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Research Article

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Induction of Nitric Oxide Synthase by Cyclic AMP in Rat Vascular Smooth Muscle Cells

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Abstract

By measurements of NO_2^-/NO_3^- (NOx) production and Northern blot analysis, we studied the effects of a membrane-permeable cAMP derivative, 8-bromo-cAMP, on the expression of inducible nitric oxide synthase (iNOS) gene and the synthesis of NOx in cultured rat vascular smooth muscle cells (VSMCs). 8-bromo-cAMP stimulated NOx production and increased steady-state levels of iNOS mRNA in rat VSMC in a time- and dose-dependent manner. N^G-monomethyl-L-arginine, a NOS inhibitor, completely blocked the 8-bromo-cAMP–induced NOx production, whose effect was partially, but significantly reversed by an excess L-arginine, but not by D-arginine. Compounds that increase intracellular cAMP levels (cholera toxin, forskolin, and 3-isobutyl-1-methylxanthine), all stimulated NOx production. Dexamethasone inhibited the stimulated NOx production, as well as the induction of iNOS mRNA by cAMP. Both actinomycin D and cycloheximide completely blocked the stimulated NOx production by cAMP. Actinomycin D abolished the cAMP-induced iNOS mRNA, whereas cycloheximide remarkably increased iNOS mRNA levels in the presence and absence of 8-bromo-cAMP (superinduction). Actinomycin D, but not dexamethasone, completely abolished the cycloheximide-induced iNOS mRNA. The half-life of cAMP-induced iNOS mRNA was ~ 2 h, whereas no decay in the cycloheximide-induced iNOS mRNA was observed during 12 h. These results demonstrate that iNOS gene is upregulated by cAMP and the superinduction of iNOS mRNA is attributable to increased mRNA stability in rat VSMC. (*J. Clin. Invest.* 1994. 93:543–549.) Key words: nitric oxide synthase • induction • cyclic AMP • vascular smooth muscle cells

Introduction

Endothelium-derived relaxing factor has been identified as nitric oxide (NO)[1] or closely related molecule(s). NO is synthesized from the guanidino nitrogen atom of L-arginine by the catalytic action of enzymes, collectively known as NO syn-

thases (NOS)[1]. At least two types of enzymes exist; one is Ca^{2+}/calmodulin-dependent and constitutive (c) NOS mainly present in brain (2) and vascular endothelial cells (3, 4), and the other is Ca^{2+}/calmodulin-independent and cytokine-inducible (i) NOS mainly present in macrophages (5) and vascular smooth muscle cells (VSMCs) (6). Recently, two isoforms of cNOS have been cloned from rat brain (7) and vascular endothelium (8–10). iNOS has also been cloned from activated mouse macrophage cell line (RAW 264.7) (11, 12). cNOS and iNOS, both of which are structurally homologous to cytochrome P450 reductase, have the recognition sites for NADPH, flavin adenine dinucleotide, flavin mononucleotide, and calmodulin. In contrast to the possible phosphorylation site for cAMP-dependent protein kinase (PKA) in brain and endothelial cNOS isoforms, macrophage iNOS lacks the consensus sequence.

Macrophage iNOS is activated by bacterial LPS and several cytokines, such as IL-1β, TNF-α, and IFN-γ (6, 13, 14). It has been reported that these cytokines increase accumulation of cGMP in rabbit and rat VSMC, possibly via NO production (6, 15). In endotoxin shock, it has been suggested that excessive production of NO stimulated by LPS and cytokines contributes to the development of profound hypotension and hyporesponsiveness of exogenous vasoconstrictors. The induction of iNOS requires de novo protein synthesis over several hours. However, whether a classical intracellular second messenger like cAMP is involved in the process of this induction in VSMC remains unknown. It also remains undetermined whether posttranscriptional modification affects iNOS mRNA stability in the cells. Therefore, the present study was aimed to investigate whether iNOS gene is regulated by cAMP in cultured rat vascular smooth muscle cells, and whether the expression of iNOS mRNA is regulated posttranscriptionally.

Methods

Materials. 8-bromoadenosine 3′:5′-cyclic monophosphate (8-bromo-cAMP), cholera toxin, forskolin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, as well as L- and D-arginine were obtained from Sigma Chemical Co. (St. Louis, MO), N^G-monomethyl-L-arginine (L-NMMA) from Calbiochem-Novabiochem Corp. (La Jolla, CA), actinomycin D and cycloheximide from Wako Pure Chemical (Osaka, Ja-

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1. *Abbreviations used in this paper:* AMD, actinomycin D; CHX, cycloheximide; cNOS, Ca^{2+}/calmodulin-dependent and constitutive nitric oxide synthase; DEX, dexamethanose; GADPH, glyceraldheyde-3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; iNOS, Ca^{2+}/calmodulin-independent and cytokine-inducible nitric oxide synthase; L-NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; NOx, nitrite and nitrate; PKA, cAMP-dependent protein kinase; VSMC, vascular smooth muscle cell.

pan), DME from Flow Laboratories (Irvine, Scotland), FBS from Cell Culture Laboratories (Cleveland, OH), deoxycytidine $5'$-[^{32}P]-triphosphate ([^{32}P]dCTP) from DuPont NEN Research Products (Boston, MA). All other compounds were reagent or molecular biology grade. cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from the American Type Culture Collection (ATCC no. 57091) (Rockville, MD).

Cell culture and incubation. VSMCs were isolated from thoracic aorta of adult Wistar rats by the enzymatic dissociation technique and cultured in DME containing 10% FBS and antibiotics at 37°C in a humidified atmosphere of 95% air/5% CO_2 as previously described (16). Subcultured VSMCs from the 10th to 20th passages were used in the experiments.

To study the production of nitrite and nitrate (NOx), confluent VSMCs (5×10^6 cells) in six-well dishes (Costar Corp., Cambridge, MA) were usually incubated for 12 h unless otherwise stated. After incubation, media were removed for NOx measurement.

Determination of NOx (NO_2^-/NO_3^-). Concentrations of NOx in cultured media were determined by an autoanalyzer (TCI-NOx 1000; Tokyo Kasei Kogyo Co., Tokyo, Japan) as recently reported (17). Briefly, samples were passed through a copperized cadmium reduction column where NO_3^- was reduced to NO_2^-, which reacts with Griess reagent (1% sulfonamide/0.1% N-1-naphthylethylenediamine dihydrochloride/5% HCl) to form a purple azo dye. The absorbance at 540 nm was detected by a flow-through visible spectrophotometer (model S/3250; Somakogaku, Tokyo, Japan).

Preparation of cDNA probe for iNOS. iNOS cDNA probe for Northern blot analysis was prepared by PCR as reported (18). Antisense and sense oligonucleotides of PCR primers for mouse iNOS were synthesized according to the sequences (GATCAGGAACCT-GAAGCCCC and GCCCTTTTTTGCCCCATAGG) corresponding to amino acid residues 942–948 and 1127–1134, respectively (12). The PCR products were amplified using mouse liver total RNA by 40 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1.5 min, and extension at 72°C for 2 min. The expected size of PCR product (577 bp) was ligated into the pGEM-3Zf($^-$) plasmid vector (Promega Biotec, Madison, WI) by T/A cloning method (19). Nucleotide sequence of the subcloned cDNA was determined by the dideoxy chain termination method (20), which coincided with that of iNOS as reported (12). An insert of BamHI-EcoRI restriction fragment of iNOS plasmids was used as a probe for Northern blot analysis.

RNA preparation and Northern blot analysis. Confluent VSMC monolayers (1.5×10^7 cells) in 60-mm culture dishes (Falcon Labware, Oxnard, CA) preincubated in serum-free DME for 24 h were usually incubated in 2 ml fresh serum-free DME with or without compounds for 6 h. For measurement of iNOS mRNA stability, cells were incubated with either 8-bromo-cAMP (10^{-3} M) or cycloheximide (1

µg/ml) for 6 h, after which actinomycin D (1 µg/ml) was added to stop transcription, and the cells were harvested for the indicated times.

Total cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (21). Northern blot analysis was carried out essentially as previously described (22). Total RNA (20 µg) was separated by formaldehyde/1.1% agarose gel electrophoresis and transferred to a nylon membrane (Magnagraph; Micron Separations Inc., Westborough, MA). The cDNA probes for mouse iNOS and human GAPDH were labeled with [^{32}P]dCTP (111 TBq/mmol) by random-primed labeling method. After ultraviolet wave crosslinking, RNA immobilized on the membrane was hybridized with the labeled probes in the presence of 50% formamide at 42°C > 16 h. The membrane was washed finally in 0.1 × SSPE (15 mM NaCl, 1 mM NaH_2PO_4, 0.1 mM ethylenediaminetetraacetic acid)/0.5% sodium dodecyl sulfate at 37°C, and autoradiographed with intensifying screens at -80°C. Radioactivities of the membranes were measured by an imaging analyzer (BAS2000; Fuji Photo Film Co. Ltd., Tokyo, Japan).

Statistical analysis. Data are expressed as mean±SE. Statistical analysis was performed by unpaired Student's t test. $P < 0.05$ was considered statistically significant.

Results

As shown in Fig. 1 *A*, 8-bromo-cAMP (10^{-3} M) stimulated NOx production in rat VSMC in a time-dependent manner; about 8–13-fold increases over control levels were observed during 6–24 h incubation. Northern blot analysis of rat VSMC RNA with mouse iNOS cDNA as a probe revealed a single hybridizing band corresponding to the size of mouse iNOS mRNA (4.4 kb) (Fig. 1 *B*); 8-bromo-cAMP (10^{-3} M) increased steady-state levels of iNOS mRNA after 6 h, which further increased after 24 h incubation.

8-Bromo-cAMP significantly increased NOx production in a dose-dependent manner (10^{-4}–10^{-3} M) (Fig. 2 *A*); about twofold (10^{-4} M) and fourfold (10^{-3} M) increases over control levels during 12 h incubation. 8-bromo-cAMP also dose dependently increased steady-state iNOS mRNA levels (Fig. 2 *B*); a maximal effect was induced with 10^{-3} M.

To determine whether NOS is activated by 8-bromo-cAMP, the effects of a specific NOS inhibitor (L-NMMA), L- and D-arginine on 8-bromo-cAMP-induced NOx production were studied (Fig. 3). L-NMMA (10^{-3} M) completely blocked the 8-bromo-cAMP–induced NOx production, whose effect was partially but significantly ($P < 0.05$) reversed by an excess

A

B
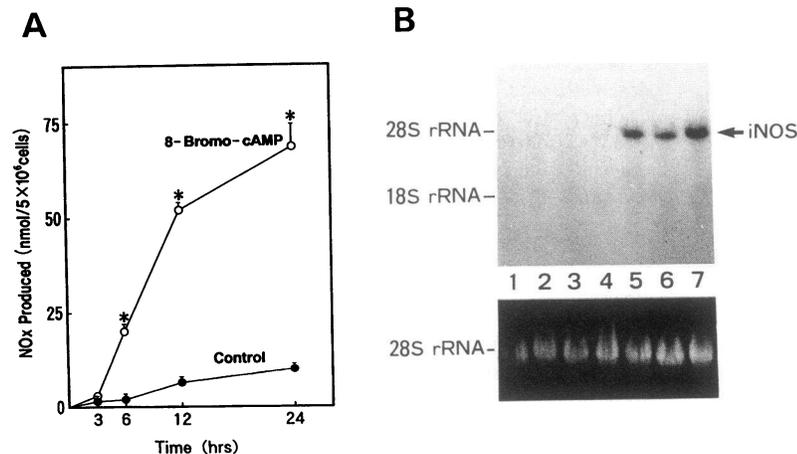
Figure 1. Effect of 8-bromo-cAMP on NOx production and iNOS mRNA expression in rat vascular smooth muscle cells as a function of time. (*A*) VSMCs were incubated with (o) or without (●) 8-bromo-cAMP (10^{-3} M) for the time indicated for measurement of NOx. Each point is the mean of three experiments; bar indicates SE. *$P < 0.05$ vs control. (*B*) Northern blot analysis of iNOS mRNA; total cellular RNA (20 µg) was hybridized with cDNA for mouse iNOS as a probe (*upper panel*) and 28S ribosomal RNA loaded in each lane is shown as an internal standard (*lower panel*). Lane *1*, control; lane *2*, 8-bromo-cAMP (10^{-3} M) 1 h; lane *3*, 3 h; lane *4*, 6 h; lane *5*, 12 h; lane *6*, 24 h.

A

8- Bromo- cAMP

B
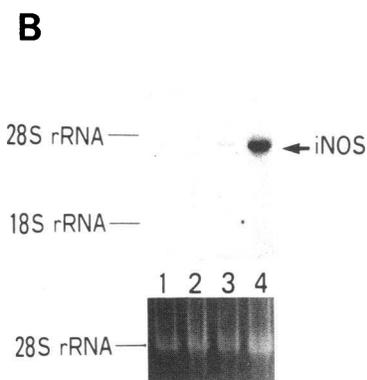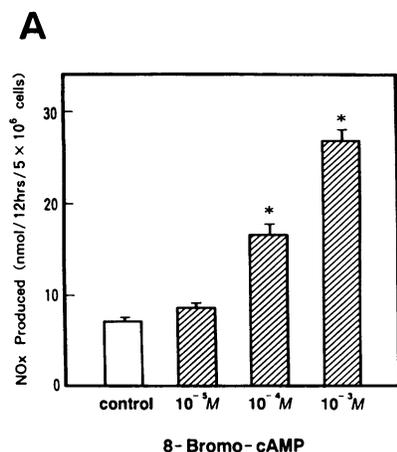
Figure 2. Dose response effect of 8-bromo-cAMP on NOx production and iNOS mRNA expression in rat vascular smooth muscle cells. (*A*) VSMCs were incubated with various concentrations (10^{-5}–10^{-3} M) of 8-bromo-cAMP for 12 h for measurement of NOx and 6 h for Northern blot analysis. Each column is the mean of three experiments; bar indicates SE. *P < 0.05 vs control. (*B*) Northern blot analysis of iNOS mRNA (*upper panel*) and 28S ribosomal RNA (*lower panel*) are shown in the same as in Fig. 1. Lane *1*, control; lane *2*, 8-bromo-cAMP (10^{-5} M); lane *3*, 8-bromo-cAMP (10^{-4} M); lane *4*, 8-bromo-cAMP (10^{-3} M).

(10^{-2} M) L-arginine, but not by D-arginine. L-arginine (5 × 10^{-3} M) also failed to fully reverse the inhibitory effect by lower concentration (5 × 10^{-4} M) of L-NMMA on cAMP-induced NOx production (data not shown).

To determine whether an intracellular cAMP affects NOx production by cultured VSMC, several compounds that increase intracellular cAMP levels, such as IBMX, forskolin, and cholera toxin, were tested. IBMX (10^{-4} M), forskolin (10^{-5} M), and cholera toxin (100 ng/ml) stimulated NOx production by 2.5-, 3-, and 6-fold above control levels, respectively (Fig. 4).

Dexamethasone (10^{-7} M) completely blocked the 8-bromo-cAMP–induced NOx production (Fig. 5 *A*) and reduced the cAMP-induced iNOS mRNA (Fig. 5 *B*). Both actinomycin D (1 μg/ml) and cycloheximide (1 μg/ml) completely blocked the 8-bromo-cAMP–induced NOx production (Fig. 6 *A*). Northern blot analysis showed that actinomycin D completely abolished the induction of iNOS mRNA by 8-bromo-cAMP, whereas cycloheximide remarkably increased the basal, as well as the stimulated iNOS mRNA expression (Fig. 6 *B*). Cycloheximide dose dependently (0.1-1 μg/ml) increased steady-state levels of iNOS mRNA during 6 h incubation with

the maximal effective dose of 1 μg/ml (Fig. 7). Actinomycin D (1 μg/ml) completely abolished the cycloheximide-induced iNOS mRNA, but dexamethasone (10^{-7} M) failed to affect the cycloheximide-induced iNOS mRNA (Fig. 8).

To determine whether cAMP and cycloheximide increase steady-state iNOS mRNA levels by decreasing its degradation rate, we studied the decay in iNOS mRNA levels induced by 8-bromo-cAMP (10^{-3} M) or cycloheximide (1 μg/ml) for 6 h before the addition of actinomycin D (1 μg/ml) (Fig. 9). The half-life of iNOS mRNA induced by 8-bromo-cAMP was ~ 2 h. However, the cycloheximide-induced upregulation of iNOS mRNA remained unaltered during 12 h.

Discussion

The present study has clearly demonstrated that 8-bromo-cAMP, a membrane-permeable cAMP derivative, increased NOx production and steady-state iNOS mRNA levels in rat VSMC in a time- and dose-dependent manner (6–24 h and 10^{-4}–10^{-3} M, respectively). The time courses of NOx production and iNOS mRNA induction by cAMP appear to be similar; expression of iNOS mRNA and NOx production were detected after 6 h and increased during 12–24 h. Such a long time
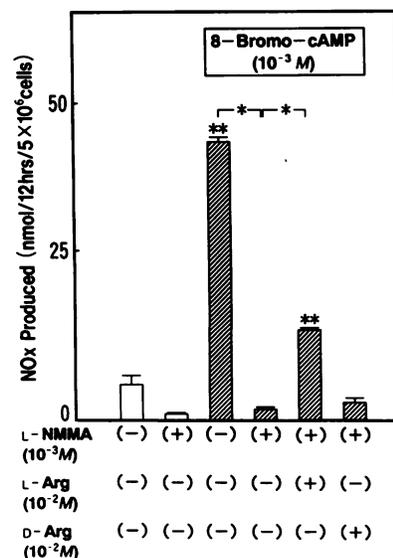
Figure 3. Effects of N^{G}-monomethyl-L-arginine, L- and D-arginine on 8-bromo-cAMP–induced NOx production in rat vascular smooth muscle cells. VSMCs were incubated with (□) or without (□) 8-bromo-cAMP (10^{-3} M) in the absence and presence of L-NMMA (10^{-3} M), L- and D-arginine (10^{-2} M) for 12 h; NOx released into medium was measured. Data are plotted in the same as in Fig. 2 (*n* = 3). *P < 0.05 between groups, **P < 0.05 vs control.
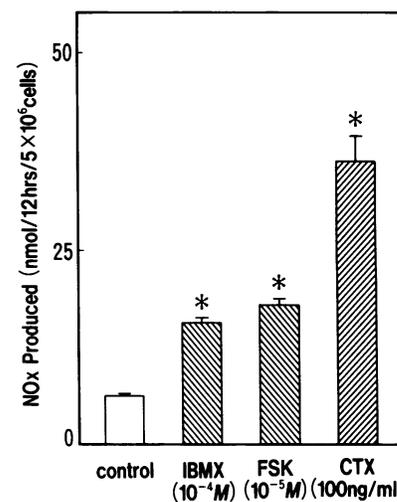
Figure 4. Effect of cAMP-generating agonists on NOx production in rat vascular smooth muscle cells. VSMCs were incubated with 10^{-4} M IBMX, 10^{-5} M forskolin (*FSK*), and 100 ng/ml cholera toxin (*CTX*) for 12 h; NOx released into medium was measured. Data are plotted in the same as in Fig. 2 (*n* = 3). *P < 0.05 vs control.

A

NOx Produced (nmol/12hrs/5×10⁴cells)

50

25

0

| 8-Bromo-cAMP (10⁻³M) | (−) | (+) | (−) | (+) |
| Dexamethasone (10⁻⁷M) | (−) | (−) | (+) | (+) |

B

28S rRNA —

18S rRNA —

1 2 3 4

28S rRNA —
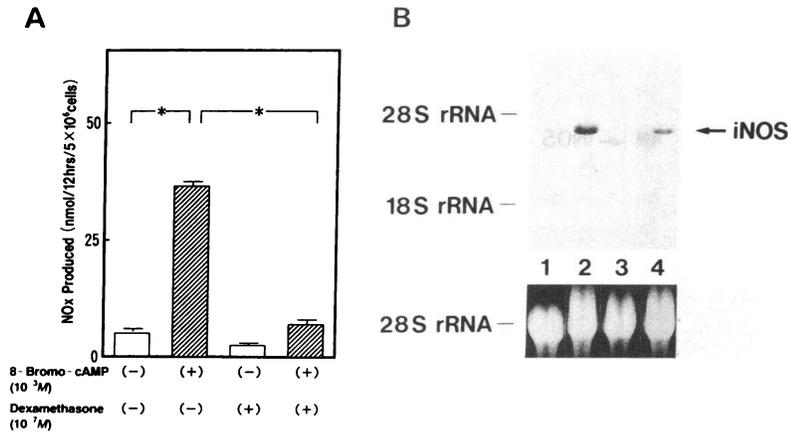
← iNOS

Figure 5. Effect of dexamethasone on 8-bromo-cAMP–induced NOx production and iNOS mRNA expression in rat vascular smooth muscle cells. (*A*) VSMCs were incubated with (▨) or without (□) 8-bromo-cAMP (10^{-3} M) in the absence and presence of 10^{-7} M dexamethasone (DEX) for 12 h for measurement NOx and for 6 h for Northern blot analysis. Data are plotted in the same as in Fig. 2 (*n* = 3). *P < 0.05 between groups. (*B*) Northern blot analysis of iNOS mRNA (*upper panel*) and 28S ribosomal RNA (*lower panel*) are shown. Lane *1*, control; lane *2*, 8-bromo-cAMP; lane *3*, DEX; lane *4*, 8-bromo-cAMP plus DEX.

required for the induction of iNOS mRNA and subsequent NOx production by cAMP in rat VSMC appears to be comparable to that of the cytokines-induced production of NO by mouse macrophages (13) and rabbit VSMCs (6).

The inhibitory effect by L-NMMA, a stereospecific NOS inhibitor, on the 8-bromo-cAMP–induced NOx production and partial reversal of this effect by an excess L-arginine, but not by D-arginine, as demonstrated in this study, is consistent with the notion that cAMP directly stimulates NOS activity to generate NO by catalyzing L-arginine as a substrate in VSMC. However, the reason why an excess substrate of L-arginine could not completely reverse L-NMMA–induced inhibition of NOx production by cAMP remains unknown. Such a partial reversal effect by L-arginine on L-NMMA–mediated inhibition of NOx production by cytokines has already been observed in several cultured cells, including rat VSMC (18), porcine endocardial cells (23), and human hepatocytes (24). Recently, it has been reported that L-NMMA can act as an irreversible inactivator of macrophage iNOS by covalent binding of its metabolite(s) to the enzyme (25). Thus, it is possible to speculate that L-NMMA may interact with iNOS molecule too tightly to be replaced by excess L-arginine in rat VSMC.

The present study has also shown that induction of iNOS mRNA and NOx production by 8-bromo-cAMP were inhibited by dexamethasone. These data are in accordance with the previous reports that dexamethasone inhibits the cytokine-in-

duced NOS activity and NOx generation in mouse macrophage (26) and porcine endothelial cells (27). However, the present result with incomplete inhibition on induction of iNOS mRNA, but complete blockade on NOx production by dexamethasone implies that glucocorticoids may have posttranslational inhibitory effect on iNOS enzyme activity other than iNOS gene expression. In sepsis syndrome, many chemical mediators, such as cytokines, leukotrienes, and prostaglandins, have been considered to be involved in the development of endotoxin shock (28). The induction of iNOS in the vessel wall including macrophages, endothelial cells, and VSMC may contribute to the development of hypotension and hyporesponsiveness to vasoconstrictors associated with endotoxin shock. In fact, we have recently shown that dexamethasone inhibits the IL-1β–induced iNOS mRNA and NOx production in cultured rat VSMC (18). Furthermore, it has been recently reported that dexamethasone has inhibitory effect on the cytokine-induced iNOS mRNA in rat hepatocytes (29). Taken together, the present results lend strong support to the observation that glucocorticoids can prevent hypotension and restore the vascular responsiveness in endotoxin shock (30).

It has been reported that cAMP plays an intracellular role as a second messenger for IL-1α and IL-1β in human natural killer–like cell line (YT) and murine thymocytes (31). Therefore, it is suggested that cAMP may be one of the intracellular second messengers for IL-1β in rat VSMC to induce iNOS

A

NOx Produced (nmol/12hrs/5 × 10⁵ cells)

| AMD (1 μg/ml) | CHX (1 μg/ml) |

30

20

10

0

8 Bromo cAMP (10⁻³M) (−X+) (−X+) (−X+)

B

28SrRNA —

18SrRNA —

1 2 3 4 5 6

18SrRNA —
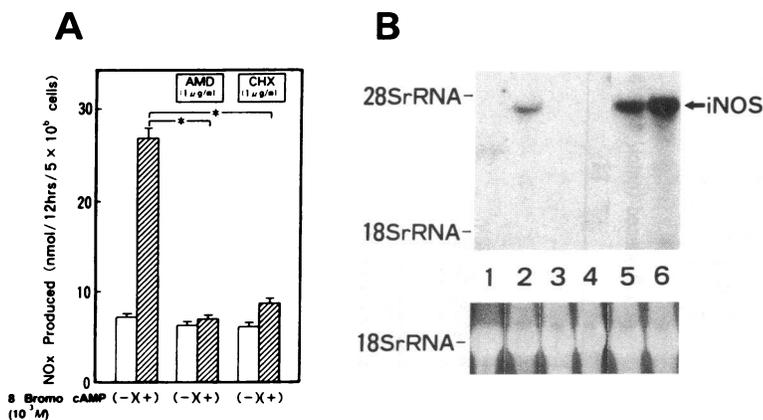
← iNOS

Figure 6. Effects of actinomycin D and cycloheximide on 8-bromo-cAMP–induced NOx production and iNOS mRNA expression in rat vascular smooth muscle cells. (*A*) VSMCs were incubated with (▨) or without (□) 8-bromo-cAMP (10^{-3} M) in the absence and presence of 1 μg/ml each of actinomycin D (*AMD*) and cycloheximide (*CHX*) for 12 h for measurement of NOx and for 6 h for Northern blot analysis. Data are plotted in the same as in Fig. 2 (*n* = 3). *P < 0.05 between groups. (*B*) Northern blot analysis of iNOS mRNA (*upper panel*) and 18S ribosomal RNA (*lower panel*) are shown. Lane *1*, control; lane *2*, 8-bromo-cAMP; lane *3*, AMD; lane *4*, 8-bromo-cAMP plus AMD; lane *5*, CHX; lane *6*, 8-bromo-cAMP plus CHX.
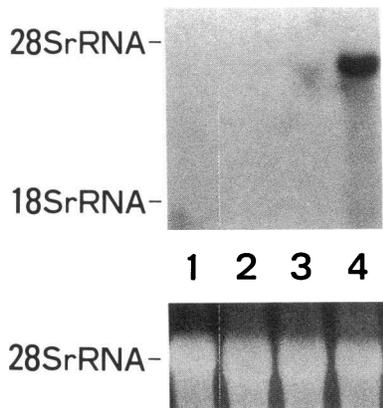
Figure 7. Dose response effect of cycloheximide on iNOS mRNA expression in rat vascular smooth muscle cells. VSMCs were incubated with various concentrations (0.01–1 μg/ml) of cycloheximide (CHX) for 6 h. Northern blot analysis of iNOS mRNA (*upper panel*) and 28S ribosomal RNA (*lower panel*) are shown. Lane *1*, control; lane *2*, CHX (0.01 μg/ml); lane *3*, CHX (0.1 μg/ml); lane *4*, CHX (1 μg/ml).

mRNA and iNOS synthesis. Our study has further shown that actinomycin D, an RNA transcriptional inhibitor, completely inhibited iNOS mRNA induction and NOx production by cAMP, suggesting the importance of cAMP in the transcriptional regulation of iNOS gene. Cycloheximide, a protein synthesis inhibitor, also completely inhibited the cAMP-induced NOx production. Surprisingly, cycloheximide alone remarkably and dose dependently increased the steady-state levels of iNOS mRNA (superinduction), whose effect was abolished by previous addition of actinomycin D, but not of dexamethasone. Furthermore, the decay in the cycloheximide-induced upregulation of iNOS mRNA after the addition of actinomycin D was not observed during 12 h. Molecular cloning of mouse iNOS (12) revealed that the 3′-noncoding region of the mouse iNOS mRNA contains "AUUUA" motif known as a selective mRNA destabilizing sequence (32). An outstanding feature of the specific mRNA degradation process directed by this motif is its sensitivity to mRNA translation inhibitor, cycloheximide. Taken together, it is suggested that cycloheximide stabilizes iNOS mRNA by inhibiting the synthesis of a labile
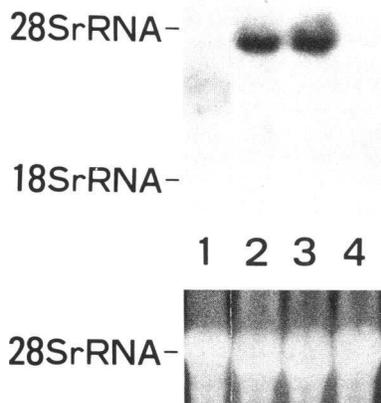
Figure 8. Effects of dexamethasone and actinomycin D on cycloheximide-induced iNOS mRNA expression in rat vascular smooth muscle cells. VSMCs were incubated with 1 μg/ml CHX in the absence and presence of 10^{-7} M dexamethasone or 1 μg/ml actinomycin D for 6 h. Northern blot analysis of iNOS mRNA (*upper panel*) and 28S ribosomal RNA (*lower panel*) are shown. Lane *1*, control; lane *2*, CHX; lane *3*, CHX plus DEX; lane *4*, CHX plus AMD.

protein(s) responsible for its degradation, leading to a marked accumulation of iNOS mRNA.

It has been reported that cNOS activated by Ca^{2+}-mobilizing agents, such as bradykinin, endothelin-3, and Ca^{2+} ionophore, can be augmented by forskolin and phosphodiesterase inhibitor in rodent neuronal cell line (33) and by cholera toxin in porcine endothelial cells (34). Conversely, there are some reports showing that neither a selective cAMP-phosphodiesterase inhibitor (AH21-132), prostacyclin, nor chlorophenylthio-cAMP affect the release of NO from bovine endothelial cells (35) or the enzyme activity of purified rat brain cNOS (7). However, there is no information yet available on the regulation of iNOS by cAMP. This is the first report that cAMP plays a key role in the regulation of iNOS gene in rat VSMC. Upregulation of iNOS mRNA by cAMP is most likely caused by decreased mRNA destabilization and/or increased transcription. To address the former possibility, the decay of cAMP-induced iNOS mRNA was measured by Northern blot analysis. The half-life of the message was ∼ 2 h, which is comparable to that of LPS-induced iNOS mRNA in murine macrophage (∼ 3 h) (36). Thus, the enhanced expression of iNOS mRNA by cAMP is least likely caused by increased mRNA stability. However, to answer the latter possibility nuclear run-on transcription analysis is needed for the transcriptional control of iNOS gene by cAMP.

It is generally recognized that the second messenger cAMP mediates its hormonal induction of numerous genes through a specific PKA that phosphorylates a 43-kD nuclear phosphoprotein, termed "cAMP response element binding protein," which is a transcriptional factor which binds to a consensus palindromic motif "TGACGTCA", termed cAMP response element, to initiate gene transcription (37, 38). Thus, the question as to whether iNOS gene contains *cis*-acting cAMP response element sequence in the promoter region through which cAMP regulates transcriptional activity needs to be determined by a chloramphenicol acetyltransferase or a luciferase reporter assay.

The present study has shown that various cAMP-generating agonists, including phosphodiesterase inhibitor (IBMX), adenylate cyclase activator (forskolin), and stimulatory guanine nucleotide binding protein regulatory protein activator (cholera toxin), all stimulated NOx production in rat VSMC. These data lend strong support to the contention that an intracellular cAMP plays a second messenger role in the induction of iNOS gene in rat VSMC. VSMC has various receptors for vasodilators, such as β-adrenergic agonist, calcitonin gene-related peptide, and prostacyclin, through which these agonists can increase intracellular cAMP levels. These agonists cause an immediate vasorelaxation via cAMP-dependent mechanism; PKA phosphorylates membrane Ca^{2+}-ATPase (Ca^{2+} pump) to decrease intracellular Ca^{2+} levels and myosin light chain kinase to decrease myosin light chain kinase activities (39, 40). Our data provide an additional and possible mechanism for the sustained vasorelaxation by these vasodilators via NO production because NO stimulates soluble guanylate cyclase to increase intracellular cGMP levels in VSMC, which in turn activates Ca^{2+} pump leading to vasorelaxation (13, 41). Thus, it is reasonable to speculate that cAMP-induced relaxation of vascular smooth muscle may be mediated by a cross-talk of signal transduction system via cAMP- and cGMP-dependent pathways.

A

8-Bromo-cAMP
(10^{-3}M)

iNOS

GAPDH

0 1 3 6 (hrs)

CHX
(1μg/mℓ)

iNOS

GAPDH

0 1 6 12 (hrs)

B
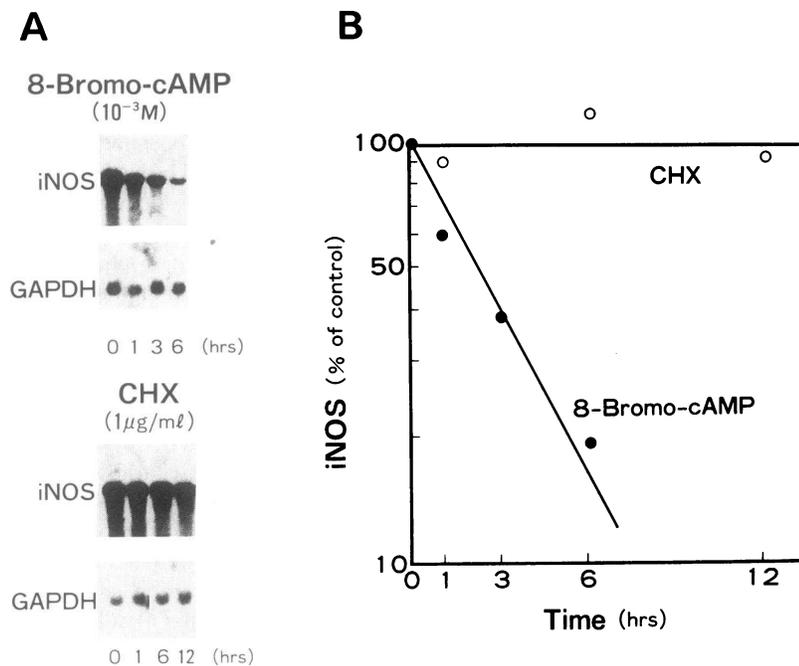
Figure 9. Decay in 8-bromo-cAMP- and cyclohexi-mide-induced iNOS mRNA expression in rat vascular smooth muscle cells. VSMCs were incubated with 10^{-3} M 8-bromo-cAMP or 1 μg/ml CHX for 6 h before addition of 1 μg/ml actinomycin D and harvested at the time indicated. (A) Northern blottings of VSMC treated with 8-bromo-cAMP (top) and CHX (bottom) with cDNAs for iNOS (upper lane) and GAPDH (lower lane) as probes are shown. (B) The relative percentage of iNOS mRNA to GAPDH mRNA measured by an imaging analyzer in log scale is plotted against time. ●, 8-bromo-cAMP; o, CHX.

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