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Tumor Suppressor p53 But Not cGMP Mediates NO-Induced Expression of p21$^{Waf1/Cip1/Sdi1}$ in Vascular Smooth Muscle Cells

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ABSTRACT

Cyclin-dependent kinase inhibitor p21$^{Waf1/Cip1/Sdi1}$ has been suggested to be involved in the antiproliferative effect of nitric oxide (NO) in vascular smooth muscle cells (VSMCs). To elucidate the mechanism underlying NO-induced p21 expression, we investigated the roles of tumor suppressor p53 and the guanylate cyclase-cGMP pathway. The induction of p21 by the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) seemed to be due to transactivation because SNAP elevated the activity of p21 promoter but did not stabilize p21 mRNA and protein. Because SNAP did not stimulate the deletion mutant of p21 promoter that lacked p53 binding sites, we tested the involvement of p53. The expression level of p53 was down-regulated after mitogenic stimulation, whereas it was sustained in the presence of SNAP. SNAP markedly stimulated DNA binding activity of p53. Furthermore, SNAP failed to induce p21 in VSMCs obtained from p53-knock out mice and in A431 cells that contained mutated p53. The antiproliferative effect of SNAP also was attenuated in these cells. NO stimulates guanylate cyclase and its product cGMP has been shown to inhibit VSMC proliferation. However, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, a guanylate cyclase inhibitor, did not prevent SNAP-induced p21 expression. 8-Bromo-cGMP, 3-isobutyl-1-methylxanthine, and their combination did not induce p21. Although 8-bromo-cGMP had a small antiproliferative effect, the elevation of cGMP concentration induced by SNAP was little throughout the G_1 phase. The antiproliferative effect of SNAP was not attenuated by Rp-8-bromoguanosine-3′,5′-monophosphorothioate, an inhibitor of cGMP-dependent protein kinase. These results suggested that NO induces p21 through a p53-dependent but cGMP-independent pathway.

Nitric oxide (NO) is antiproliferative for vascular smooth muscle cells (VSMCs) in vitro and in vivo. Chemical NO donors reversibly inhibit the proliferation of cultured VSMCs (Garg and Hassid, 1989; Kariya et al., 1989; Assender et al., 1992; Estrada et al., 1997; Ishida et al., 1997; Yu et al., 1997). Intimal VSMC hyperplasia after balloon injury is inhibited by supplementation with L-arginine, the metabolic precursor of NO (McNamara et al., 1993; Tarry and Makhoul, 1994), and by introduction of endothelial NO synthase (eNOS) gene (von der Leyen et al., 1995). Conversely, mice with a targeted disruption of eNOS display an increase in arterial wall thickness accompanied by cellular hyperplasia, suggesting that endogenous NO is a negative regulator of VSMC proliferation (Rudic et al., 1998).

In an investigation of the effects of *S*-nitroso-*N*-acetylpenicillamine (SNAP), a NO-releasing agent, on the cell cycle of VSMCs, we found that the G_1 inhibition induced by NO may result from the induction of p21$^{Waf1/Cip1/Sdi1}$, a cyclin-dependent kinase inhibitor (Ishida et al., 1997). However, the mechanism underlying NO-mediated p21 induction remains to be determined.

p21 was discovered from genes induced by the tumor suppressor p53 (El-Deiry et al., 1993). p53 accumulates after cells are exposed to DNA-damaging stimuli, such as irradiation and alkylating agents, and induces several genes, including p21, by functioning as a transcription factor (Agar-

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ABBREVIATIONS: NO, nitric oxide; VSMCs, vascular smooth muscle cells; eNOS, endothelial nitric oxide synthase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; iNOS, inducible nitric oxide synthase; sGC, soluble guanylate cyclase; CHX, cycloheximide; IBMX, 3-isobutyl-1-methylxanthine; Rp-GMPS, Rp-8-bromoguanosine-3′,5′-monophosphorothioate; Rp-AMPS, Rp-8-bromoadenosine-3′,5′-monophosphorothioate; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; G_0, quiescent state; TdR, thymidine; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase.

wal et al., 1998). Cytotoxic levels of NO released from high concentrations of NO donors or generated by inducible NO synthase (iNOS) have been reported to increase the level of p53 (Meßmer et al., 1994; Forrester et al., 1996; Ho et al., 1996; Ambs et al., 1997). However, it is unclear whether lower concentrations of NO, which inhibit cell proliferation but do not cause cytotoxicity or apoptosis, activate p53. Therefore, we investigated whether p53 is involved in the up-regulation of p21 and the inhibition of cell proliferation induced by NO.

It is established that NO-induced smooth muscle relaxation is mediated by the soluble guanylate cyclase (sGC)-cGMP pathway (Murad, 1986; Walter, 1989). However, it is a matter of some controversy whether the antiproliferative effect of NO also is mediated by this pathway. Indeed, some studies showed that cGMP analogs and phosphodiesterase inhibitors inhibited VSMC proliferation (Abell et al., 1989; Garg and Hassid, 1989; Kariya et al., 1989; Furuya et al., 1991; Assender et al., 1992; Yu et al., 1997). However, others have implied cGMP-independent mechanisms. NO donors were able to inhibit the proliferation of BALB/c3T3 fibroblasts in spite of the lack of sGC activity (Garg and Hassid, 1990). VSMC proliferation was not inhibited by cGMP analogs and cGMP-specific phosphodiesterase inhibitors (Garg and Hassid, 1990; Estrada et al., 1997). Therefore, we also examined whether the sGC-cGMP pathway is involved in the p21 expression and antiproliferative effect induced by NO.

Materials and Methods

Chemicals. SNAP was purchased from Research Biochemicals Inc. (Natick, MA). Cycloheximide (CHX), actinomycin D, 8-bromo-cGMP, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, MO). Rp-8-bromoguanosine-3′,5′-monophosphorothioate (Rp-GMPS) and Rp-8-bromoadenosine-3′,5′-monophosphorothioate (Rp-AMPS) were purchased from Biolog Life Sciences Institute (Bremen, Germany). 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was purchased from Wako Pure Chemicals Industries (Osaka, Japan). Other common chemicals were of reagent grade.

Cell Culture. VSMCs obtained from human umbilical arteries as described in Ishida et al. (1997) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% (v/v) fetal bovine serum (FBS) (Life Technologies, Rockville, MD), 5 ng/ml human recombinant basic fibroblast growth factor (Amersham Pharmacia Biotech, Uppsala, Sweden), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B (growth medium). Cells synchronized in a quiescent state (G_0) by serum starvation for 48 h were stimulated with growth medium to reenter the cell cycle. Cell numbers were determined with a Coulter counter (Z1; Coulter Electronics, Luton, Beds, UK). VSMC line P53LMACO1 cells obtained from thoracic aorta of p53-knock out mice (Ohmi et al., 1997) were kindly provided by Yoshiaki Nonomura, Teikyo University, Tokyo, Japan. P53LMACO1 cells and VSMCs from wild-type mice (C57BL/6J strain) were cultured in DMEM containing 10% FBS on dishes coated with collagen type I-C (Iwaki Glass, Chiba, Japan). A431 cells obtained from Riken Cell Bank (Saitama, Japan) also were maintained in DMEM supplemented with 10% FBS.

DNA Synthesis Assay. DNA synthesis was assessed by the level of [^{3}H]thymidine ([^{3}H]TdR) incorporation as described in Ishida et al. (1997).

Immunoprecipitation and Western Blotting. For detection of p21, cell lysates were immunoprecipitated and analyzed by Western blotting as described in Ishida et al. (1997). For p53, cells were lysed in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40 (Nacalai Tesque, Kyoto, Japan), 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM NaF, 200 μM Na_3VO_4, 20 μg/ml phenylmethylsulfonyl fluoride (PMSF), and 20 μg/ml leupeptin (RIPA buffer). Lysates were sonicated for 10 s and rocked on ice for 1 h, followed by a centrifuge at 16,000*g* for 20 min to remove insoluble pellets. The protein concentrations were determined by the modified Lowry's method with Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins (20 μg/lane) were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) (10%), electroblotted onto a polyvinylidene difluoride membrane, and immunoblotted with an antihuman p53 monoclonal antibody (2 μg/ml, DO-7; PharMingen, San Diego, CA). To normalize the amounts of proteins applied to SDS-PAGE, the membranes were reprobed with anti-α-tubulin monoclonal antibody (2 μg/ml, DM1A; Oncogene Research Products, Cambridge, MA).

Northern Blotting. cDNA of p21 was prepared as described in Ishida et al. (1997). Total cellular RNAs (10 μg/lane) were electrophoresed and analyzed by Northern blotting as described in Ishida et al. (1997).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). RT-PCR was performed with Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech). Total cellular RNAs (1 μg) were used for the RT reaction and the products were amplified by 25 thermal cycles with DNA Thermal Cycler 480 (Perkin-Elmer Cetus Instruments, Norwalk, CT). PCR primers for p53 were synthesized based on the GenBank database (5′-ATGGAGGAGCCGCAGTCAGA-3′ and 5′-CACTCGGATAAGATGCTGAG-3′). PCR products were electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide. Amplified DNAs were identified by sequencing.

Transfection and Luciferase Assay. The 2.4-kilobase human genomic p21 promoter, which contained the transcriptional initiation site at its 3′ end, was excised with *Hin*dIII from wild-type waf1 promoter (WWP)-Luc (El-Deiry et al., 1993) and subsequently cloned into the *Hin*dIII site of PGV-B2, a firefly luciferase reporter vector (Toyo Ink Mfg. Co., Tokyo, Japan). To construct a plasmid containing a deletion mutant of the promoter lacking p53 consensus sequences, a 1.3-kilobase *Dra*I/*Hin*dIII fragment excised from WWP-Luc was cloned between the *Sma*I/*Hin*dIII sites of PGV-B2. These plasmids were designated PGV-WWP and PGV-deletion mutant (DM), respectively. Cells seeded in 12-well plates (5×10^5 cells/well) were cultured in growth medium for 24 h. After three washes with antibiotic-free DMEM containing 0.1% bovine serum albumin, the cells were incubated in the same medium for 45 h for synchronization in G_0. Plasmid DNAs (600 ng/well) were transiently transfected into the cells with LipofectAMINE PLUS reagent (Life Technologies) as described in the manufacturer's protocol. Simultaneously, pRL-simian virus 40 (SV40) (60 ng/well, Toyo Ink. Mfg. Co.), which contained *Renilla* luciferase gene with an SV40 promoter, was cotransfected as a control for transfection efficiency. Transfected cells were incubated in growth medium in the absence or presence of SNAP (100 μM) for 18 h. After two washes with phosphate-buffered saline, the firefly and *Renilla* luciferase activities were measured sequentially with a double luciferase assay system (Toyo Ink Mfg. Co.) and a luminometer (Dia-Iatron, Tokyo, Japan). Firefly luciferase activities were normalized to those of *Renilla* luciferase.

Electrophoretic Mobility Shift Assay. To prepare nuclear extracts, cells were suspended in the hypotonic buffer [10 mM HEPES/KOH (pH 7.9), 10 mM KCl, 100 μM EDTA, 0.1% Nonidet P-40 (v/v), 1 mM dithiothreitol, 500 μM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM Na_3VO_4] and incubated for 10 min on ice. After centrifugation at 3000*g* for 1 min, the pelleted nuclei were resuspended in the extraction buffer [50 mM HEPES/KOH (pH 7.9), 420 mM KCl, 5 mM $MgCl_2$, 100 μM EDTA, 1 mM dithiothreitol, 500 μM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM Na_3VO_4, and 20% glycerol] and incubated for 30 min on ice. The lysates were centrifuged at 16,000*g* for 15 min and resultant supernatants were used as nuclear extracts. Double-stranded oligonucleotides containing a p53

consensus sequence 5′-TCAGGAACATGTCCCAACATGTTGAGC-3′ were labeled at 5′-ends with ^{32}P by T4 polynucleotide kinase, and purified with Sephadex G-50 (ProbeQuant G-50 Micro Columns; Amersham Pharmacia Biotech). For the DNA binding reactions, nuclear extracts (10 μg of protein) were incubated with the labeled DNA probe (~5 × 10^4 cpm) and poly(dI-dC)·poly(dI-dC) (2 μg; Amersham Pharmacia Biotech) in the binding buffer [12.5 mM HEPES/KOH (pH 7.9), 105 mM KCl, 1.25 mM $MgCl_2$, 250 μM EDTA, 25 μM dithiothreitol, 12.5 μM PMSF, 500 ng/ml aprotinin, 500 ng/ml leupeptin, 250 μM Na_3VO_4, and 5% glycerol] for 1 h on ice. For the competition experiments, a 100-fold molar excess of unlabeled oligomers was added before the addition of labeled probe. To identify the protein bound to DNA, nuclear extracts were preincubated with an antihuman p53 monoclonal antibody (5 μg of PAb421; Oncogene Research Products) on ice for 1 h before adding the labeled probe. Protein-DNA complexes were electrophoresed on 6% native polyacrylamide gel in 0.5× Tris, boric acid, and EDTA buffer [1× Tris, boric acid, and EDTA: 89 mM Tris, 89 mM boric acid, and 2 mM EDTA (pH 8.0)] at 4°C. Dried gels were analyzed for radioactivity with a bioimage analyzer BAS-2500 (Fuji Photo Film Co., Tokyo, Japan).

cGMP Assay. To extract cGMP, cells (~1.5 × 10^5) were frozen at −80°C after the culture medium was replaced with 1 ml of ice-cold pure ethanol. Cells were centrifuged at 2000*g* for 5 min at 4°C, and the supernatant was transferred to a new tube. A 200-μl aliquot was completely evaporated with a vacuum concentrator (Speed Vac Plus SC210A; Savant, Holbrook, NY), and dissolved in water. cGMP concentrations were determined with a radioimmunoassay kit (Yamasa Shoyu Co., Chiba, Japan).

Statistics. Results are expressed as means ± S.D. of the number of observations. Statistical significance was assessed by Student's *t* test for paired or unpaired values.

Results

p53 Is Involved in NO-Induced p21 Expression and Growth Arrest. SNAP stimulates the induction of p21 expression in VSMCs (Ishida et al., 1997). To examine whether NO stabilizes p21 protein and mRNA, we measured the rates of their degradation with CHX, a protein synthesis inhibitor, and actinomycin D, an RNA synthesis inhibitor, respectively. The protein and mRNA, which had been accumulated by incubation with growth medium for 3 h, were degraded as cells were incubated with CHX and actinomycin D, respectively (Fig. 1). SNAP (100 μM) had no significant effect on their rates of degradation. Therefore, it is unlikely that this compound induces p21 expression by stabilizing the protein and mRNA.

To determine whether SNAP stimulates the transcription of p21 gene, we assayed p21 promoter activity (Fig. 2). In cells transfected with PGV-WWP, which contained a 2.4-kilobase human p21 promoter, SNAP elevated the luciferase activity by 2.04-fold. However, SNAP had no significant effect in cells transfected with PGV-DM, in which the distal 1.1 kilobase containing p53 consensus sequences was deleted.

We then examined the effect of CHX on expression of p21 mRNA induced by SNAP to determine whether de novo protein synthesis is required for this expression. As shown in Fig. 3, CHX had no significant effect, suggesting that p21 expression induced by SNAP does not require newly synthesized proteins.

Because involvement of p53 in the NO-mediated induction of p21 expression was implied, we analyzed the expression levels of p53 by Western blotting (Fig. 4, a and b). The expression level of p53 protein decreased after mitogenic stimulation, whereas it was sustained in the presence of SNAP. However, there was no significant difference in the expression levels of p53 mRNA between controls and SNAP-treated cells (Fig. 4c).

Whether SNAP activates p53 to bind DNA was examined

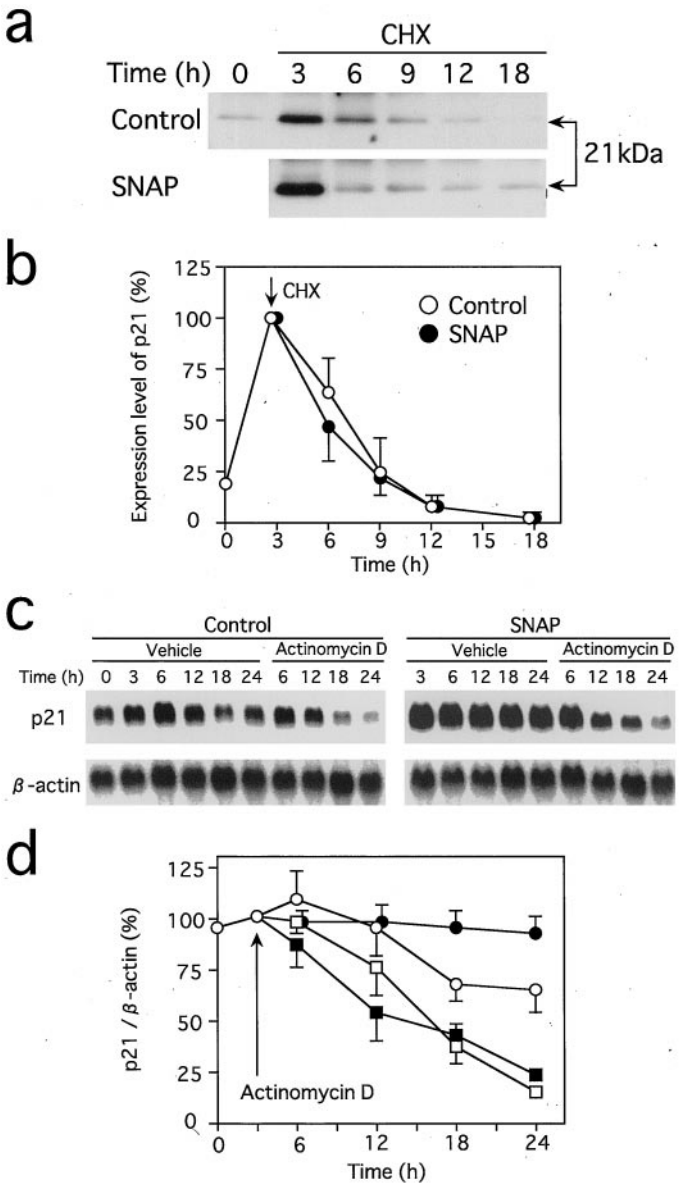

Fig. 1. Effects of SNAP on the rates of degradation of p21 protein and mRNA. a, G_0 cells were stimulated with growth medium in the absence or presence of SNAP (100 μM). CHX (1 μM) was added 3 h after stimulation. Cell lysates prepared at the indicated times were immunoprecipitated with a polyclonal antibody to p21 (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). Precipitated proteins were fractionated by SDS-PAGE (12.5%) and immunoblotted with a monoclonal antibody to p21 (1 μg/ml 6B6; PharMingen). b, degrees of intensity of blots obtained in (a) were quantified by a bioimage analyzer. Values were standardized to those obtained at 3 h and are shown as means ± S.D. (n = 3). ○, vehicle-treated cells; ●, SNAP-treated cells. c, G_0 cells were stimulated with growth medium in the absence or presence of SNAP (100 μM). Actinomycin D (300 nM) was added 3 h after stimulation. Total cellular RNAs were extracted at the indicated times. Equal amounts of RNA (10 μg/lane) were fractionated by electrophoresis and hybridized with ^{32}P labeled cDNA probes for p21 and β-actin. d, data obtained in (c) were quantified. The levels of p21 normalized to those of β-actin were standardized to the values obtained at 3 h and are shown as means ± S.D. (n = 3). ○, no addition; ●, SNAP; □, actinomycin D; and ■, actinomycin D + SNAP.

by electrophoretic mobility shift assay (Fig. 5). The level of the protein-DNA complex was elevated by more than 3-fold in SNAP-treated cells compared with vehicle-treated cells. Specificity of the binding was confirmed by the disappearance of the shifted band in the presence of a 100-fold excess of unlabeled probes. The protein bound to DNA was identified as p53 because pretreatment with an anti-p53 antibody but not a nonimmunized IgG prevented the shift.

To confirm that p53 is involved in SNAP-mediated induction of p21 expression, we examined the effect of SNAP on a p53-null VSMC line (P53LMACO1), which was obtained from thoracic aorta of p53-knock out mice (Fig. 6a). In VSMCs obtained from wild-type mice, SNAP greatly increased the amount of p21. In contrast, SNAP did not change the level of p21 in P53LMACO1 cells. SNAP also did not stimulate p21 expression in p53-mutated human epidermoid carcinoma A431 cells (Fig. 6b).

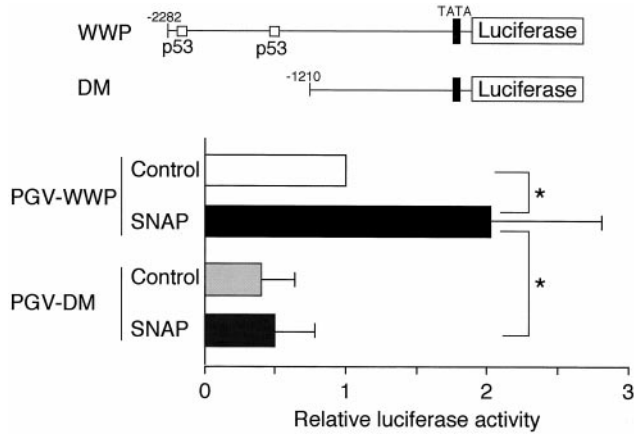

Fig. 2. p21 Promoter assay. G_0 cells cotransfected with PGV-WWP or PGV-DM and pRL-SV40 were stimulated with growth medium in the absence or presence of SNAP (100 μM) for 18 h. Luciferase activities were determined as described in *Materials and Methods*. Firefly luciferase activities were normalized to those of *Renilla* to eliminate differences in transfection efficiencies. The activities are expressed as fold increases compared with the activity in the cells transfected with PGV-WWP and incubated without SNAP. Data represent means ± S.D. ($n = 3$). *$P < .05$.

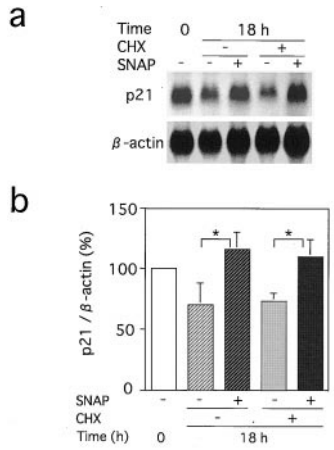

Fig. 3. Effect of protein synthesis inhibitor on expression of p21 mRNA induced by SNAP. a, G_0 cells were stimulated with growth medium for 18 h. SNAP (100 μM) and CHX (1 μM) were added at 3 h after mitogenic stimulation. Total cellular RNAs extracted at 0 and 18 h were analyzed by Northern blotting for p21 and β-actin. b, data obtained in (a) were quantified. The levels of p21 normalized to those of β-actin were standardized to the values obtained at time zero and are shown as means ± S.D. ($n = 3$). *$P < .05$.

Therefore, we examined whether SNAP inhibits the growth of P53LMACO1 and A431 cells, in which SNAP did not induce p21. SNAP completely inhibited the [^{3}H]TdR incorporation of VSMCs from wild-type mice; however, the inhibitory effect of SNAP was partial in P53LMACO1 cells and A431 cells (Fig. 6, c and d).

NO-Induced p21 Expression and Growth Arrest Are Independent of cGMP. Because NO activates sGC to generate cGMP by binding to the heme moiety of the enzyme (Murad, 1986; Walter, 1989), we investigated whether the sGC-cGMP pathway is involved in NO-induced p21 expression.

Initially, we examined the effect of ODQ, a specific inhibitor of sGC (Garthwaite et al., 1995), on the p21 expression. G_0 cells were preincubated with ODQ for 30 min before mitogenic stimulation, and the same concentration of ODQ was added to the growth medium. We measured intracellular cGMP levels by radioimmunoassay in the presence of IBMX, a phosphodiesterase inhibitor, to confirm that ODQ really inhibits sGC (Fig. 7a). ODQ significantly inhibited the elevation of cGMP levels induced by SNAP at 1 and 18 h after mitogenic stimulation. Nevertheless, ODQ did not inhibit the ability of SNAP to stimulate p21 expression (Fig. 7, b and c). On the contrary, ODQ tended to enhance p21 expression, although this effect did not reach statistical significance.

Before examining whether cGMP stimulates p21 expression, we measured cGMP levels to determine whether cGMP is accumulated after the addition of SNAP (Fig. 8a). The cGMP levels in control cells did not change throughout the observation period. When cells were treated with IBMX for

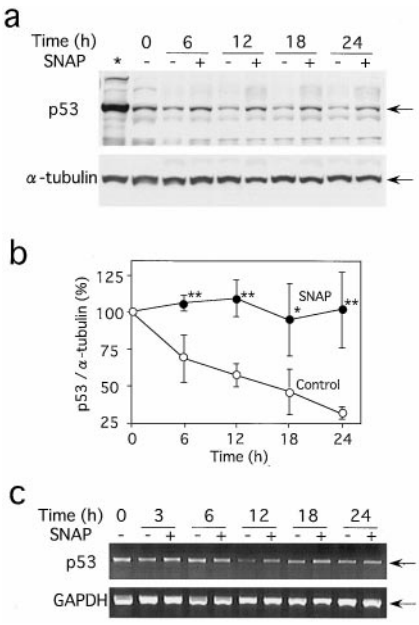

Fig. 4. Effect of SNAP on the expression of p53. a, G_0 cells were stimulated with growth medium in the absence or presence of SNAP (100 μM). Cells were lysed in RIPA buffer at the indicated times. Proteins (20 μg/lane) were fractionated by SDS-PAGE (10%) and blotted with an anti-p53 or an anti-α-tubulin antibody. *, an A431 cell extract (10 μg) was applied as a positive control. b, expression levels of p53 normalized to those of α-tubulin were plotted as percentages of the values obtained at time zero. Data represent means ± S.D. ($n = 4$). *$P < .05$, **$P < .01$. ○, vehicle-treated cells; ●, SNAP-treated cells. c, G_0 cells were stimulated with growth medium in the absence or presence of SNAP (100 μM). Total cellular RNAs extracted at the indicated times were analyzed for p53 by RT-PCR as described in *Materials and Methods*.

30 min before cell lysis, SNAP markedly increased the cGMP levels, with the maximal effect being obtained at 6 h after stimulation. IBMX treatment alone had no significant effect on cGMP levels (data not shown). cGMP production was still accelerated in SNAP-treated cells at 18 to 24 h, indicating that SNAP added once at time zero retained the ability to generate effective amounts of NO until 24 h. When measured in the absence of IBMX, however, the elevation of cGMP levels in response to SNAP was very small compared with that measured in the presence of IBMX. In particular, there was no increase in cGMP concentration in the late G_1 phase (12–18 h), where SNAP markedly up-regulated p21 and inhibited the cell cycle (Ishida et al., 1997). It seemed that, although SNAP stimulated sGC throughout a 24-h period, the cleavage of cGMP to 5′-GMP by phosphodiesterase also was accelerated simultaneously, resulting in a limited accumulation of intracellular cGMP.

Then we tested whether p21 is up-regulated by cGMP by examining the effects of 8-bromo-cGMP, a membrane-permeable cGMP analog, and IBMX on p21 expression by Western blotting (Fig. 8, b and c). SNAP markedly up-regulated the expression of p21, whereas 8-bromo-cGMP, IBMX, and their combination did not stimulate its expression. Moreover, IBMX did not enhance the effect of SNAP but rather significantly attenuated it.

Finally, we tested whether cGMP inhibits VSMC proliferation with 8-bromo-cGMP (Fig. 9a). SNAP (>100 μM) markedly suppressed [^{3}H]TdR incorporation, whereas 8-bromo-cGMP had little, if any, effect even at 1 mM and a significant inhibitory effect only at 100 μM. To examine whether cGMP-dependent protein kinase (PKG) or cAMP-dependent protein kinase (PKA) is involved in the antiproliferative effect of SNAP, specific inhibitors of these kinases were used (Fig. 9b). However, neither Rp-GMPS, an inhibitor of PKG, nor Rp-AMPS, an inhibitor of PKA, attenuated the effect of SNAP on [^{3}H]TdR incorporation.

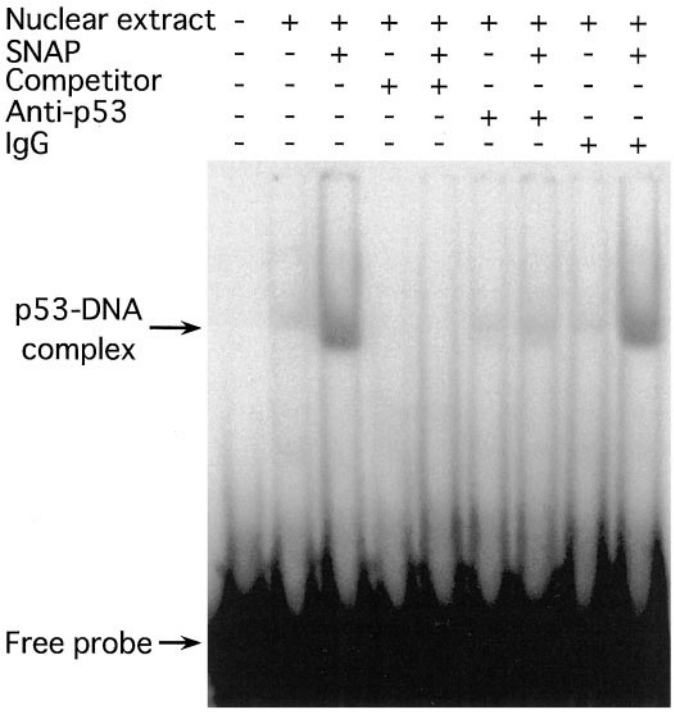

Fig. 5. Electrophoretic mobility shift assay for p53. Nuclear extracts (10 μg) prepared from VSMCs stimulated with growth medium for 18 h in the absence or presence of SNAP (100 μM) were incubated with ^{32}P labeled double-stranded oligonucleotides containing a p53 binding site of human p21 promoter as described in *Materials and Methods*. For competition experiments, a 100-fold molar excess of unlabeled oligonucleotides (Competitor) was added before the addition of radiolabeled probes. To identify the protein associating with DNA, some of the nuclear extracts were preincubated with an anti-p53 monoclonal antibody (5 μg) or a nonimmunized mouse IgG (5 μg).

Discussion

p21 is induced by p53-dependent or independent mechanisms (Gartel et al., 1996). DNA-damaging stimuli induce

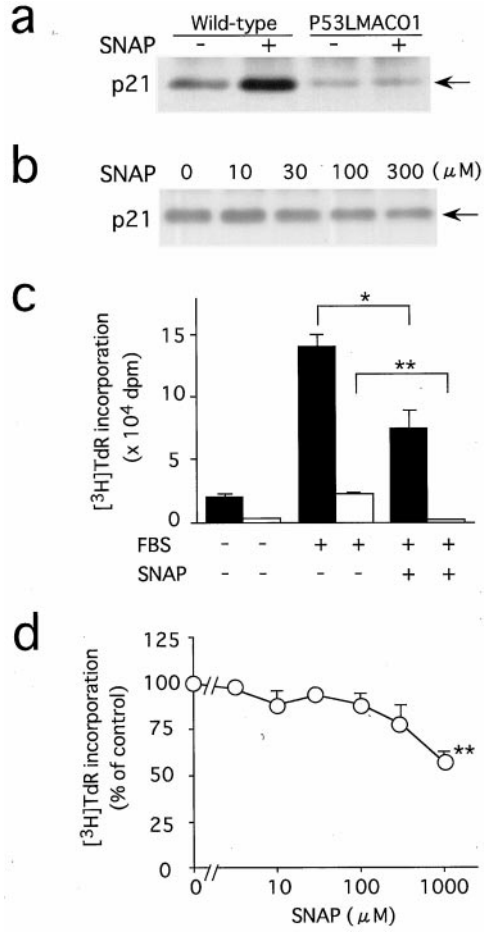

Fig. 6. Effects of SNAP on p21 expression and DNA synthesis in p53-deficient and mutated cells. a, after serum starvation for 48 h, VSMCs from wild-type mice (C57BL/6J strain) and P53LMACO1 cells were stimulated with DMEM supplemented with 10% FBS in the absence or presence of SNAP (100 μM) and harvested at 24 h. Immunoprecipitation and Western blotting for p21 were performed using an anti-p21 polyclonal antibody. b, quiescent A431 cells were stimulated with DMEM supplemented with 10% FBS for 24 h in the presence of various concentrations of SNAP. Immunoprecipitation and Western blotting were performed as described in the legend for Fig. 1a. c, after serum starvation for 48 h, wild-type VSMCs and P53LMACO1 cells were incubated with [^{3}H]TdR in DMEM supplemented with 10% FBS in the absence or presence of SNAP (100 μM). Incorporated radioactivities were measured at 30 h. Data represent means ± S.D. (n = 3). *P < .05, **P < .01. Open columns, wild-type VSMCs, and filled columns, P53LMACO1 cells. d, Quiescent A431 cells were incubated with [^{3}H]TdR in DMEM supplemented with 10% FBS in the presence of various concentrations of SNAP. Radioactivities were measured at 24 h and are shown as percentages of values obtained in cells incubated without SNAP. Data represent means ± S.D. (n = 3). **P < .01.

p53 to promote transcription of a number of proteins involved in regulation of the cell cycle, DNA repair, and apoptosis, such as p21, Gadd45, and Bax (Agarwal et al., 1998). Indeed, p53 is induced by cytotoxic levels of NO, which are released from high concentrations of NO donors or generated by iNOS (Meßmer et al., 1994; Forrester et al., 1996; Ho et al., 1996; Ambs et al., 1997). But p53-dependent induction of p21 is not limited to cytotoxic stimuli; for instance, the induction of p21 by depleting ribonucleotides is dependent on p53 but not accompanied by cytotoxicity (Linke et al., 1996).

Our study suggested that p53 is involved in the expression of p21 induced by NO because SNAP up-regulated p53 probably by post-transcriptional mechanisms, activated p53-specific DNA binding, failed to stimulate a deletion mutant of p21 promoter that lacked p53 consensus sequences, and did not induce p21 in VSMCs obtained from p53-knock out mice or in A431 cells carrying mutated p53. An involvement of p53 also was suggested in the inhibition of cell proliferation in-

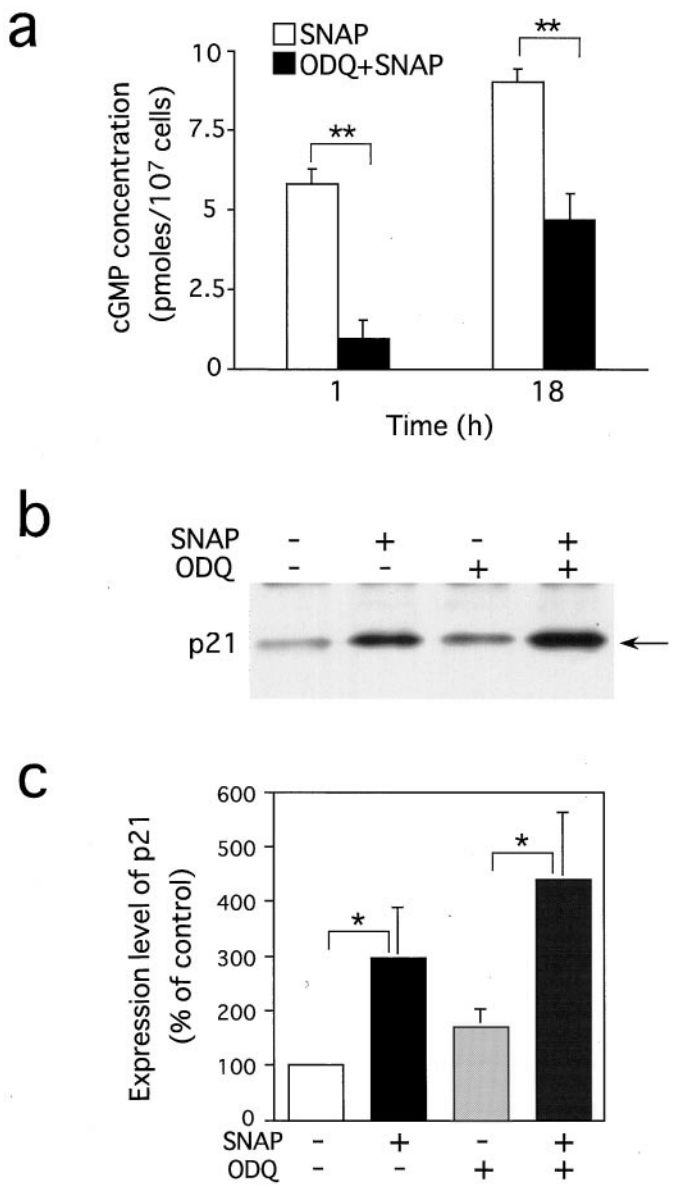

Fig. 7. Effect of an sGC inhibitor on SNAP-induced p21 expression. a, G_0 cells were stimulated with growth medium containing SNAP (100 μM) for indicated times. IBMX (100 μM) was added for 30 min before harvest. Cells represented by filled columns were pretreated with ODQ (10 μM) for 30 min before mitogenic stimulation, and the same concentration of ODQ was added to the growth medium. Intracellular cGMP concentrations were determined by radioimmunoassay as described in *Materials and Methods*. Data represent means ± S.D. (n = 3). $^{**}P < .01$. Open columns, SNAP and filled columns, ODQ + SNAP. b, G_0 cells were stimulated with growth medium for 18 h in the absence or presence of SNAP (100 μM) and analyzed for p21 expression by Western blotting. ODQ was added as described in (a). c, degrees of intensity of the blots obtained in (b) were quantified and are shown as means ± S.D. (n = 3). $^{*}P < .05$.

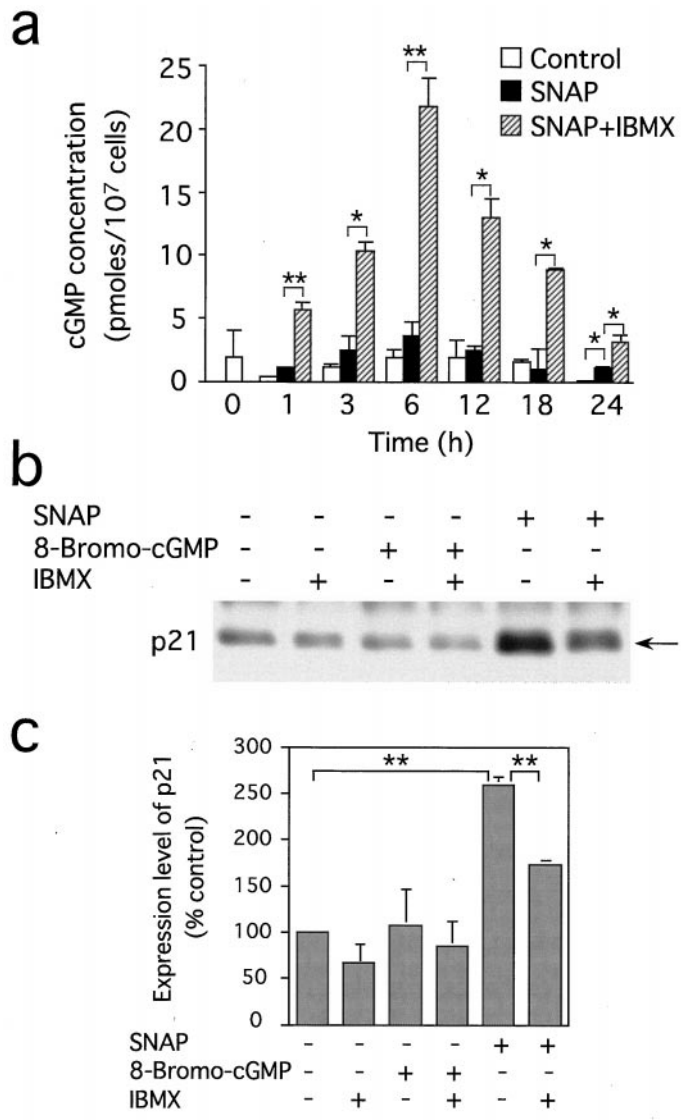

Fig. 8. Relation between SNAP-induced p21 expression and cGMP pathway. a, G_0 cells were stimulated with growth medium in the absence or presence of SNAP (100 μM), and intracellular cGMP concentrations were determined by radioimmunoassay at the indicated times. Cells represented by hatched columns were treated with IBMX (100 μM) for 30 min before extraction. Data from one representative experiment are shown. Similar results were obtained from three independent experiments. $^{*}P < .05$, $^{**}P < .01$. Open columns, control (vehicle-treated); filled columns, SNAP; and hatched columns, SNAP + IBMX. b, G_0 cells were stimulated with growth medium in the absence or presence of SNAP (100 μM), 8-bromo-cGMP (1 mM), and IBMX (100 μM), which were added alone or in combination. Cell lysates prepared at 18 h were analyzed for p21 expression by Western blotting. c, degrees of intensity of the blots obtained in (b) were quantified and are presented as means ± S.D. (n = 3). $^{**}P < .01$.

duced by NO because the antiproliferative effect of SNAP was attenuated in cells lacking p53 or containing mutated p53. The reason that NO partially retained the ability to inhibit proliferation in cells without functioning p53 may be that p21 induction is not the only way for NO to inhibit the cell cycle. In fact, NO has been suggested to inhibit cell proliferation by inhibiting the activity of ribonucleotide reductase, an essential enzyme for DNA synthesis (Roy et al., 1995), consistent with our finding that SNAP interrupted the smooth muscle cell cycle at at least two points located in the G_1 and S phases (Ishida et al., 1997).

SNAP did not appear to change the level of p53 in our previous study (Ishida et al., 1997), in which we lysed cells with a relatively mild detergent (0.5% Nonidet P-40) to avoid interference with subsequently performed immunoprecipitation. In the present study, we used a lysis buffer containing strong detergents to extract nuclear proteins more efficiently and immunoblotted whole-cell lysates instead of immunoprecipitates. The discrepancy between our results may have resulted from the difference in the efficiencies to extract p53 bound to DNA.

In other cell species, p53 has been suggested to be involved in NO-mediated p21 induction. Among several human cancer cell lines, NO gas induced p21 expression in cells containing wild-type p53 but not in cells lacking p53 or containing mutant p53 (Ho et al., 1996). In PC12 cells, the activation of p21 promoter by nerve growth factor was mediated by NO and partially but not totally depended on up-regulated p53 because responsiveness remained in a deletion mutant of the p21 promoter, in which a p53 consensus sequence was removed (Poluha et al., 1997). However, their mutant still contained the proximal p53 binding site that we deleted in the mutant promoter used in the present study, which may explain the incomplete loss of responsiveness.

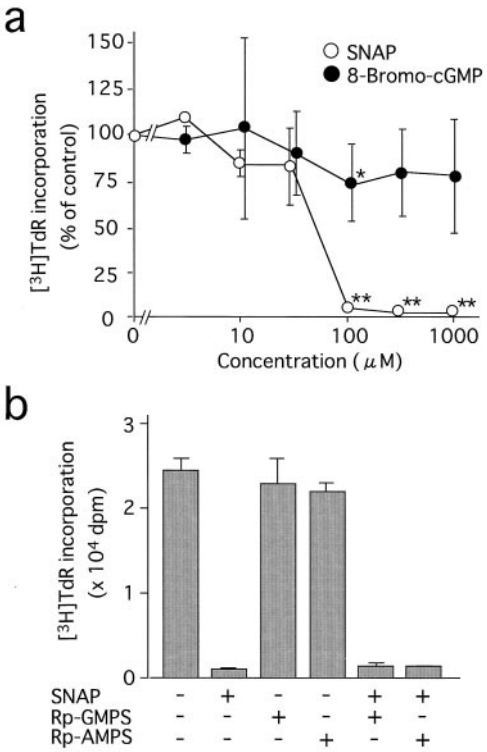

Fig. 9. Relation between antiproliferative effect of SNAP and cGMP pathway. a, G_0 cells were incubated with [^{3}H]TdR in growth medium in the presence of various concentrations of SNAP (○) or 8-bromo-cGMP (●). Incorporated radioactivities were measured at 24 h and are shown as percentages of values in cells incubated in the absence of the compounds. Data represent means ± S.D. (n = 3). *P < .05, **P < .01 versus the values obtained in the absence of the compounds. b, G_0 cells were labeled with [^{3}H]TdR in growth medium in the absence or presence of SNAP (100 μM), Rp-GMPS (25 μM), and Rp-AMPS (50 μM). Incorporated radioactivities were measured at 24 h. Data represent means ± S.D. (n = 3).

It is still unclear whether the up-regulation of p21 can be explained simply by the increase in the expression level of p53. SNAP prevented the decrease in the amount of p53 after mitogenic stimulation, resulting in the retention of p53 at levels 2- to 3-fold higher than control cells. However, the SNAP-induced elevation of DNA binding activity of p53 was more prominent. Therefore, the activity of p53 may be regulated not only by the amount of protein but also by other post-transcriptional mechanisms. Recent evidence suggests that the function of p53 is regulated by several modifications such as phosphorylation, glycosylation, acetylation, changes in redox states, and the association with regulatory proteins such as Mdm2 (Agarwal et al., 1998).

NO modifies the function of a variety of proteins by nitration, nitrosation, and nitrosylation. Such proteins so far reported include some enzymes such as ribonucleotide reductase and epidermal growth factor receptor tyrosine kinase (Roy et al., 1995; Estrada et al., 1997) and transcription factors such as activator protein-1, cAMP response element-binding protein, and nuclear factor-κB (Lander, 1997). In particular, NO modifies the thiol groups of cysteins contained in these transcription factors, which are important for DNA binding. The DNA-binding domain of p53 also contains several cystein residues that play important roles in binding to DNA (Cho et al., 1994). Recently, NO was found to modify the conformation and function of p53 (Rainwater et al., 1995; Calmels et al., 1997). High concentrations of SNAP (2–5 mM) increased the level of p53 in nuclei but significantly decreased DNA binding activity, whereas lower concentrations of SNAP (250–500 μM) stimulated DNA binding (Calmels et al., 1997). Further studies are needed to determine whether structural changes of p53 contribute to the up-regulation of p21 induced by NO.

Recent in vivo study suggested that p53 plays a significant role in the regulation of cell proliferation during the formation of atherosclerotic lesions (Guevara et al., 1999). The development of aortic atheromas in response to a high-fat diet was accelerated in mice deficient in p53 and apo-E compared with mice lacking apo-E only, being accompanied by a significant increase in the rate of cell proliferation. On the other hand, iNOS has been shown to be expressed in macrophages, smooth muscle cells, and T lymphocytes in human and rabbit atherosclerotic lesions (Buttery et al., 1996; Esaki et al., 1997; Luoma et al., 1998). Because we revealed that an NO donor activates p53 to induce the expression of p21 in VSMCs in vitro, NO generated in vivo by iNOS also may activate the p53-p21 pathway to inhibit excessive VSMC proliferation and thereby prevent development of atherosclerotic lesions. In fact, in vivo iNOS gene transfer has been reported to inhibit intimal hyperplasia in injured arteries of rats and pigs (Shears et al., 1998).

Our results also suggested that NO-induced p21 expression is independent of the sGC-cGMP system. Pretreatment with the sGC inhibitor ODQ did not inhibit p21 expression and 8-bromo-cGMP, IBMX, and their combination did not

induce p21. Furthermore, it may be particularly important that SNAP had no significant effect on intracellular cGMP concentration during the G_1 phase, although cGMP production, determined by measuring cGMP levels in the presence of IBMX, was certainly accelerated by SNAP. This implied that SNAP not only stimulated cGMP production but also accelerated hydrolysis of cGMP by phosphodiesterase. Considering that the SNAP-induced up-regulation of p21 is sustained for at least 30 h after mitogenic stimulation (Ishida et al., 1997), it is difficult to explain the up-regulation of p21 simply by the elevation of cGMP.

Smooth muscle relaxation is mediated by the sGC-cGMP pathway (Murad, 1986; Walter, 1989). NO stimulates sGC to convert intracellular GTP to cGMP by forming a nitrosylheme at the active center of this enzyme. However, it is unlikely that the sGC-cGMP system plays a predominant role in inhibition of VSMC proliferation induced by NO. The role of this system seemed to be small in the inhibition of G_1/S transition induced by NO because 8-bromo-cGMP resulted in only a slight inhibition of [^{3}H]TdR incorporation and Rp-GMPS did not attenuate the antiproliferative effect of SNAP.

Our results agree with previous reports that have demonstrated that 8-bromo-cGMP and zaprinast, a cGMP-specific phosphodiesterase inhibitor, are not able to inhibit cell proliferation (Garg and Hassid, 1990; Estrada et al., 1997). Interestingly, according to a recent article (Chiche et al., 1998), *S*-nitrosoglutathione, an NO donor, and 8-bromo-cGMP did not inhibit the proliferation of VSMCs, in which the expression of PKG had decreased after the cells were passaged in culture, but they significantly inhibited the proliferation in cells infected with adenovirus encoding PKG Iβ to restore the kinase activity. Chiche et al. (1998) proposed that an abundant expression of PKG in VSMCs in intact blood vessels increases cellular sensitivity to the antiproliferative effect of NO and cGMP.

In addition, PKA has been suggested to be partially responsible for the antiproliferative effect of NO in VSMCs (Cornwell et al., 1994). In our cells, however, an involvement of PKA is also unlikely because Rp-AMPS did not attenuate the antiproliferative effect of SNAP. Moreover, the antiproliferative effect of *S*-nitrosoglutathione on VSMCs infected with adenovirus encoding PKG was not blocked by KT5720, a PKA-selective inhibitor (Chiche et al., 1998).

Collectively, our results and those of others suggest that NO inhibits VSMC proliferation through two distinct mechanisms. At low concentrations such as are produced by eNOS, NO inhibits proliferation by the sGC-cGMP-PKG pathway, whereas relatively high concentrations of NO released from chemical NO donors (10^{-5}-10^{-4} M), two to three orders of magnitude higher than that required for smooth muscle relaxation, can inhibit proliferation by activating p53 to induce p21 expression independently of cGMP.

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