Role of Phosphodiesterase 3 in NO/cGMP-Mediated Antiinflammatory Effects in Vascular Smooth Muscle Cells

Toru Aizawa, Heng Wei, Joseph M. Miano, Jun-ichi Abe, Bradford C. Berk, Chen Yan

Abstract—Atherosclerosis involves cellular immune responses and altered vascular smooth muscle cell (VSMC) function. Nitric oxide (NO)/cGMP is uniquely capable of inhibiting key processes in atherosclerosis. In this study, we determined the effects of NO/cGMP and their molecular mechanisms in the regulation of NF-κB–dependent gene expression in VSMCs. We found that cGMP-elevating agents such as the NO donor S-nitroso-N-acetylpenicillamine (SNAP) and C-type natriuretic peptide (CNP), reduced TNF-α–induced NF-κB–dependent reporter gene expression in rat aortic VSMCs in a cGMP-dependent manner. The effects of SNAP and CNP on NF-κB are mediated by cAMP-dependent protein kinase (PKA) but not cGMP-dependent protein kinase (PKG) based on the findings that the selective PKA inhibitor, PKI, abolished the effects of SNAP and CNP on NF-κB, whereas the PKG inhibitor Rp-8-Br-PET-cGMP had no effect. Inhibition of cGMP-inhibited cAMP-hydrolyzing phosphodiesterase 3 (PDE3) blocked SNAP- and CNP-elicted effects on NF-κB–dependent transcription. Furthermore, cGMP analogues such as 8-pCPT-cGMP, which selectively activates PKG but does not inhibit PDE3, had no effect on NF-κB–mediated transcription. Activation of PKA by SNAP or cAMP-elevating agents not only inhibited TNF-α–induced NF-κB–dependent reporter gene expression but also reduced endogenous NF-κB–dependent adhesion molecule and chemokine expression. These results suggest that SNAP and CNP exert inhibitory effects on NF-κB–dependent transcription by activation of PKA via cGMP-dependent inhibition of PDE3 activity. Therefore, PDE3 is a novel mediator of inflammation in VSMCs. (Circ Res. 2003;93:406–413.)

Key Words: nitric oxide ■ nuclear factor-κB ■ cGMP ■ phosphodiesterases

Many investigators define the progression of the atherosclerotic lesion as a chronic inflammatory process in the vessel wall. Vascular endothelial cells and vascular smooth muscle cells (VSMCs) are both important targets for inflammatory cytokines and themselves are capable of producing significant amounts of cytokines, chemokines, and adhesion molecules. The expression of adhesion molecules and chemokines by VSMC may facilitate the accumulation of transmigrated leukocytes within the vascular wall. The role of the transcription factor, nuclear factor-κB (NF-κB), in inflammation has been well documented. Many of the genes that are regulated by NF-κB in VSMCs encode for inflammatory molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1), which may lead to atherosclerosis.

In the vasculature, NO not only functions as a vasodilator, but also inhibits several key events in atherosclerosis. Several lines of evidence suggest that NO serves as an antiatherogenic molecule. The development of atherosclerotic lesions is accelerated by NO synthase (NOS) inhibitors and attenuated by overexpression of endothelial NOS (eNOS). Deletion of eNOS gene in the background of ApoE deficient mice results in hypertension and increased atherosclerosis. Many cellular effects of NO are cGMP-dependent. Although PKG is a well-known cGMP target, cGMP also interacts with several other molecules in the cell, such as cGMP-gated ion channels and the guanine nucleotide exchange factor CNras-GEF. In addition, the effects of cGMP could involve modulation of cAMP levels and PKA activity via stimulating PDE2 or inhibiting PDE3 activity. High concentrations of cGMP may also directly stimulate PKA as well. Moreover, several cGMP-independent NO mechanisms have also been defined, such as S-nitrosylation of cysteine residues of target proteins. Therefore, due to the multiple effector molecules of NO and cGMP, the mechanisms by which NO and cGMP regulate VSMC function have been controversial.

Phosphodiesterases (PDEs) play critical roles in controlling intracellular cAMP and cGMP. PDEs constitute a large superfamily of enzymes grouped into 11 broad families based on their distinct kinetic properties, regulatory mechanisms, and sensitivity to selective inhibitors. Four major families of PDEs have been identified in VSMCs, including Ca2+/calmodulin-stimulated PDE1, cGMP-inhibited PDE3, cAMP-specific PDE4, and cGMP-specific PDE5. PDE3 and PDE4 account for the majority of the cAMP-hydrolyzing PDE activity (>80%) in VSMCs. PDE3 binds to both cAMP and...
cGMP with high affinities but the $V_{\text{max}}$ for hydrolyzing cGMP is at least 10-fold lower than that for cAMP, which allows cGMP to act as a potent competitive inhibitor of cAMP hydrolysis by PDE3.12

The purpose of this study was to determine the mechanisms by which NO/cGMP regulates NF-κB–mediated gene expression in VSMCs. We found that NO and CNP, via cGMP, inhibits NF-κB–dependent transcriptional activity in rat aortic VSMCs. The effects of NO and CNP are mediated by cGMP-dependent activation of PKA, but not of PKG. PDE3, a cGMP-inhibited cAMP-hydrolyzing PDE, plays an important role in the cross-activation of PKA by cGMP. The finding that PDE3 plays a role in mediating the effect of NO/cGMP in the inhibition of NF-κB–dependent gene expression suggests that PDE3 inhibitors may be therapeutically useful in the prevention of atherosclerosis.

Materials and Methods

Reagents
SNAP, ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one), and retro-combinant PKGlo were purchased from Calbiochem and CNP was from Sigma. 8-Br-cAMP, Sp-8-CPT-cAMP, 8-Br-cGMP, 8-pCPT-cGMP, and Rp-8-Br-PET-cGMP were purchased from Biolog. Milrinone, cilostamide, and rolipram were purchased from Biomol. cGMP, and Rp-8-Br-PET-cGMP were purchased from Biolog.

Cell Culture
Rat aortic VSMCs were isolated by enzymatic dissociation method and maintained as described previously.14 Cells (passage 5 to 12) were growth-arrested for 48 hours before the indicated treatments. Cell identity was confirmed by smooth muscle marker α-actin immunostaining. More than 98% of cells were positive for α-actin.

Dual Luciferase Reporter Assay
Cells were seeded in 12-well plates (1.0 5 10⁵ cells/well) overnight and transiently transfected with NF-κB–luciferase reporter plasmid (Promega), control luciferase construct pRL-TK (Promega), and/or other DNA constructs as indicated using FuGENE6 Transfection Reagent (Roche). The total DNA is 1 µg per well. Transfected cells were serum-starved for 48 hours followed by exposure to tumor necrosis factor-α (TNF-α; Roche) for 6 hours. Firefly and Renilla luciferase activities in cell extracts were measured using Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity was then calculated by normalizing NF-κB promoter-driven firefly luciferase activity to control Renilla luciferase activity. Data from all experiments are presented as the relative luciferase activity (mean ± SE) from at least three independent sets of experiments, each with triplicate measurements.

cGMP and PDE Assays
Cyclic GMP levels were measured using a cGMP colorimetric kit (Assay Designs, Inc) as described in the manufacturer’s protocol. PDE assays were performed according to established procedures.15 All assays were performed at 30°C using either 1 µmol/L cAMP or 1 µmol/L cGMP as substrates.

Western Blot Analysis
Western blot analysis was performed as we described previously.15 Polyclonal antibody against PKG1α (Stressgen), VCAM-1, α-actin (Santa Cruz), VASP (Alexis), and phosphospecific monoclonal antibody against phospho-Ser239 VASP (Alexis) were used.

ELISA
VSMCs were stimulated with TNF-α for 8 hours. MCP-1 protein levels in the cultured media were measured by a Rat MCP-1 ELISA kit (Pierce) based on the manufacturer’s protocol.

Relative Quantitative RT-PCR
Total RNA isolation, first-strand cDNA synthesis, and relative quantitative reverse transcription–polymerase chain reaction (RT-PCR) using Ambion’s competitor technology were performed as we described.16 Ambion’s competitor technology allows to modulate the amplification of 18S rRNA in the same linear range as the RNAs under studies when amplified under the same condition. The following primers were used for PCR analysis: VCAM-1, 5'-CGTACCTGCCTAAGTGATGG-3' (sense) and 5'-GTGCTCTCCTCTGTGACGC T-3' (antisense); MCP-1, 5'-ATGCAGGTCTCTGTCACG-3' (sense) and 5'-CTAGTTCCTCTGTCACTAT-3' (antisense).

Statistical Analysis
Data are shown as mean ± SE. Differences were analyzed with one-way analysis of variance (ANOVA). Comparison of two populations was made by Student’s unpaired t test. A value of $P<0.05$ was considered statistically significant.

Results

Effects of cGMP-Elevating Agents and PKG Activators on TNF-α–Induced NF-κB–Dependent Transcription
To determine the effect of NO and cGMP on TNF-α–induced NF-κB activation, we measured NF-κB–dependent reporter gene luciferase activity in rat aortic VSMCs. As shown in Figure 1A, NF-κB–dependent luciferase activity was stimulated by TNF-α. TNF-α activation of NF-κB was significantly inhibited by the cGMP-elevating agents SNAP (a NO donor) and CNP, which are activators of soluble guanylyl cyclase (GC) and particulate GC, respectively (Figure 1A). Although cGMP mediates most known effects of NO, some cGMP-independent NO effects have also been reported.8 To determine the role of cGMP in SNAP-mediated regulation of NF-κB activation, we used ODQ, which specifically inhibits NO-sensitive soluble GC but not particulate GC. As expected, ODQ blocked the inhibitory effect of SNAP, but not CNP, on TNF-α–induced luciferase activity (Figure 1A). In addition, cGMP levels were greatly increased by SNAP or CNP and ODQ inhibited cGMP-elevation induced only by SNAP (Figure 1B). Given that cGMP is the only known mediator of CNP, these observations suggest strongly that cGMP mediates the effects of NO and CNP on NF-κB–dependent gene expression in VSMC.

To examine if cGMP activation of PKG mediates the effects of SNAP and CNP on NF-κB, we used two cGMP analogues that are PKG activators, 8-Br-cGMP and 8-pCPT-cGMP (a more cell-permeable and PDE-resistant analogue than 8-Br-cGMP). Interestingly, both cGMP analogues had no significant effect on TNF-α–induced luciferase activity (Figure 1C). These results suggest that the effects of SNAP and CNP are unlikely to be mediated by activation of PKG.

To further examine the role of PKG in regulation of NF-κB–dependent transcriptional activity, the PKG-specific inhibitor Rp-8-Br-PET-cGMP was used. Inhibition of PKG did not alter SNAP or CNP inhibition of NF-κB–dependent luciferase activity (Figure 1D), confirming that PKG is not
involved in SNAP- and CNP-mediated regulation of NF-κB–dependent transcriptional activity. The effectiveness and specificity of Rp-8-Br-PET-cGMP to inhibit PKG was confirmed by specific inhibition of PKG-dependent but not PKA-dependent phosphorylation of VASP (vasoconstriction-stimulated-phosphoprotein) (data not shown). These results support strongly the hypothesis that cGMP but not PKG, mediates the effects of NO and CNP on NF-κB–dependent gene expression in VSMC.

Expression of PKG in Subcultured Rat Aortic VSMCs

It has been reported that PKG expression may gradually decline to nearly undetectable levels when adult rat aortic VSMCs are subcultured in vitro. To determine whether the PKG-independence of SNAP- and CNP-mediated effects on NF-κB was due to the lack of PKG, the expression level of PKG was examined by Western blot analysis in our subcultured rat aortic VSMCs isolated by the enzymatic dissociation method. As shown in Figure 2, PKGI was highly expressed in our subcultured rat aortic VSMCs up to passage 15 in contrast to rat aortic VSMCs isolated by explant method, consistent with the previous observation. This result suggests that the PKG-independence of SNAP and CNP is not due to the lack of PKG.

Role of PKA in SNAP- and CNP-Mediated Inhibition of NF-κB-Dependent Transcription

In addition to regulating PKG, cGMP may modulate PKA activity directly or indirectly. To determine the role of PKA in SNAP- or CNP-mediated inhibition of NF-κB, we inhibited PKA by transducing VSMCs with adenovirus-mediated PKA inhibitor PKI (Ad-PKI). PKI, a 75-amino acid heat-stable protein, binds to the catalytic subunit of PKA and inhibits PKA activity with high affinity and high specificity, providing more selective inhibition of PKA than pharmacological inhibitors. The inhibitory effects of SNAP and CNP on NF-κB–dependent luciferase activity were significantly
blocked by the overexpression of Ad-PKI but not Ad-LacZ (Figure 3A). These results suggest that activation of PKA is important for the effects of SNAP and CNP on NF-κB-dependent transcriptional activity.

To further examine the role of PKA activation in regulation of NF-κB-dependent transcription, PKA was also activated by specifically increasing intracellular cAMP using two cAMP analogues (8-Br-cAMP and Sp-8-CPT-cAMP) and two adenylyl cyclase activators (isoproterenol and forskolin). As shown in Figure 3B, activation of PKA significantly inhibited NF-κB-dependent luciferase activity. In addition, overexpression of the constitutively active PKA catalytic subunit (PKAc) also significantly inhibited NF-κB-dependent luciferase activity (Figure 3C). These results suggest that activation of PKA is necessary and sufficient to negatively modulate NF-κB-dependent gene expression in rat aortic VSMCs.

**Differential Effects of cGMP-Elevating Agents and cGMP Analogues on PKA Activation in Rat Aortic VSMCs**

These experiments suggest that the difference in NF-κB-dependent transcription between cGMP analogues versus native cGMP-elevating agents (such as SNAP and CNP) is a function of PKA activation. To determine the effects of SNAP, CNP, and 8-pCPT-cGMP on PKA activation, we measured phosphorylation of VASP, a well-characterized substrate for both PKA and PKG (Figure 4A).18 However, at high levels of cyclic nucleotides or their analogues, both sites may be phosphorylated by either protein kinase (Figure 4A).18 Phosphorylation of Ser157 leads to a shift in the apparent molecular weight from 46 to 50 kDa on two adenylyl cyclase activators (isoproterenol and forskolin). As shown in Figure 3B, activation of PKA significantly inhibited NF-κB–dependent luciferase activity. In addition, overexpression of the constitutively active PKA catalytic subunit (PKAc) also significantly inhibited NF-κB–dependent luciferase activity (Figure 3C). These results suggest that activation of PKA is necessary and sufficient to negatively modulate NF-κB–dependent gene expression in rat aortic VSMCs.
 SDS-PAGE, which is detectable by Western blot analysis using anti-VASP antibody (Figure 4B). Phosphorylation of Ser239 can be detected using a phospho-VASP monoclonal antibody that recognizes the 46-kDa band phosphorylated on Ser239 alone, as well as the 50-kDa band phosphorylated on both Ser239 and Ser157 (Figure 4B). Therefore, we could determine which of the two sites was phosphorylated by Western blot using the combination of Ser239 phosphospecific VASP antibody and total VASP antibody.

As shown in Figure 4C, in control VSMCs, there is low level of PKA and PKG activity (lane 1). The phosphorylation levels of VASP at Ser 157 and Ser239 were differentially upregulated by 8-pCPT-cGMP, CNP, or SNAP (lanes 2 to 4). Sp-8-CPT-cAMP, a PKA activator, increased VASP phosphorylation primarily at PKA–preferable Ser157 (lane 9 versus 10), which was inhibited in the presence of the PKA inhibitor, PKI (lane 10 versus 11). 8-pCPT-cGMP, a PKG activator, increased VASP phosphorylation primarily at Ser239 and to a much lesser extent at Ser157 (lanes 2 versus 1 and 13 versus 12). Both Ser239 and Ser157 phosphorylation were mediated by PKG because they were inhibited by the PKG inhibitor Rp-8-Br-PET-cGMP (lane 14 versus 13) but not by the PKA inhibitor PKI (lane 6 versus 2), indicating that 8-pCPT-cGMP activates PKG but not PKA. CNP and SNAP significantly increased both Ser157 and Ser239 phosphorylation (lanes 3 and 4 versus 1). Ser157 phosphorylation but not Ser239 phosphorylation was significantly inhibited by PKA inhibitor PKI (lanes 7 versus 3 and 8 versus 4), indicating that SNAP and CNP are able to increase PKA-dependent phosphorylation in addition to PKG-dependent phosphorylation of VASP. White et al. have previously shown using direct measurements of PKA activity that NO donors stimulated cross-activation of PKA in coronary arteries although the precise molecular mechanism that mediates cross-activation of PKA was not examined. Taken together, by using a combination of PKA- or PKG-selective inhibitors and measuring VASP phosphorylation, we have found that 8-pCPT-cGMP, SNAP, and CNP differentially regulate PKA and PKG activation. 8-pCPT-cGMP preferentially activates PKG, whereas SNAP and CNP activate both PKA and PKG.

**Role of PDE3 in Mediating the Effects of SNAP and CNP on NF-κB–Dependent Transcription**

Activation of PKA by cGMP-elevating agents (specifically SNAP and CNP) may occur via two mechanisms: (1) the direct stimulation of PKA by high concentrations of cGMP and/or (2) indirect stimulation of PKA through inhibition of cGMP-inhibited cAMP-hydrolyzing PDE3 resulting in increased intracellular cAMP. We hypothesized that the effects of SNAP and CNP on regulation of NF-κB are mediated by inhibition of PDE3 and activation of PKA. To test this hypothesis, we pharmacologically eliminated PDE3 function in cells by using two structurally different PDE3-selective inhibitors (milrinone and cilostamide). The concentrations of milrinone and cilostamide used in this study have no significant inhibitory effects on other cAMP-hydrolyzing PDEs such as PDE1 and PDE4 present in VSMC (see online Figure S1, available in the online data supplement at http://www.circresaha.org). Rolipram, a selective inhibitor of PDE4 (cGMP-independent), was used as a negative control. PDE3 and PDE4 account for the majority of the cAMP-hydrolyzing PDE activity in VSMCs (Table). Inhibition of PDE3 (with milrinone or cilostamide) or PDE4 (with rolipram) dose-dependently decreased TNF-α–induced NF-κB–dependent reporter gene expression (Figure 5A), consistent with the roles of PDE3 and PDE4 in regulation of intracellular cAMP levels in VSMC. As shown in Figure 5B, SNAP, CNP, and isoproterenol inhibited TNF-α–induced NF-κB–dependent

<table>
<thead>
<tr>
<th>cAMP-PDE Activity, pmol/min per mg Total Protein</th>
<th>PDE3 Activity</th>
<th>PDE4 Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cAMP-PDE Activity</td>
<td>56.8±0.08</td>
<td>36.8±0.07</td>
</tr>
</tbody>
</table>

Activities are mean±SE of cAMP-hydrolyzing PDE activities in the total cell lysates in the absence or presence of milrinone (3 μmol/L) or rolipram (10 μmol/L). PDE3 or PDE4 activity is assayed by milrinone-inhibited or rolipram-inhibited PDE activity, respectively.

**Figure 5.** PDE3 mediates the inhibitory effects of SNAP and CNP inhibition of TNF-α–induced NF-κB transcriptional activity. A, VSMCs transfected with NF-κB–Luc reporter plasmid were pretreated with different concentrations of milrinone, cilostamide, and rolipram as indicated and stimulated with TNF-α (10 ng/mL) for 6 hours followed by luciferase assays. B, Transfected VSMCs were pretreated without or with PDE3 inhibitors milrinone (30 μmol/L), or cilostamide (10 μmol/L), or rolipram (30 μmol/L) for 30 minutes. Cells were then treated with CNP (100 nmol/L), SNAP (100 μmol/L), or isoproterenol (1 μmol/L) for 30 minutes and stimulated with TNF-α. *P<0.05, **P<0.01 vs TNF-α. #P<0.01 vs TNF-α. N.S. indicates no significant difference. C, Cells were treated with different concentrations of milrinone or cilostamide as indicated in the presence or absence of Ad-PKI transduction. Western blot using anti-VASP antibody was performed.
analogue 8-Br-cAMP or SNAP significantly inhibited TNF-α expression by quantitative RT-PCR. Activation of PKA by the cAMP analogue 8-pCPT-cGMP (a selective PKG activator that has no inhibitory effects on PDE3) had no effect on NF-κB-mediated transcription (Figure 1C) further supports the role of PDE3 in the cGMP-induced inhibition of NF-κB activation.

Based on the knowledge that PDE3 is a cAMP-hydrolyzing PDE, we predicted that inhibiting PDE3 activity should increase cAMP and thereby activate PKA. We measured cAMP levels in total cell lysates after treatment with SNAP, CNP, or milrinone. We were not able to detect a significant increase in cell lysates of cAMP levels by SNAP, CNP, or milrinone (data not shown). Similar observations have been reported previously for the effects of NO donors as well as milrinone on cAMP in VSMCs. Failure to detect cAMP is probably because cAMP raised via inhibition of PDE3 is concentrated in a subcellular compartment and not detected in the total cell lysate. However, PKA-dependent VASP phosphorylation was increased after inhibition of PDE3 with both milrinone and cilostamide (Figure 5C). This finding indicates that PKA was activated and suggests that “local” cAMP levels were increased.

**Effects of PKA and PKG on Endogenous Inflammatory Molecule Expression**

To determine the effect of cAMP on expression of endogenous genes regulated by NF-κB, we measured the expression of VCAM-1 and MCP-1, which are known to be regulated by NF-κB in VSMC. TNF-α increased VCAM-1 (Figure 6A) and MCP-1 (Figure 6B) mRNA levels, as assayed by relative quantitative RT-PCR. Activation of PKA by the cAMP analogue 8-Br-cAMP or SNAP significantly inhibited TNF-α-induced VCAM-1 and MCP-1 mRNA levels (Figures 6A and 6B). In contrast, activation of PKG by the cGMP analogue 8-pCPT-cGMP (Figures 6A and 6B) or 8-Br-cGMP (data not shown) had no effect, consistent with effects on exogenous NF-κB–dependent reporter gene expression. In addition, the change in VCAM-1 protein expression is consistent with the change in VCAM-1 mRNA as measured by Western blot analysis (Figure 6C). TNF-α–induced MCP-1 protein expression was inhibited by 8-Br-cAMP and SNAP, consistent with the mRNA data. However, MCP-1 protein expression was also inhibited by the PKG-activators 8-pCPT-cGMP and 8-Br-cGMP, which differs from the change in mRNA expression. This observation suggests that cGMP likely regulates MCP-1 protein synthesis, stability, or secretion via a PKG-dependent mechanism, in addition to regulat-

**Discussion**

In this study, we have demonstrated that the inhibitory effects of endogenous cGMP-elevating agents such as SNAP and CNP on NF-κB–dependent transcription are mediated by activation of PKA, not PKG. SNAP- and CNP-induced activation of PKA and inhibition of NF-κB activation are mediated specifically by inhibition of cAMP-hydrolyzing/cGMP-inhibited PDE3 in a cGMP-dependent manner (Figure 7). Our findings are consistent with previous studies suggesting cGMP cross-activation of PKA in VSMCs. Our study using a more comprehensive investigation establishes PDE3 as a critical mediator in NO and cGMP signaling in VSMCs.

The current state of knowledge regarding the mechanisms for cGMP and PKG regulation of VSMC functions is not...
cohesive. One possible explanation is that VSMCs from different species, vascular beds, isolation methods, or subcultured passages may behave differently. For example, the expression of PKG is highly variable among different VSMCs. We and others have demonstrated that rat aortic VSMCs isolated by enzymatic dissociation often maintain higher PKG levels than cells isolated by the explant method (Figure 2). Lack of PKG may switch the target of cGMP, but our observation of PKG-independence in regulation of NF-κB–dependent gene expression makes it unlikely that PKG expression levels play a role. Another potential explanation is that there are multiple targets for cGMP. PKG appears to be the primary receptor protein for cGMP and is believed to be a major mediator in cGMP-dependent regulation of VSMC contractility. However, the role of PKG in other cGMP-mediated VSMC effects remains poorly defined. Our present observations indicate strongly that the activation of PKA and not PKG is the mechanism by which NO and CNP regulate NF-κB–dependent gene expression in rat aortic VSMCs.

cGMP-dependent activation of PKA could be either a direct effect of cGMP on PKA as suggested by Sausbier et al. or an indirect effect via inhibition of cGMP-inhibited cAMP-hydrolyzing PDE3 that increases cAMP and activates PKA. The direct activation of PKA has not yet been confirmed in vivo although Sausbier et al. have speculated that the concentration of cGMP raised by NO donors should be sufficient to activate PKA based on the $K_v$ value for cGMP to activate PKA in vitro. Nevertheless, our finding that the inhibition of PDE3 activity by specific inhibitors blocked the ability of SNAP and CNP to regulate NF-κB supports the model in which PDE3–mediated increase of cAMP and activation of PKA is the major mechanism for SNAP- and CNP-mediated regulation of NF-κB. Failure to detect a significant change in cAMP on SNAP or CNP stimulation is probably due to the fact that the rise in cAMP, secondary to inhibition of PDE3, is highly compartmentalized and insufficient to raise total cellular cAMP. Future techniques to detect cAMP elevations in small subcellular compartments will be necessary to address this problem. This explanation is supported by previous work and our current observations that the change of cAMP due to the inhibition of PDE3 with PDE3 selective inhibitors was barely detected. Our observations of changes in PKA-dependent VASP phosphorylation on SNAP, CNP, or milrinone treatment suggest that VASP phosphorylation appears to be more sensitive than the measurement of cyclic nucleotide levels.

The effects of cAMP on NF-κB activity are dependent on cell types. For example, in myeloid cells, erythroleukemia cells, and osteoblastic cells, cAMP has been demonstrated to stimulate NF-κB activity. In contrast, elevated cAMP and activation of PKA have been shown to inhibit NF-κB–mediated transcription induced by TNF-α in monocytes and vascular endothelial cells. In the present study, we observed that activation of PKA via stimulation of cAMP synthesis by various stimuli (such as isoproterenol and forskolin) or using cAMP analogues significantly inhibited NF-κB–mediated transcription. Overexpression of the constitutively active PKA catalytic subunit also inhibited NF-κB–mediated transcription. Our observations indicate that activation of PKA inhibits NF-κB–mediated transcription in VSMCs.

The role of PKG in the regulation of NF-κB might vary among different cell types. It has been reported that PKG activation positively regulates NF-κB activity in a few nonvascular cell types. For example, in T-lymphocytes, it has been shown that PKG phosphorylates the p65 subunit of NF-κB, which mediates the PKG-dependent increase in the transactivating activity of p65 from the NF-κB consensus sequence. Interestingly it has been recently shown that PKG can be activated by PKC in a cyclic nucleotide-independent manner in HeLa epithelial cells and HEK-293 fibroblasts. The PKC-mediated activation of PKG appears to be different from cGMP analogue-mediated activation of PKG. Therefore, it is possible that in certain cell types, PKG activation by different mechanisms may lead to different PKG-mediated effects. In nonvascular cell types, activation of PKG may lead to an increase in NF-κB–dependent gene expression as demonstrated in VSMCs. The role of PKG in regulating NF-κB activity is not well studied. In the present study, we demonstrated that activation of PKG via cGMP analogues did not significantly modulate TNF-α–stimulated NF-κB activity (Figure 1A). The occurrence and role of cGMP-independent activation of PKG in regulation of NF-κB in VSMC needs to be further determined.

The progression of the atherosclerotic lesion is believed to be a chronic inflammatory process as well as being due to vascular remodeling in the vessel wall. Cell proliferation, migration, and the expression of inflammatory molecules have all been found to be increased in VSMCs of atherosclerotic lesions in both human and experimental animals. Our findings in this study suggest that the inhibition of PDE3 activity mediates the inhibitory effect of NO/cGMP in VSMC inflammatory responses. Inhibition of PDE3 has also been shown to mediate the inhibitory effect of NO in VSMC mitogenesis, and the PDE3 inhibitor cilostamide suppressed arterial intimal hyperplasia in a rat balloon injury model.

Given that NO is an important antiatherogenic molecule in vivo, inhibition of PDE3 activity with PDE3-selective inhibitors may be an effective antiatherogenic strategy. It has

![Figure 7. Schematic representation of the role of PDE3 in NO/cGMP-dependent inhibition of NF-κB pathway. cGMP elevated by NO donor or natriuretic peptide inhibits PDE3 activity, which leads to increase of cAMP and activation of PKA. PKA inhibits NF-κB transcription activity and downstream target gene expressions.](http://circres.ahajournals.org/doi/figure/10.1161/01.RES.0000153109.72096.a1)
already been shown that the PDE3 inhibitor cilostazol (an analogue of cilostamid) is an effective drug for the clinical treatment of symptoms of intermittent claudication due to peripheral arterial disease, which itself, is believed to be a manifestation of systemic atherosclerosis. It has also been reported that PDE3 activity and expression are significantly increased in the aorta of atherosclerosis-prone insulin-resistant rats. Together these findings indicate that PDE3 may play an important role in vascular diseases such as atherosclerosis and therefore suggest that PDE3 inhibitors may be therapeutically useful for the treatment and prevention of atherosclerosis.

Acknowledgments

This work was supported in part by American Heart Association Research Grant 0030302T (to Dr Yan), NIH HL-63462 (to Dr Berk), and a grant from the Japanese Heart Foundation, Bayer Yakuhin Research Grant Abroad (to Dr Aizawa). We thank Dr Joseph A. Beavo and James Surapishitchat for helpful comments on the manuscript.

References

Role of Phosphodiesterase 3 in NO/cGMP-Mediated Antiinflammatory Effects in Vascular Smooth Muscle Cells

Toru Aizawa, Heng Wei, Joseph M. Miano, Jun-ichi Abe, Bradford C. Berk and Chen Yan

*Circ Res.* 2003;93:406-413; originally published online August 14, 2003;
doi: 10.1161/01.RES.0000091074.33584.F0

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/93/5/406

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2003/09/03/93.5.406.DC1
SUPPLEMENT 1

Figure S1. Effects of cilostamide and milrinone on various cAMP-hydrolyzing PDEs present in VSMC. PDE1A1, 4D3, and PDE3A2 were transiently expressed in COS-7 cells and assayed for PDE activity in cell lysates in vitro in the presence of various concentrations of cilostamide or milrinone.