Potentiation of nitric oxide-induced apoptosis in p53−/− vascular smooth muscle cells

MELINA R KIBBE,1 JIANRONG LI,1 SUHUA NIE,1 BYUNG MIN CHOI,1 IMRE KOVESDI,2 ALENA LIZONOVA,2 TIMOTHY R BILLIAR,1 AND EDITH TZENG1
1Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania 15261; and 2GenVec Inc., Gaithersburg, Maryland 20878

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Kibbe, Melina R, Jianrong Li, Suhua Nie, Byung Min Choi, Imre Kovesdi, Alena Lizonova, Timothy R Billiar, and Edith Tzeng. Potentiation of nitric oxide-induced apoptosis in p53−/− vascular smooth muscle cells. Am J Physiol Cell Physiol 282: C625–C634, 2002. First published October 3, 2001; 10.1152/ajpcell.00119.2001.—The functional role of p53 in nitric oxide (NO)-mediated vascular smooth muscle cell (VSMC) apoptosis remains unknown. In this study, VSMC from p53−/− and p53+/+ murine aortas were exposed to exogenous or endogenous sources of NO. Unexpectedly, p53−/− VSMC were much more sensitive to the proapoptotic effects of NO than were p53+/+ VSMC. Furthermore, this paradox appeared to be specific to NO, because other proapoptotic agents did not demonstrate this differential effect on p53−/− cells. NO-induced apoptosis in p53−/− VSMC occurred independently of cGMP generation. However, mitogen-activated protein kinase (MAPK) pathways appeared to play a significant role. Treatment of the p53−/− VSMC with S-nitroso-N-acetylpenicillamine resulted in a marked activation of p38 MAPK and, to a lesser extent, of c-Jun NH2-terminal kinase, mitogen-activated protein kinase kinase (MEK) 1/2, and p42/44 (extracellular signal-regulated kinase, ERK). Furthermore, basal activity of the MEK-p42/44 (ERK) pathway was increased in the p53+/− VSMC. Inhibition of p38 MAPK with SB-203580 or of MEK1/2 with PD-98059 blocked NO-induced apoptosis. Therefore, p53 may protect VSMC against NO-mediated apoptosis, in part, through differential regulation of MAPK pathways.

mitogen-activated protein kinase; guanosine 3′,5′-cyclic monophosphate, p38; c-Jun NH2-terminal kinase; p42/44

APOTOPSIS OF VASCULAR SMOOTH MUSCLE CELLS (VSMC) is essential for angiogenesis and blood vessel formation but also serves an important role in several different vascular pathologies including atherosclerosis, intimal hyperplasia following vascular injury, and vascular remodeling. In these various processes, apoptosis has both beneficial and detrimental consequences. For example, in experimental models of atherosclerosis as well as in human arterial specimens, VSMC apoptosis has been detected within the atherosclerotic plaques (12). This programmed cell death may affect the size and stability of these lesions by leading to plaque regression or to destabilization of the fibromuscular lesion, resulting in plaque rupture (18). After vascular injury, VSMC apoptosis is evidenced at both early and late time points and may represent a mechanism by which the vasculature regulates overall neointimal thickness after damage to the arterial wall. Lastly, after vein grafting, medial VSMC undergo apoptosis upon exposure to arterial blood flow, and this may ultimately stimulate the remodeling process that occurs in the vein graft wall (24).

Nitric oxide (NO) is an important mediator of VSMC apoptosis. While NO is vasoprotective with respect to inhibition of platelet aggregation, leukocyte chemotaxis, VSMC proliferation and migration, and endothelial cell (EC) apoptosis (for review, see Ref. 15), NO can also induce apoptosis in VSMC under certain conditions (8, 22). After vascular injury, it may induce apoptosis, resulting in additional reduction of overall neointimal mass and maintenance of vascular patency. Alternatively, NO-induced apoptosis can be deleterious to the vasculature. Inducible NO synthase (iNOS) has been detected in atherosclerotic plaques and has been implicated in the initiation of VSMC apoptosis that may weaken the plaque and initiate plaque rupture (2, 5). Therefore, given the diverse roles of NO in inducing apoptosis in the vasculature, understanding how NO regulates this process is important to devise safe therapies directed at either enhancing or minimizing this proapoptotic effect.

The regulatory signals of the apoptosis cascade are extremely complex. p53 is one of the central regulators of apoptosis and is pivotal in designating whether an injured cell should undergo cell cycle arrest for DNA repair or proceed to apoptosis when the genomic damage is too extensive (27). While much remains unknown about the regulation of p53 and its ability to determine cell fate, several studies have demonstrated that p53 is involved in regulating VSMC apoptosis (3, 4). Furthermore, NO has been shown to upregulate and stabilize p53, and this increase in p53 expression has been associated with increased VSMC apoptosis.

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Address for reprint requests and other correspondence: M. R. Kibbe, Dept. of Surgery, Univ. of Pittsburgh, 677 Scaife Hall, Pittsburgh, PA 15261 (E-mail: kibbemr@msx.upmc.edu).

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(13). Conversely, p53 can downregulate iNOS expression and thereby control NO synthesis (6).

On the basis of evidence that p53 expression and NO production are intimately linked and given the importance of each of these molecules to VSMC apoptosis, we conducted studies to determine the contribution of p53 to NO-induced apoptosis. Surprisingly, we found that the absence of p53 renders VSMC more susceptible to NO-induced apoptosis than were p53 competent cells. The antiapoptotic effect of p53 appears to be conferred by the differential expression and activation of the mitogen-activated protein kinase (MAPK) pathways in response to NO.

**MATERIALS AND METHODS**

**Cell culture.** VSMC were cultured from thoracic aortas of p53−/− N4 and wild-type C57BL/6 × SV129 N5 mice (Taconic Laboratories, Germantown, NY) using the explant method. Cultured cells had the characteristic billets-and-valleys appearance and were routinely >95% pure by SMC α-actin staining (Dako, Carpinteria, CA). Cells were cultured in DMEM (low glucose)/Ham’s F-12 (1:1 vol/vol; BioWhittaker; Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; BioWhittaker), 100 U/ml penicillin (Life Technologies, Rockville, MD), 100 μg/ml streptomycin (Life Technologies), and 4 mM L-glutamine (Life Technologies) and maintained in a 37°C, 95% air-5% CO2 incubator.

For some experiments, VSMC were pretreated for 1 h with the following compounds before exposure to S-nitroso-N-acetylpenicillamine (SNAP) or H2O2 (25 μM) or N-acetyl-L-cysteine (250 μM). Cells were then treated with TNF-α (40 U/ml), IFN-γ (40 U/ml), or LPS (10 μg/ml). Cells were then incubated in fresh media for 6 h. Immediately after infection, cells were cultured in media with 10% FBS and tetrahydrobiopterin (10 μM; Schirck, Jona, Switzerland) for 24 h.

**Antisense oligonucleotides.** VSMC were plated for 24 h and then transfected with 1 μM of phosphorothioate-modified p21 antisense (5’-AGGATTGGACATGGTT-3’) or p21 sense (5’-ACCATGTCCAATCTC-3’) oligonucleotides in lipofectin at a concentration of 37°C with AdiNOS (9) was constructed and prepared as previously described (25).

**In vitro transduction of VSMC.** VSMC (passages 3–8) were plated for 24 h and then infected for 4 h at 37°C with AdiNOS or an adenoviral vector carrying the β-galactosidase gene (AdLacZ) using a multiplicity of infection of two. Immediately after infection, cells were cultured in media with 10% FBS and tetrahydrobiopterin (10 μM; Schirck, Jona, Switzerland) for 24 h.

**Adenoviral vectors.** An E1- and E3-deleted adenoviral vector carrying the human iNOS cDNA (AdiNOS) (9) was constructed and prepared as previously described (25).

**Western blot analysis.** VSMC were collected by scraping and were resuspended in buffer A (20 mM Tris with 100 μM phenylmethylsulfonyl fluoride (PMSF; Sigma), 1 μM leupeptin (Sigma), and 1 μM sodium orthovanadate (Sigma)). For some experiments, whole cell samples were converted to lysis and membrane bound fractions by three cycles of freezing and thawing. Protein was quantified with the bicinchoninic acid protein assay (Pierce, Rockford, IL). Samples (20–40 μg protein) were subjected to SDS-PAGE on 8, 10, or 13% gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were hybridized with rabbit polyclonal anti-Bcl-2, anti-poly(ADP-ribose)-polymerase (PARP), anti-p21 antibodies (1:1,000; Santa Cruz, Santa Cruz, CA), rabbit polyclonal anti-phospho-p42/44, p42/44, phosphoryc-Jun NH2-terminal kinase (JNK), JNK, and phospho-activating transcription factor (ATF)-2 antibodies (1:1,000; New England Biolabs, Beverly, MA) or a rabbit polyclonal anti-actin antibody (1:1,000, Sigma) followed by horseradish peroxidase-linked secondary antibody (1:10,000, Pierce). Proteins were visualized by using chemiluminescence reagents according to the manufacturer's instructions (Supersignal Substrate, Pierce).

**Lactate dehydrogenase measurements.** Lactate dehydrogenase (LDH) release in cultured media was measured by using an automated procedure on a Technicon RA-500 autoanalyzer.

**Quantitative MAPK assays.** The following protocol was provided by the New England Biolabs. VSMC were collected, rinsed with ice-cold phosphate-buffered saline (PBS), and resuspended in 1× lysis buffer (New England Biolabs) containing 1 mM PMSF. The cells were sonicated four times for 5 s each and centrifuged at 13,000 rpm for 10 min at 4°C, and the whole cell lysate was then transferred to a new tube. Protein lysate (150 μg) was mixed with immobilized phospho-p38 monoclonal antibody (p38 assay kit; New England Biolabs) or a phospho-mitogen-activated protein kinase kinase (MEK) 1/2 polyclonal antibody (MEK assay kit; New England Biolabs) overnight at 4°C to immunoprecipitate phospho-p38 and phospho-MEK1/2, respectively. The samples were then centrifuged and washed twice with lysis buffer and twice with kinase buffer. Immunoprecipitated proteins were resuspended in 50 μl of the kinase reaction mixture for 30 min at 30°C with one of the following substrates: ATP-2 fusion protein for phospho-p38 or nonphosphorylated p42/44 for phospho-MEK1/2 (New England Biolabs). The kinase reaction was terminated with 2× SDS-PAGE sample buffer. The samples were then boiled for 5 min and loaded onto a SDS-PAGE gel for Western blot analysis.

**FACS analysis.** To analyze both adherent and floating cells, VSMC were trypsinized (0.25%; Life Technologies) and combined with the medium from the respective wells, centrifuged, washed, and permeabilized with 70% ethanol for 30 min at 4°C. Cells were treated with DNase-free RNase (5 μg/ml; Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C and then stained with propidium iodide (50 μg/ml; Sigma, St. Louis, MO). The DNA content was analyzed by fluorescence-activated cell sorting (FACS) analysis (FACScan; Becton Dickinson, Bedford, MA) using Lysis II cell cycle analysis software (Becton Dickinson). A total of 1 × 104 cells were counted for each sample.

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VSMC cultured on coverslips were fixed in 4% paraformaldehyde at 4°C for 1 h. After permeabilization in 0.1% Triton X-100 (Sigma) for 20 min and several rinses in PBS, the VSMC were incubated with the TUNEL reaction mixture for 1 h at 37°C in the dark. VSMC were rinsed in PBS and then mounted on a slide and viewed under a fluorescent microscope (Olympus, Tokyo, Japan).

Hoechst staining. VSMC were rinsed with Hanks’ balanced salt solution (Life Technologies), exposed to Hoechst 33258 (2 μg/ml; Sigma) for 30 s, rinsed, and coverslipped with gelvatol. Images were collected with an Olympus Provis microscope.

Statistical analysis. Results are expressed as means ± SE. Differences between groups were analyzed by using one-way analysis of variance (ANOVA) with the Student-Newman-Keuls post hoc test for all pairwise comparisons (SigmaStat; SPSS, Chicago, IL). Statistical significance was assumed when P < 0.05.

RESULTS

**p53**−/− VSMC are sensitive to NO-induced cell death. Both **p53**−/− and **p53**+/+ VSMC were treated with increasing concentrations of the NO donor SNAP. **p53**−/− VSMC underwent a significantly greater level of cell death upon exposure to NO than did matched **p53**+/+ VSMC (Fig. 1A) in both time- and concentration-dependent fashions (Fig. 1B). After 32 h of treatment with 1 mM SNAP, ~25% of the **p53**−/− VSMC contained hypodiploid fragmented DNA as measured by FACS analysis, whereas wild-type cells showed minimal increase above baseline levels.

The effect of endogenous NO production was tested by expression of iNOS in the VSMC by using AdiNOS. The **p53**−/− VSMC underwent a similar but more dramatic cell death after infection with AdiNOS compared with cells treated with SNAP (Fig. 1C). The apoptosis observed after iNOS gene transfer was inhibited with administration of the NOS inhibitor L-NIO, indicating that this effect was NO specific. The more dramatic apoptotic effect of iNOS gene transfer is most likely the result of sustained NO production over the 24-h duration of the experiment. In cells treated with SNAP, the donor was administered only at the beginning of the experiment, and the half-life of SNAP is on the order of 7–14 h. Therefore, NO release decreases with time, and this may explain the longer period of SNAP treatment required to elicit apoptosis.

For the subsequent mechanistic studies, we used 1 mM SNAP as the source of NO to simplify our approach. This concentration was chosen because it re-

Fig. 1. Both exogenous and endogenous sources of nitric oxide (NO) preferentially induce cell death in **p53**−/− vascular smooth muscle cells (VSMC). A: phase-contrast microscopy of **p53**+/+ and **p53**−/− VSMC 32 h after exposure to S-nitroso-N-acetylpenicillamine (SNAP). B: **p53**+/+ and **p53**−/− VSMC were exposed to increasing concentrations of SNAP over varying lengths of time. Percent apoptosis was determined by measuring the hypodiploid DNA content by fluorescence-activated cell sorting (FACS) analysis. C: **p53**+/+ and **p53**−/− VSMC were infected with an adenoviral vector carrying the human inducible NO synthase (iNOS) gene (AdiNOS) for 4 h. Twenty-four hours later, hypodiploid DNA content was determined by FACS analysis. AdLacZ, adenoviral vector carrying the β-galactosidase gene; NIO, L-N5-(1-iminoethyl)ornithine dihydrochloride. Data are representative of 3–5 experiments.
producing a cell death rate of ~25% by 32 h. However, it should be noted that even SNAP at concentrations as low as 25 μM induced significantly more cell death in p53−/− VSMC compared to wild-type cells (Fig. 1B).

To confirm that NO was initiating apoptosis in p53−/− VSMC, we examined the morphological changes of the nuclei. Upon exposure to SNAP, the nuclei of p53−/− VSMC exhibited nuclear condensation and fragmentation (Fig. 2A, d and e) as well as nuclear blebbing (Fig. 2A, f), all of which are characteristic of apoptosis. TUNEL assay also revealed positive end nick labeling in p53−/− VSMC exposed to SNAP but not in similarly treated wild-type cells (Fig. 2B, d vs. b). Bcl-2, Bcl-xl, and PARP expression also were evaluated using Western blot analysis. While there was no significant difference in the expression of Bcl-xl, there was increased Bcl-2 expression in p53−/− VSMC compared with p53−/− VSMC at baseline as well as after SNAP exposure (Fig. 3A). SNAP-treated p53−/− VSMC were associated with PARP cleavage to the 85-kDa fragments, which is associated with apoptosis (Fig. 3B). Further supporting the etiology of p53−/− cell death as apoptosis, no increase in LDH release was detected at 32 h, a time point associated with a 25% increase in cells.

Fig. 2. NO-induced cell death in p53−/− VSMC shows nuclear characteristics consistent with apoptosis. A: p53−/− VSMC were exposed to media alone (a–c) or SNAP (1 mM; d–f) for 32 h. The nuclei were stained with Hoechst 33258 and visualized with fluorescent microscopy. SNAP treatment resulted in nuclear condensation (d and e), fragmentation (d and e), and blebbing (f). Magnification: a and d, ×20; b and e, ×40; c and f, ×100. B: p53−/− (a and c) and p53−/− VSMC (b and d) were treated with media alone (a and b) or SNAP (1 mM; c and d) for 32 h. TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) positive cells (arrows) were detected in the p53−/− VSMC exposed to SNAP. Magnification: ×20. Data are representative of 3 experiments.
containing hypodiploid DNA (Fig. 3C). At later time points, cell death by both FACS analysis and by LDH release increased in both p53+/+ and p53−/− cells, suggesting that necrosis and apoptosis both may be involved with prolonged exposure to SNAP.

Lastly, we investigated whether the apoptotic effect of NO on p53−/− VSMC was specific to NO. Both p53+/+ and p53+/+ VSMC were treated with known proapoptotic agents. Cell death induced by actinomycin D, calyculin A, cantharidin, diethyl maleate, and Fas was not statistically different between wild-type and knockout VSMC (Fig. 3D). Cyclohexamide did induce greater cell death in p53+/+ than in p53−/− VSMC, which fits the accepted proapoptotic role of p53. Thus the ability of NO to differentially induce apoptosis in p53 deficient cells may be specific to NO.

NO-induced apoptosis in p53−/− VSMC is independent of cGMP. To determine whether the NO-induced cell death is mediated through soluble guanylate cyclase (sGC)-cGMP signaling, p53−/− and p53+/+ VSMC were pretreated with the sGC inhibitor ODQ (40 μM) and then exposed to SNAP (1 mM). At the concentration used, ODQ completely inhibited cGMP release after SNAP treatment (17). However, it did not inhibit SNAP-induced apoptosis in p53−/− VSMC (Fig. 4).

p38 MAPK and MEK1/2 inhibitors, SB-203580 and PD-98059, respectively, inhibit NO-induced apoptosis in p53−/− VSMC. We examined the role of other signaling pathways known to be activated by NO, namely, the MAPK pathways. VSMC were pretreated for 1 h with either SB-203580, an inhibitor of phospho-p38 MAPK, or PD-98059, an inhibitor of MEK1/2 that lies upstream of p42/44 (extracellular signal-regulated kinase, ERK), before exposure to SNAP. The inhibitors alone did not alter cell viability. However, they inhibited NO-induced apoptosis in p53−/− VSMC (Fig. 5, A and B), indicating that the MAPK pathways are involved in regulating this differential effect of NO on p53+/+ and p53−/− VSMC.

p53+/+ VSMC express higher baseline levels of p42/44. To determine whether MAPK pathways regulate NO-induced apoptosis in p53−/− VSMC, cells were treated with SNAP and cell lysates were analyzed for the expression and activities of the different MAPK.

Fig. 3. Cell death in p53−/− VSMC is consistent with apoptosis and may be specific to NO. p53+/+ and p53−/− VSMC were exposed to media alone or SNAP (1 mM) and were collected 32 h after treatment. Western blot analysis of Bcl-2 and Bcl-xl (A; in duplicate) and poly(ADP-ribose) polymerase (PARP) expression (B; in triplicate) was performed. C: lactate dehydrogenase (LDH) release was determined. D: effect of known proapoptotic agents on p53−/− and p53+/+ VSMC was determined by FACS analysis: actinomycin D (0.01 μg/ml), cyclohexamide (50 μg/ml), calyculin A (50 nM), cantharidin (4 μM), diethyl maleate (5 mM), and Fas (1 μg/ml). Data are representative of 3–5 experiments.
Wild-type cells demonstrated higher basal levels of MEK1/2 activity that diminished after 15 min of SNAP exposure (Fig. 6A). p53−/− VSMC showed an increase in MEK1/2 activity 5 min after SNAP exposure, with subsequent return to baseline (Fig. 6A). When a quantitative MEK1/2 assay was performed, a mild but significant increase in activity (1.2-fold) was detected in p53−/− cells at 5 min after SNAP treatment, whereas a gradual reduction in activity (0.7-fold) to 60 min was detected in the wild-type cells (Fig. 6B; \( P < 0.001 \)).

Finally, we examined p42/44, one of the downstream effectors of MEK1/2, through Western blot analysis. Levels of p42/44 protein were equivalent in p53−/− and p53+/+ cells and were unchanged by SNAP treatment. However, p53+/+ VSMC expressed higher levels of phosphorilated p42/44 at baseline compared with p53−/− cells, and exposure to SNAP reduced this expression. In p53−/− VSMC, SNAP induced an increase in phosphorilated p42/44 (Fig. 6C). These results correlate with the findings from the kinase assays, indicating that p53+/+ VSMC express greater MEK-p42/44 MAPK activity at baseline that is then inhibited by NO; in contrast, NO transiently activates the MEK-p42/44 pathway in p53−/− VSMC.

**Activation of JNK by NO in p53−/− cells.** Similar to the effect NO had on the MEK-p42/44 pathway in p53−/− VSMC, SNAP induced a small increase in both the phosphorylated form of JNK, as measured by Western blot analysis (Fig. 7A) and JNK kinase assay (\( P < 0.001 \); Fig. 7B), in the knockout cells. However, SNAP did not significantly alter JNK activity in wild-type cells.

**Activation of p38 MAPK by NO in p53−/− cells.** The p38 MAPK pathway has been shown to regulate proapoptotic signaling in a variety of cell types (23). Our studies indicated that p38 plays a major role in NO-mediated cell death in p53-deficient cells. SNAP treatment of these cells induced a dramatic increase in p38 activity at 5 min (\( P = 0.016 \) vs. 0 min; Fig. 8) with return to basal levels by 15 min (\( P = 0.012 \) vs. 5 min; Fig. 8). Subsequently, p38 activity increased again at 30 and 60 min but to a lesser extent. In contrast, p53+/+ VSMC possessed higher basal p38 activity, and NO significantly reduced this activity (Fig. 8).

**NO upregulates p21 expression in p53−/− VSMC.** Because prior studies have shown that NO can activate MAPK pathways and that MAPK pathways can regulate p21 expression, we examined the role of p21 in...
NO-induced apoptosis in p53−/− VSMC. NO induced a dramatic increase in p21 protein levels in a time- and concentration-dependent manner in the knockout cells (Fig. 9A). Levels of p21 were essentially unchanged in wild-type cells. However, inhibiting p21 expression with p21 ASO did not inhibit NO-induced apoptosis in p53−/− VSMC (Fig. 9B). This would suggest that p21 protein is increased after NO exposure in knockout cells but is not integral to mediating NO-induced apoptosis.

DISCUSSION

Although much research has focused on the role of VSMC proliferation in the vascular healing response and in the pathogenesis of atherosclerosis, VSMC apoptosis may contribute to the pathogenesis of these processes. Apoptosis has been documented in both human disease and animal models of atherosclerosis, and apoptosis of neointimal and medial VSMC is known to occur after vascular injury and may limit overall intimal hyperplasia. NO, known to inhibit intimal hyperplasia after injury (for review, see Ref. 16), has been shown to induce VSMC apoptosis (13) in vitro and has been implicated in apoptosis of VSMC after injury (5). NO has been reported to induce apoptosis in a variety of cell types, including cardiac myocytes (1), fibroblasts (14), thymocytes (10), endometrial cells (20), and multiple different tumor cell types (26), among others. Many studies have shown that this NO-induced apoptosis is often mediated through the creation of DNA damage that then induces the expression of the tumor suppressor gene p53. However, the role of p53 in NO-mediated VSMC apoptosis has not been well characterized. In this study, we present evidence that, contrary to the accepted paradigm, the absence of p53 renders VSMC more susceptible to NO-induced apoptosis. Cells cultured from p53−/− mice underwent higher levels of apoptosis after exposure to both endogenous and exogenous sources of NO compared with wild-type cells. The susceptibility of p53−/− cells to NO appears to involve the differential regulation of MAPK pathways (Fig. 10).

Loss of wild-type p53 activity is traditionally associated with resistance to apoptosis and malignant transformation. However, in the past several years, our understanding of the role of p53 in determining cellular responses to genotoxic agents has evolved. On the one hand, it is known that p53 may enhance sensitivity to programmed cell death via transcription-independent events as well as through the regulation of proapoptotic and antiapoptotic genes (21). Conversely, p53 is capable of imposing resistance to apoptosis by promoting growth arrest, DNA repair, and cellular differentiation and by enhancing the expression of antiapoptotic genes (21). Cell culture studies revealed that the role of p53 in apoptosis is cell-type specific as well as cell-context specific (29). Some cell types derived from p53−/− animals are more sensitive to genotoxic
stresses such as ultraviolet and ionizing irradiation and certain chemotherapeutic agents (Taxol) (11, 28), whereas other cell types from the same animals are resistant to these same agents (29). Therefore, the exact role of p53 in determining cell fate is complex and multifaceted.

One possible explanation for the ability of NO to induce apoptosis in p53−/− VSMC may be related to the proliferative state of the cell. p53−/− VSMC proliferate at much faster rates than do matched wild-type cells, presumably because of impaired cell cycle regulation and the inability to undergo appropriate G1 cell cycle arrest (data not shown). Normally, when a cell sustains DNA damage, p53 induces cell cycle arrest. Cell cycle arrest prevents replication or segregation of damaged DNA and facilitates repair. The inability to undergo cell cycle arrest can permit cells with damaged DNA to undergo misrepair with propagation of mutations and resultant immortalization of the cell. Alternatively, DNA damage may be so severe that mutations accumulate to the point that cellular demise is inevitable and the cell undergoes apoptosis. Hence, rapidly proliferating cells may be more susceptible to genotoxic agents such as NO because of the insufficient amount of time spent in G0/G1 to undergo DNA repair. In support of this latter hypothesis, we found that p53−/− VSMC displayed significant cellular heterogeneity and pleomorphism and a higher aneuploid DNA content compared with wild-type cells (data not shown). Hence, the impairment of cell cycle regulation by the absence of p53 may ultimately confer susceptibility to apoptosis.

The most accepted NO signaling pathway in VSMC is that involving cGMP. However, cGMP is not involved in the enhanced apoptotic response to NO in knockout VSMC. Another potential signaling pathway is one involving the cell cycle inhibitor p21. We have shown that NO-treated VSMC demonstrate a significant increase in p21 protein expression (17). Similarly, p21 expression was stimulated by NO in time- and concentration-dependent fashions in p53−/− cells but not in wild-type cells. While there was a clear association between p21 expression and NO-mediated apoptosis in the knockout cells, p21 was not involved in this response, as evidenced by persistent sensitivity to NO after p21 expression was blocked with ASO. One potential role of enhanced p21 expression in NO-treated p53−/− cells may be to promote cell cycle arrest in the absence of p53.

The differential effect of NO on p53−/− and p53+/+ cells appears to be intimately linked to the MAPK pathways. The link between NO and the MAPK pathways was established by Lander et al. (19), who reported that NOx species induced a rapid and transient

![Fig. 7. SNAP transiently activates c-Jun NH2-terminal kinase (JNK) in p53−/− VSMC.](http://ajpcell.physiology.org/)
activation of all three MAPK pathways in Jurkat T cells. Subsequently, we have shown that NO activates MAPK pathways in VSMC (17). What was evident in our study is that inhibitors of the MAPK pathways reversed the proapoptotic effect of NO in p53−/− cells. While the inhibitors are not specific to a single MAPK pathway and may inhibit non-MAPK pathways at the doses used, the results would suggest that one or all of these pathways might be involved. When the different MAPK pathways were individually evaluated, we found that basal MEK1/2, p42/44, and p38 activities were decreased in p53−/−/H11002/VSMC compared with p53+/−/VSMC. The activities of these kinases were all quickly suppressed by NO in p53−/−/cells and increased in p53−/− cells. p38 activation in NO-treated knockout cells was the most dramatic.

The roles of the different MAPK pathways have been defined but are ever evolving. Traditionally, the p38 and JNK pathways have been considered proapoptotic, whereas the p42/44 pathway has been associated with cell proliferation. However, the balance between the basal activity of these pathways and their inducibility by various genotoxic agents may prove to be the factor that determines the fate of different cells. The increased basal activity of p42/44 and p38 in p53−/− cells may be the result of stabilization of these kinases by p53. The basal activation of these MAPK pathways may repress the expression of proapoptotic genes or enhance the expression of antiapoptotic gene products such as Bcl-2, as evidenced in p53-competent cells. What also was different between the p53−/− and p53+/+ cells was that all MAPK pathways were activated by NO in the knockout cells but inhibited in the wild-type cells. It is possible that the basal MAPK activity level of the cell may lead to different thresholds for subsequent activation of the enzymes by certain genotoxic agents such as NO. NO induced only a low-level activation of MEK-p42/44 as well as JNK but a much more dramatic activation of p38 in knockout VSMC but not in wild-type cells. The greater activation of p38 and the activation of JNK may overcome the antiapoptotic effects of MEK-p42/44 and resulted in apoptosis in knockout cells. Finally, MAPK may confer stability to p53 in wild-type cells, thereby allowing appropriate cell cycle arrest and repair when exposed to NO. In support of this hypothesis, Fuchs et al. (7) have shown that p53 stability is affected by JNK in MCF-7 cells. Further studies are required to explore all these hypotheses.

In conclusion, we found that the absence of p53 enhances the sensitivity of VSMC to NO-induced apoptosis. This sensitivity is not dependent on cGMP or p21 but appears to be mediated through altered basal activation of MAPK pathways as well as the ability of NO to activate these pathways in p53−/− and p53+/+ cells. Further studies delineating the role of p53 in regulating the MAPK pathways may yield important information about the function of p53. However, it is clear that the ability of NO to induce apoptosis in VSMC is more complex than originally thought.
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