known stimulators for RAW264.7 to release NO whereas IL-1β and INF-γ are effective in VSMC (Busse and Mulsch, 1990; Koide *et al.*, 1993a). Among the several agents that are capable of modulating NO production, cAMP is a potent inducer of VSMC (Koide *et al.*, 1993b; Imai *et al.*, 1994). Here we show that cAMP is a novel agent that functions as a bifunctional modulator of NOS II induction in RAW264.7 cells. cAMP induces NOS II gene expression at low concentrations and suppresses it at high concentrations in the cells. Endogenous cAMP production was more prominent in VSMC than in RAW264.7 cells (Fig. 2), but no suppression was observed in VSMC that had been stimulated with IL-1β. High levels of cAMP decreased the levels of the NOS II mRNA and the corresponding protein, resulting in a decrease in NOS II activity in RAW264.7 cells (Figs. 1 and 3). Thus, the lowering of NO production by high levels of cAMP were the result of suppression of the NOS II gene expression.

Both mouse and rat NOS II genes have been cloned and their regulatory mechanisms investigated by several groups (Xie *et al.*, 1993; Lowenstein *et al.*, 1994; Martin *et al.*, 1994; Xie *et al.*, 1994; Spink *et al.*, 1995; Eberhardt *et al.*, 1996). Consensus binding sequences for many transcriptional factors including NF-κB and IRF-1 have been identified in the 5′-regulatory region of the mouse gene (Xie *et al.*, 1993; Kamijo *et al.*, 1994; Xie *et al.*, 1994). This region contains two binding sites for NF-κB, a transcription factor, which can be activated in response to stimuli such as TNF, IL-1β, LPS, and reactive oxygen species (ROS) (Galea and Feinstein, 1999). NF-κB binds the downstream κB site which is located at -85 to -75 after stimulation with LPS in RAW264.7 cells, but is not essential for the induction of NOS II by IL-1β in VSMC (Spink *et al.*, 1995). The upstream κB site, which is located at -971 to -962, on the other hand, appears to be essential for activation by these cytokines in VSMC. These data herein also show that the targeted disruption of the gene for IRF-1, a transcriptional factor that can be activated by IFN-γ stimulation, suppressed cytokine-stimulated NOS II gene induction (Kamijo *et al.*, 1994) and that the consensus sequences for IRF-1, located at -923 to -913 within the cytokine-responsive elements, bind nuclear proteins when VSMC is activated by IFN-γ and IL-1β (Martin *et al.*, 1994). These upstream sequences actually bind nuclear proteins that were extracted from VSMC stimulated with IL-1β and TNF (Xie *et al.*, 1994).

When the effects of cAMP-elevating agents on the binding of NF-κB to these probes were examined, they had no effect on binding ability (data not shown). This is consistent with the data on renal mesangial cells stimulated with IL-1β and cAMP (Eberhardt *et al.*, 1998). It was also shown that cAMP-activatable NO release was not affected by treatment with pyrrolidine dithiocarbamate, an inhibitor of NF-κB activation. Therefore, it is not likely that NF-κB is involved in cAMP-activated NOS II induction or its suppression. Thus, the mechanism by which cAMP suppresses NOS II expression remains unclear at present. The promoter assay using the mouse NOS II 5′-flanking region failed to confirm the inhibitory effect of high concentrations of cAMP, suggesting that the responsive

element to the inhibitory stimuli of cAMP exist in a region other than the one examined in this study. Moreover, the enhancing effect of the promoter activity of the CAT constructs was much less in VSMC than RAW264.7 cells. This also suggests the presence of another enhancing element in the gene. One possible candidate for the cAMP-dependent mediator is the cAMP-responsive element modulator (CREM), which is induced by activation of the cAMP signaling pathway and is involved in the enhancement in gene transcription. Its alternative product, inducible cAMP early repressor (ICER), represses the CRE-responsive gene expression (Sassone-Corsi, 1998). The involvement of ICER in the cAMP-mediated transcriptional attenuation of cytokine gene expression has, in fact, been reported in the case of macrophages (Bodor and Habener, 1998). The NOS II gene may contain a second, as-yet-unidentified CRE-like element that binds CREM/ICER only when cAMP levels reach sufficiently high levels.

Chen *et al.*, reported the mechanism of LPS-induced NO in RAW264.7 cells (Chen *et al.*, 1999). They suggested that LPS increases intracellular cAMP levels via COX-2 induction and prostaglandin E2 production, resulting in protein kinase A activation, NF-κB activation, NOS II expression, and NO production. It may occur at a low level of cAMP concentration in RAW264.7 cells. However, the mechanism by which high-concentration cAMP suppresses NOS II expression remains unclear. Arginase is also regulated by cAMP in RAW264.7 cells (Morris *et al.*, 1998; Gotoh and Mori, 1999). When cAMP was further added, arginase was induced, and NO production was much decreased. As NO is synthesized by NO synthase from arginine, a common substrate of arginase, these two enzymes compete for arginine. Arginase may play some important roles at a high level of cAMP concentration in RAW264.7 cells.

In atherosclerotic plaque and certain inflammations such as pancreatitis, colitis, and rheumatics, NO produced from macrophages or its metabolites are suspected to be a causative factor. The suppression of NOS II expression by high concentrations of cAMP may be mediated by a mechanism similar to that of cAMP-dependent immunosuppression in macrophages (Kammer, 1988). Because the production of cytokines and prostaglandins, which enhance inflammatory processes including NO production, impair surrounding tissues under inflammatory conditions, this immunosuppressive effect by high concentrations of cAMP may function as a feedback regulator for the immunoreaction of macrophages under severe inflammatory conditions.

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ABBREVIATIONS

BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; CRE, cAMP-responsive element; CREM, cAMP-responsive element modulator; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; ICER, inducible cAMP early repressor; IgG, immunoglobulin G; IFN-γ; interferon-γ; IL-1β, interleukin-1β; IRF, interferon regulatory factor; LPS, lipopolysaccharide; NO, nitric oxide; NOS, NO synthase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SV40, simian virus 40; TNF, tumor necrosis factor; VSMC, vascular smooth muscle cells.

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Address reprint requests to:
Dr. Naoyuki Taniguchi
Department of Biochemistry
Osaka University Medical School
2-2 Yamadaoka, Suita
Osaka 565-0871, Japan

E-mail: proftani@biochem.med.osaka-u.ac.jp

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