Nitric Oxide–Induced Downregulation of Cdk2 Activity and Cyclin A Gene Transcription in Vascular Smooth Muscle Cells

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Background—Nitric oxide (NO) inhibits vascular smooth muscle cell (VSMC) proliferation and neointima formation after balloon injury. However, the molecular mechanisms underlying NO-mediated growth arrest are poorly understood. In the present study, we examined the effects of the NO donors sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) on cell cycle activity in VSMCs.

Methods and Results—Stimulation of quiescent rat VSMCs with serum leads to an increase in cyclin-dependent kinase (cdk)2 kinase activity that correlates with a marked induction of cyclin A protein expression. The addition of SNP or SNAP to VSMC cultures at the time of serum stimulation abrogates the induction of cdk2 activity without suppressing protein levels of cdk2 or cyclin E. These NO donors block serum-stimulated upregulation of cyclin A mRNA and protein and repress the serum induction of cyclin A promoter activity in VSMCs.

Conclusions—The addition of the nitric oxide donors SNP or SNAP to mitogen-stimulated VSMCs prevents activation of cdk2, a key regulator of the G1 and S phases of the cell cycle. These NO donors do not affect the expression of cdk2 protein but block the mitogen-induced expression of cyclin A, an activating subunit of cdk2. SNP and SNAP also repress the mitogen-stimulated activation of the cyclin A promoter. These data suggest that the antiproliferative effect of NO on VSMCs results, at least in part, from the repression of cyclin A gene transcription. (Circulation. 1998;97:2066-2072.)

Key Words: nitric oxide □ muscle, smooth □ genes

Vascular smooth muscle cell proliferation contributes to the restenotic lesion that develops after balloon angioplasty and is of major importance in the development of in-stent restenosis and bypass graft occlusion. NO is released from the endothelium and is believed to function as a physiological regulator of vessel tone and VSMC proliferation. The restoration of endothelial cell NO synthase activity in denuded rat carotid arteries through gene transfer results in increased vascular reactivity and a reduction in neointima formation. Likewise, the long-term oral administration of L-arginine, a precursor of NO, enhances vascular NO activity and reduces the size of vascular lesions that result from injury on hypercholesterolemia. Despite the widely recognized involvement of NO in the regulation of VSMC proliferation, the molecular mechanisms underlying NO-mediated VSMC growth arrest are poorly understood.

Cell cycle progression is regulated by the periodic activation of a family of cdk's. Activation of cdk's requires their association with specific cyclin regulatory subunits. The cdk2/cyclin E and cdk2/cyclin A holoenzymes are important regulators of the G1 and S phases of the cell cycle, respectively. Microinjection of anti–cyclin E antibody prevents fibroblasts from entering S phase, and overexpression of cyclin E induces the phosphorylation of the retinoblastoma protein and shortens the G1 phase. Cyclin A expression typically peaks in S phase and is required for DNA replication and progression through S phase. Precedently, we demonstrated the induction of cdk2/cyclin E and cdk2/cyclin A complexes during the fibroproliferative response to balloon injury in the rat carotid artery, and others have shown that antisense cdk2 oligonucleotides inhibit neointima formation.

In the present study, we tested the hypothesis that the antiproliferative effect of NO in VSMCs is mediated, at least in part, by the inhibition of cdk2 activity. The addition of the NO donors SNP or SNAP to VSMCs effectively inhibited mitogen-stimulated cdk2 activity, but this treatment had no effect on cdk2 protein levels. NO donors did not alter cyclin E expression levels, but they blocked the induction of cyclin A by mitogens. These donors also blocked the mitogen induction of cyclin A mRNA and promoter activity; therefore, these data suggest that the transcriptional repression of cyclin A gene is one mechanism by which NO inhibits VSMC proliferation.
Northern Blotting

Northern blot analysis was performed with 20 μg of total RNA prepared with the use of RNeasy B (Biotex Laboratories, Inc) as described previously. After transfer to a Hybond-N membrane (Amersham) and UV cross-linking, the blot was hybridized with a human cyclin A cDNA probe generated by random primer labeling (Boehringer-Mannheim Biochemicals) according to the manufacturer’s instructions. After hybridization, the blots were washed for 20 minutes at 65°C in 0.2× SSC (1× SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate)/0.1% SDS and exposed to Kodak X-ray film.

Methods

cdk2 Assay

Primary rat aortic VSMCs were isolated essentially as described by Mader et al. Cells were starved for 24 hours in DMEM supplemented with 0.5% FBS, after which cells were switched to DMEM supplemented with 10% FBS for 18, 36, or 48 hours with or without the presence of the indicated amount of SNAP or SNP. Cells were lysed in lysis buffer (50 μmol/L Tris, pH 8.0, 250 mmol/L NaCl, 0.5% Nonidet P-40, 1 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride). Insoluble materials were cleared through centrifugation at 4°C for 10 minutes at 12,000 rpm. Protein concentrations were determined with the Bradford assay (BioRad). For cdk2 kinase assay, 20 μg of protein extracts was precleared with Protein A agarose, after which 0.5 μg of anti-cdk2 antibodies (Santa Cruz Biotechnology) were added, incubated for 2 hours, and precipitated by the addition of Protein A agarose. After being washed three times with lysis buffer and three times with cdkt2 kinase buffer (250 μmol/L Tris, pH 7.6, 10 mmol/L MgCl2, 1 mmol/L dithiothreitol), kinase activity of the immunopellets was assayed with the use of histone H1 as substrate (2 μg histone H1, 10 μmol/L ATP, 4 μCi [γ-32P]ATP in kinase buffer) at room temperature for 30 minutes. After electrophoresis with SDS-12% polyacrylamide gels, the gels were exposed to fixative solution (10% methanol, 10% acetic acid) for 30 minutes and dried. Gels were exposed to Kodak X-ray film overnight.

Western Blotting

Fifty micrograms of protein extracts was subjected to Western blotting with the use of anti-cdk2, anti–cyclin A, anti–cyclin E, or anti-tubulin antibodies (Santa Cruz Biotechnology) as described previously. Briefly, proteins were separated in SDS-12% polyacrylamide gels and transferred to membranes. Blots were incubated in 5% nonfat dry milk (in 1× PBS, 0.2% Tween 20) for 2 hours at room temperature and were incubated with the indicated antibodies (1:200 dilution in 2% nonfat dry milk, 0.2% Tween 20 in 1× PBS) for 3 hours. After three washes in 0.1% SDS and kinase activity was assayed by using histone H1 as substrate (see “Methods”). A representative blot is shown. Serum-deprived, quiescent VSMCs (Q) or VSMCs that were serum supplemented for 18 hours in the presence or absence of SNP were analyzed for cdk2 histone kinase activity.

Figure 1. SNAP and SNP repress mitogen-stimulated cdk2 kinase activity in VSMCs. A, Dose-dependent inhibition of cdk2-mediated histone kinase activity by SNP. Indicated rat VSMC cell extracts were immunoprecipitated with anti-cdk2 antibodies, and kinase activity was assayed by using histone H1 as substrate (see “Methods”). A representative blot is shown. Serum-deprived, quiescent VSMCs (Q) or VSMCs that were serum supplemented for 18 hours in the presence or absence of SNP were analyzed for cdk2 histone kinase activity. B, Repression of cdk2 activity by SNP was maintained for 48 hours after serum stimulation. The cdk2-dependent histone kinase activity was assayed in extracts from quiescent rat VSMCs and from VSMCs stimulated with serum for 18, 36, or 48 hours in the absence or presence of 300 μmol/L SNP. C, SNAP represses serum-stimulated cdk2 kinase activity. The cdk2-mediated histone kinase activity was assayed in extracts from quiescent or serum-stimulated VSMCs (18 hours) in the absence or presence of 300 μmol/L SNAP or SNP.

Transient Transfection

Cyclin A promoter-luciferase constructs contain the human −924/+245 and −79/+100 cyclin A promoter regions. The −54/+100 cyclin A–luciferase reporter was generated with the polymerase chain reaction with specific primers containing KpnI and BglI restriction sites and subcloned into KpnI/BglII–digested pG52 Basic (Promega). The Simian virus 40 promoter–alkaline phosphatase reporter construct, pSV2-AP, contains the reporter gene under the control of the Simian virus 5 promoter-enhancer.

Before transfections, PAC1 cells were serum starved in DMEM supplemented with 0.5% FBS for 24 hours. Transfections were carried out with Lipofectamine (GIBCO Laboratories) according to the manufacturer’s instructions. Briefly, DNA and Lipofectamine were mixed together for 40 minutes in OptiMem medium (GIBCO) and incubated with cells in OptiMem medium for 3 hours. After transfection, cells were switched to DMEM supplemented with 0.5% FBS or DMEM supplemented with 10% FBS with or without the indicated amount of 8-Br-cGMP, SNAP, or SNP for 20 hours. Cells were harvested in 1× lysis buffer (Promega) and assayed for luciferase and alkaline phosphatase activity, as described previously. Reporter gene activity was recorded with a Berthold LB 9501 luminometer (measurement time, 7.5 seconds).
Statistical Analysis

Results are expressed as mean±SEM. Statistical significance was evaluated with an unpaired Student’s t test for comparisons between two mean values and ANOVA followed by Scheffé’s procedure for more than two mean values. A value of \( P < .05 \) was interpreted to denote statistical significance.

Results

NO Donors Repress Serum Induction of cdk2 Kinase Activity in VSMCs

We first examined the effect of SNP or SNAP on cdk2 kinase activity in cultures of rat VSMCs. Protein extracts were prepared from cultures of serum-deprived VSMCs and VSMCs stimulated with 10% FBS in the presence or absence of NO donors. These extracts were immunoprecipitated with anti-cdk2 antibodies, and the kinase activity of the immunopellets was assayed with histone H1 as substrate. As shown in Fig 1A, cdk2 activity is markedly induced in serum-stimulated VSMCs. This serum-dependent increase in cdk2 activity after 18 hours of serum stimulation was completely abrogated by the presence of SNP in a dose-dependent manner. The repression by SNP was sustained for up to 48 hours after serum induction (Fig 1B). Moreover, the NO donor SNAP also abrogated the induction of cdk2 activity by serum (Fig 1C).

NO Donors Specifically Repress Cyclin A Induction

Having demonstrated that NO donors repress cdk2 activity, we performed a series of Western blot analyses to test the effect of NO donors on the expression of cdk2 and its regulatory subunits, cyclins E and A. As shown in Fig 2A, either 10% FBS or 10% FBS plus SNP had no detectable effect on the protein expression of cdk2, cyclin E, or the control protein tubulin. In contrast, cyclin A protein levels were markedly upregulated in serum-stimulated VSMCs, and the inclusion of SNP abrogated this induction. The effect of SNP on serum-stimulated cyclin A expression was dose dependent (Fig 2B). The repression of cyclin A by SNP was detected at 18 hours (Fig 2B) and at the 36- and 48-hour time points after serum addition (not shown). Similarly, SNP did not affect cdk2 or cyclin E expression at any of these other time points. Finally, cyclin A expression was also repressed by SNAP (Fig 2C). Similar to SNP, SNAP did not affect cyclin E or cdk2 protein levels. Thus, changes in the expression of cyclin A, an activating subunit of cdk2, may contribute to NO-mediated downregulation of cdk2 activity.
SNP Represses Serum Induction of Cyclin A Promoter Activity in VSMCs

Northern blot analyses were performed to determine whether the repression of cyclin A by NO donors occurred at the level of mRNA. As shown in Fig 3, cyclin A mRNA was markedly upregulated by the addition of serum to quiescent VSMCs, and the inclusion of SNP or SNAP abrogated this induction. To test whether NO donor-mediated suppression of cyclin A expression in serum-stimulated VSMCs may be achieved at the transcriptional level, we next performed transient transfection assays with plasmids containing fragments of the human cyclin A promoter fused to the luciferase reporter gene (Fig 4A). The pulmonary arterial cell line PAC1 was used for these studies because it is efficiently transfected with plasmid DNA constructs. After transfection in serum-free medium, PAC1 cells were switched to DMEM supplemented with 0.5% FBS (quiescent cells) or serum-stimulated with DMEM supplemented with 10% FBS with or without SNP, SNAP, or 8-Br-cGMP. Cell extracts were prepared 20 hours later, and luciferase activity was measured. As shown in Fig 4B, activity of the −924/+245 cyclin A promoter region is markedly upregulated by serum, and the sequence spanning from −79 to +100 is sufficient to confer this regulation. However, the −54/+100 cyclin A promoter fragment was not induced by serum. The addition of SNP, SNAP, or 8-Br-cGMP prevented the serum-dependent induction of cyclin A promoter activity. The repression of the −79/+100 promoter construct by SNP was dose dependent (Fig 4C). Treatment with serum or with serum plus SNP, SNAP, or 8-Br-cGMP had little or no effect on transcription from the Simian virus 40 promoter (Fig 4D).

Discussion

It is well documented that NO has an antiproliferative effect on VSMC proliferation in vitro and in vivo. The inhibition of VSMC proliferation by NO is associated with distinct cell cycle arrests in phases G1 and S. In the present study, we examined the effects of SNP and SNAP on the expression and activity of cdk2 and its regulatory subunits, cyclins E and A. Treatment with these NO donors prevented the serum induction of cdk2 activity in VSMCs. We found that mitogen stimulation markedly induced cyclin A expression, but cdk2 and cyclin E levels remained constant. The serum induction of both cyclin A mRNA and protein was abrogated by the inclusion of SNP or SNAP, suggesting a reduction in cyclin A expression can contribute to the NO-mediated decrease in cdk2 activity and VSMC growth arrest. Others have shown that NO donors upregulate expression of the cdk inhibitor p21, which may also contribute to VSMC growth inhibition.

To test whether NO donors may limit VSMC growth through transcriptional repression of the cyclin A gene, we examined the effect of SNP and SNAP on cyclin A promoter activity in immortalized PAC1 smooth muscle cells. In agreement with previous studies in NIH 3T3 fibroblasts and VSMCs, our transient transfection assays demonstrated that serum-dependent regulation of the cyclin A gene promoter in VSMCs is mediated by sequences extending from −79 to +100 relative to the predominant transcription start site. This promoter fragment contains a CRE and an E2F-binding site that are required for cell cycle-regulated expression of this promoter in VSMC and nonmuscle cell types. The −54/+100 cyclin A promoter fragment, which contains the E2F but not CRE site, is not sufficient for cyclin A promoter transcription in serum-stimulated VSMCs. Similarly, this construct is not induced by serum in bovine aortic endothelial cells. The present study indicates that CRE-dependent transcriptional regulation of cyclin A expression integrates both positive and negative cell growth regulatory signals. Serum activation of either the −79/+100 or −924/+245 cyclin A promoter fragment was repressed by the addition of SNP or SNAP, indicating that NO functions as a negative regulator of cyclin A gene transcription. Serum-induced expression of the cyclin A promoter was also repressed by 8-Br-cGMP, as was endogenous cyclin A expression and cdk2 activity (not shown). Further studies will be required to determine whether cGMP mediates NO-dependent repression of cyclin A promoter activity.

Accumulating evidence indicates that cdk2 is a key regulator of VSMC proliferation in vitro and in vivo. First, cdk2 activity is induced after angioplasty in the rat carotid artery, and abrogation of cdk2 function by antisense cdk2 oligonucleotides suppresses neointimal VSMC accumulation. Collectively, these results indicate that CRE-dependent transcriptional regulation of cyclin A expression integrates both positive and negative cell growth regulatory signals. Serum activation of either the −79/+100 or −924/+245 cyclin A promoter fragment was repressed by the addition of SNP or SNAP, indicating that NO functions as a negative regulator of cyclin A gene transcription. Serum-induced expression of the cyclin A promoter was also repressed by 8-Br-cGMP, as was endogenous cyclin A expression and cdk2 activity (not shown). Further studies will be required to determine whether cGMP mediates NO-dependent repression of cyclin A promoter activity.

Suppression of cdk2 activity in SNP- and SNAP-treated VSMCs correlated with reduced cyclin A protein and mRNA levels, but SNP did not diminish cdk2 or cyclin E protein expression. Similarly, inhibition of growth factor–stimulated VSMC proliferation by polymerized collagen or by overexpression of the Gax transcription factor diminishes cyclin A protein levels but has no effect on cdk2 or cyclin E protein expression. Collectively, these results indicate that cyclin A may function at one of the key regulatory points underlying integrin-, Gax-, and NO-mediated antiproliferative effects in VSMCs.
In summary, the results of the present study suggest a mechanism by which repression of cyclin A gene transcription contributes to NO-mediated downregulation of cdk2 activity and, ultimately, VSMC growth arrest. Elucidation of the networks underlying the regulation of cyclin A gene expression by NO may permit the development of new strategies to inhibit the proliferative response of VSMCs to arterial injury.

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References


