Focused PCR Screen Reveals p53 Dependence of Nitric Oxide-Induced Apoptosis and Up-Regulation of Maspin and Plasminogen Activator Inhibitor-1 in Tumor Cells

Shuhui Lim, Amos C. Hung, and Alan G. Porter

*Cell Death and Human Disease Group, Division of Cancer and Developmental Cell Biology, Institute of Molecular and Cell Biology, A*STAR (Agency for Science, Technology and Research), Singapore, Republic of Singapore*

Abstract

We investigated p53-dependent gene expression in nitric oxide (NO)-induced apoptosis of two tumor cell types. Seventy-seven putative p53-regulated genes were screened for NO-mediated expression changes. Twenty-four genes were up-regulated and three genes were down-regulated significantly by NO in human neuroblastoma cells. Genes known to be involved in apoptosis, which were up-regulated by ≥2-fold, included *FAS, CASP-1, BIK, PUMA, DR4* and the serpins maspin (SERPINB5), and plasminogen activator inhibitor-1 (PAI-1). Real-time PCR confirmed maspin and PAI-1 mRNAs exhibited the greatest NO-induced induction, which occurred in a p53-dependent manner. The substantial NO-mediated up-regulation of these serpins mRNAs correlated with large increases in their protein levels, which occurred before or coinciding with apoptosis. p53-deficient neuroblastoma cells were largely resistant to NO killing and showed much reduced maspin and PAI-1 mRNA and protein levels after NO treatment. p53 was activated by NO mainly in the nuclei of neuroblastoma cells. $p53^{-/-}$ HCT116 colon carcinoma cells were strongly resistant to NO-induced apoptosis and failed to up-regulate maspin and PAI-1 (in contrast to $p53^{+/+}$ HCT116 cells). Our results suggest that both apoptosis and induction of the two serpins by NO require the transcriptional activity of p53. Because maspin is a tumor suppressor and PAI-1 can promote senescence and regulate cell death, it will now be worth investigating whether their p53-mediated expression contributes to the NO-induced p53-dependent death of tumor cells. (Mol Cancer Res 2009;7(1):55–66)

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A.C. Hung and A.G. Porter contributed equally to this work.

Current address for A.C. Hung: Menzies Research Institute, University of Tasmania, Australia.

Requests for reprints: Lim Shuhui, Cell Division and Cancer Laboratory, Institute of Molecular and Cell Biology, 61 Biopolis Drive, Proteos, Singapore 138673, Republic of Singapore. Phone: 65-6586-9850; Fax: 65-6779-1117. E-mail: shlim@imcb.a-star.edu.sg

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Introduction

Nitric oxide (NO) promotes and controls a bewildering range of functions in cells of many tissue origins (1-3). Relatively low levels of NO mediate normal physiologic functions such as vasodilation and neurotransmission. In contrast, high levels of extracellular NO can mediate macrophage killing of target cells during infections, and overproduced intracellular NO (e.g., from NO syntheses) can induce inappropriate apoptosis or necrosis (4, 5). The ability of NO to simultaneously induce both protective and cell death–inducing responses, the balance of which is considered to determine the outcome, provides a further level of complexity (1, 6). Thus, many cells can resist cell death through the ability of moderate amounts of NO to variously up-regulate diverse antiapoptotic/repair proteins or antioxidant pathways including activator protein, neural cell adhesion molecule-140, DNA protein kinase, Bcl-X_L, Bcl-2, cyclic AMP-response element-binding protein, and Nrf2 (7-11). Conversely, excessive NO can elicit apoptosis or necrosis through a huge variety of proposed pathways and mechanisms. These may be transcription-independent—for example NO-mediated S-nitrosylation of specific amino acids in certain proteins (some of which may also be antiapoptotic; refs. 5, 12); or involve NO reacting with superoxide to generate the highly reactive-free radical peroxynitrite, which causes nonspecific oxidative DNA, protein, and lipid damage (4, 5). Alternatively, cell death may depend on NO-stimulated signaling pathways leading to gene expression, involving the c-Jun-NH_2-kinase or p38 mitogen-activated protein kinases, phosphorylated c-Jun, $p19^{ARF}$, and the tumor suppressor p53 (5, 8, 13-18).

However, it is likely that not all these pathways operate in all cell types, and many NO-dependent target genes remain to be identified that contribute to cell death. Indeed, microarray studies from several groups using different cell lines has revealed a large diverse set of mRNAs that are up- or down-regulated by NO but few common targets (2, 19, 20). For example, in human lymphoblastoid cells, a number of known p53 target genes associated with apoptosis were either up-regulated (*PIG3, FAS/CD95, GADD45, PUMA, NOXA*) or down-regulated (*XIAP, c-IAP-1, SURVIVIN*) in a p53-dependent manner after NO stimulation (20). It was not further investigated which, if any, of these genes are required to mediate NO-induced apoptosis in these cells.

The tumor suppressor p53 controls a remarkable number of physiologic functions (including metabolism, differentiation, reactive oxygen species levels) and is stabilized and activated in response to diverse stresses (DNA damage, hypoxia, oncogene

activation, drugs, nucleotide depletion; refs. 21, 22). Cells that possess a fully functional p53 pathway can either arrest and repair the damage caused by these untimely stresses or undergo p53-dependent apoptosis. p53 acts largely as a sequence-specific transcription factor during apoptosis (22-25), although in some scenarios, it may translocate to mitochondria and mediate cell death by various mechanisms independently of (or in conjunction with) new gene transcription (26). Considerable effort has gone into searching for the relevant target genes that are required for p53-dependent apoptosis, but thus far, only a few are regarded as likely proapoptotic targets in certain tissues and in some cell lines, although it is less clear to what extent they may also mediate tumor suppression. These include *BAX, PUMA, NOXA, KILLER/DR5, FAS/APO-1, BIM, BID, PIG3, PIDD,* and *APAF-1* (23-25, 27, 28).

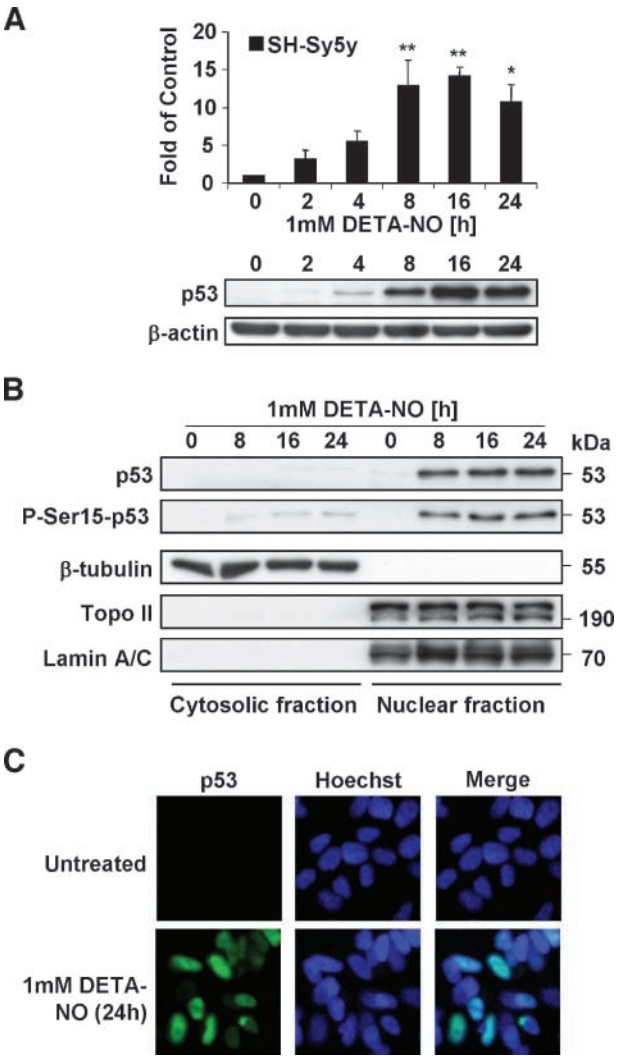


FIGURE 1. NO induces p53 serine 15 phosphorylation and p53 accumulation in the nuclei of SH-Sy5y cells. **A.** SH-Sy5y cells were treated with 1 mmol/L DETA-NO for various times. Western blot of 10% polyacrylamide gel probed with anti-p53 antibody. Bands were quantified and expressed as average fold of untreated cells ± SE (n = 3) after normalization to β-actin levels. *, $P < 0.05$; **, $P < 0.01$ compared with untreated control. **B.** SH-Sy5y cells were treated with 1 mmol/L DETA-NO for various times. Cytosolic and nuclear extracts were prepared and separated in a 10% polyacrylamide gel. The membrane was probed with anti-p53 and anti–ser15-p53 antibodies. **C.** Immunofluorescence with Alexa Fluor 488–labeled antibodies. SH-Sy5y cells were treated with 1 mmol/L DETA-NO, and the fixed cells were stained with an anti-p53 antibody. Bound antibodies were probed with Alexa Fluor 488 goat anti-mouse IgG and Hoechst 33342 nuclear stain, washed, and mounted for microscopy. Green, p53 staining; blue, nuclear staining. Pale greenish-blue, overlap between p53 and the nucleus in the merged images.

Identification of stress-regulated p53 target genes that make a significant contribution to apoptosis has been hampered by two main factors. First, *in vivo* studies have indicated that the few known p53 target genes found to contribute to apoptosis act in an unpredictable stress- and cell type–dependent fashion (23-25, 27). Second, there are hundreds of putative or known p53 target genes in several categories, including cell cycle arrest, reactive oxygen species and respiration control, genome stability, and angiogenesis, in addition to apoptosis and senescence (29-33). Regulation of genes in some of these categories (e.g., respiration or cell cycle control) might also be expected to influence susceptibility to apoptosis.

Most (but not all) studies associate NO-induced apoptosis with p53 activation, but it remains unclear whether p53 normally makes a major or minor contribution to NO-mediated cellular demise (4, 34, 35). In this article, we set out to determine the extent to which NO-induced apoptosis is p53 dependent, and did a focused reverse transcription-PCR (RT-PCR) screen to determine if any known or putative p53-regulated genes are induced or repressed by NO in a p53-dependent manner.

Results

NO Induces p53 Accumulation and p53 Ser-15 Phosphorylation Mainly in Nuclei of Cells Before and During p53-Dependent Apoptosis

We first verified the kinetics and magnitude of apoptosis induced by the NO donor diethylenetriamine NO adduct (DETA-NO) in SH-Sy5y neuroblastoma cells. Similar to our previous study (11), apoptosis was detected between 8 and 16 hours after DETA-NO treatment (Supplementary Fig. S1A). Consistent with and extending this result, we found that two other apoptosis markers, procaspase-3 and poly(ADP-ribose) polymerase, were cleaved from 8 hours onwards (Supplementary Fig. S1B and C). To determine whether the wild-type p53 in human SH-Sy5y cells is up-regulated and activated with similar kinetics to cell death, we did Western blots with p53 and phospho Ser-15 p53 antibodies. p53 protein was up-regulated as early as 2 hours after NO donor treatment, peaking between 8 and 16 hours (Fig. 1A). This up-regulation was a posttranscriptional event because p53 mRNA levels remained constant throughout (data not shown). Cell fractionation and immunofluorescence studies both showed that p53 protein and Ser-15 phosphorylated protein accumulated in the nuclei of SH-Sy5y cells (Fig. 1B and C), although a minor fraction of Ser-15–phosphorylated p53 was detectable outside the nucleus (Fig. 1B). Because phosphorylation of p53 on Ser-15 is one gain-of-function indicator (21, 36), these data together show that p53 accumulates and is activated mainly in the nucleus before and during cell death induced by NO in SH-Sy5y cells.

We next generated SH-Sy5y cells stably transfected with siRNA to p53 to quench the NO-induced p53 up-regulation. Two independent clones were generated in which p53 mRNA

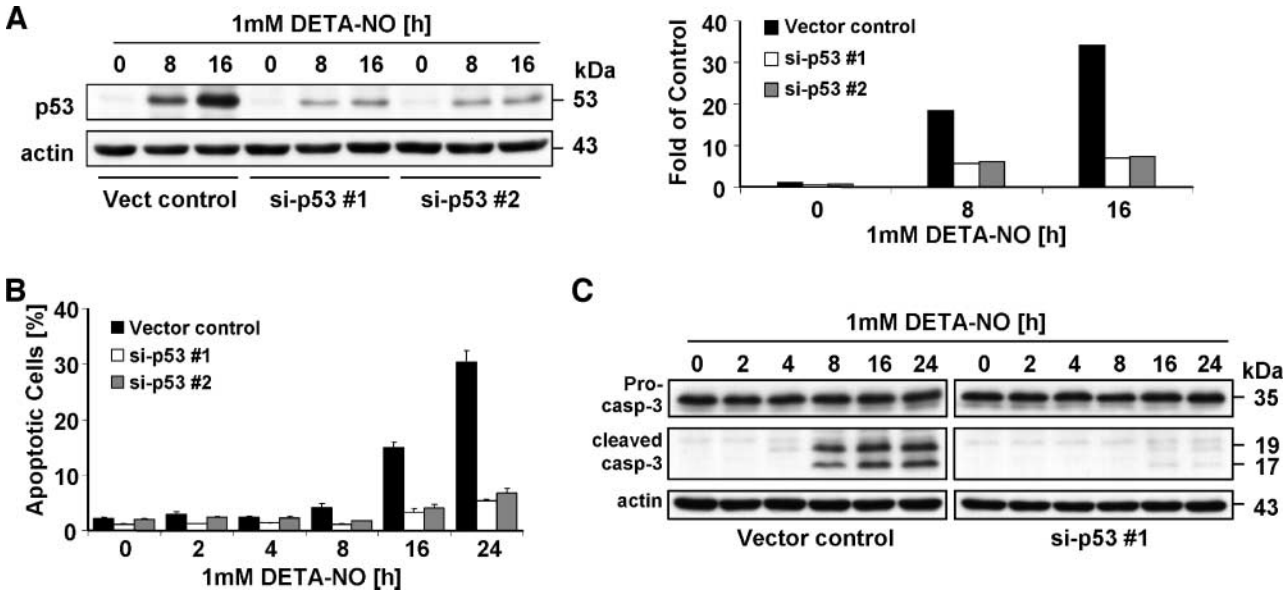


FIGURE 2. Stable SH-Sy5y cells expressing p53 siRNA are resistant to NO-induced apoptosis. **A.** Western blot analysis. SH-Sy5y vector control cells and si-p53 clones #1 and #2 were treated with 1 mmol/L DETA-NO for various times. Total cell lysates were separated in 10% polyacrylamide gels, and the membrane was probed with anti-p53 antibody (*left*). The bands were quantified and expressed as fold of untreated vector control cells after normalization to β-actin protein levels (*right*). **B.** Fluorescence-activated cell sorting analysis after PI staining. SH-Sy5y vector control cells and si-p53 clones #1 and #2 were treated with 1 mmol/L DETA-NO for various times and fixed in 70% ethanol. After rehydration, intracellular DNA was stained with PI and the percentage of apoptotic cells was determined by flow cytometry. Columns, mean (n = 3); bars, SE. **C.** SH-Sy5y vector control and si-p53 knockdown clone #1 cells were treated with 1 mmol/L DETA-NO for various times. Similar results were obtained for si-p53 clone #2 (data not shown). Total cell lysates were separated in a 10% polyacrylamide gel, which was probed with an anti–caspase-3 antibody.

levels were >70% lower (data not shown), and NO-mediated p53 protein accumulation was reduced by >80% at 16 h posttreatment (Fig. 2A). Both clones exhibited strong resistance to NO-induced apoptosis as measured by flow cytometry and procaspase-3 processing (Fig. 2B and C). These data together with two independent approaches to inhibit p53 (see later) provide evidence that nuclear p53 plays a prominent pro-apoptotic role in NO-induced cell death.

NO Induces or Represses a Subset of Putative p53-Regulated Genes in SH-Sy5y Cells

There are large numbers of known and putative p53-regulated genes (24, 25, 27, 33, 37, 38). We did semiquantitative RT-PCR for 77 of such genes in DETA-NO–treated SH-Sy5y cells. Genes were selected either because they have been shown to be involved in p53-dependent apoptosis in other systems, or are implicated in the cell cycle, reactive oxygen species generation or scavenging, respiration, genome stability, or senescence—any of which could potentially influence susceptibility to NO-induced cell death.

Cultures of SH-Sy5y cells were treated with DETA-NO, and samples were taken at various times from 2 to 24 hours after treatment for semiquantitative RT-PCR analysis using commercially validated primers. Conditions were established where DNA amplifications gave single unsaturated bands in agarose gel electrophoresis, and the analyses were done at least thrice on independently collected RNA samples. The bands were quantified by scanning and normalized to untreated samples. The fold changes in expression of 77 putative p53-regulated genes measured at different times after DETA-NO treatment are listed in Supplementary Table S1. Figure 3 shows a summary of the genes grouped according to whether they are up-regulated, down-regulated, not regulated significantly, or not detected under the RT-PCR conditions. More genes were up-regulated (total of 20) than were down-regulated (total of 3) as defined by a change in expression of 2-fold or greater at any time point. Of the 77 genes tested, 2 were up-regulated by >10-fold and 3 by 5- to 10-fold (Fig. 3). The largest group (total of 15) was up-regulated by 2- to 5-fold. Several genes that are up-regulated by 2- to 10-fold have been directly implicated in apoptosis or the regulation of cell death or senescence. These include *SERPINB5* (Maspin), *SERPINE1* (plasminogen activator inhibitor-1; PAI-1), *FAS, CASP-1, BIK, BBC3* (PUMA), and *TNFRSF10A* (TRAIL-R1; DR4). The majority of genes (total of 54; 70%) were either not regulated or detected. The three down-regulated genes (*BCL2, BIRC5*, and *SNAI1*; Supplementary Table S1) exhibited changes in expression of 2- to 3-fold, and did not seem to require p53 for their NO-mediated down-regulation (data not shown).

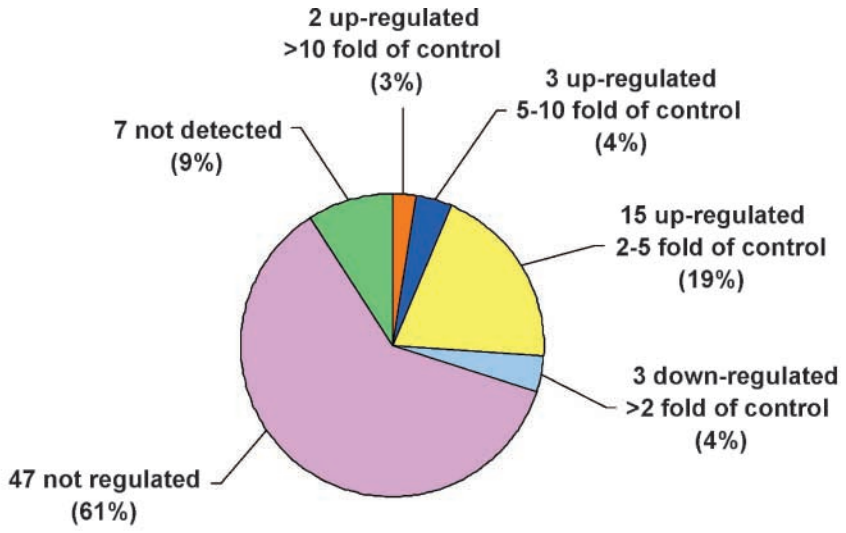
To corroborate and extend the semiquantitative RT-PCR data, we did real-time quantitative RT-PCR (Q-PCR) for all the genes that were regulated by 2-fold or greater by NO. There was an overall concordance between the semiquantitative and Q-PCR results, although in almost all cases, the magnitude of the regulation was much higher with the Q-PCR (compare Supplementary Table S1 with Fig. 4A, B, and Supplementary Fig. S2). Thus, for example, the 2 genes up-regulated by up to 10-fold by semiquantitative RT-PCR, *SERPINB5* (Maspin) and *SERPINE1* (PAI-1; Supplementary Table S1), showed up to 400- and 20-fold up-regulation, respectively, with Q-PCR

(Fig. 4A and B). Nevertheless, using both methods *maspin* and *PAI-1* mRNAs showed delayed up-regulation between 8 and 16 hours after NO donor treatment. Of the top hits, *maspin* and *PAI-1,* as well as *CASP-1* (encoding the protease caspase-1) and *SLC7A11* (encoding a cystine-glutamate transporter) were found to be up-regulated in a p53-dependent fashion (Supplementary Fig. S2; Fig. 4A and B).

Maspin and PAI-1 mRNAs and Proteins are Up-Regulated by NO in a p53-Dependent Manner in Different Tumor Cell Types

Western blot analysis for maspin and PAI-1 showed that both proteins were up-regulated by NO with kinetics rather similar to the RT-PCR and Q-PCR kinetics; and moreover, the NO-induced increases in these proteins was to a large extent p53 dependent (Fig. 4C and D). In contrast, Western blotting did not show consistent up-regulation of caspase-1 and SLC7A11 proteins by NO (data not shown). Maspin and PAI-1 have been found to be deregulated in some cancers (39-41). Maspin is a bone fide tumor suppressor (39, 42), whereas PAI-1 can mediate cellular senescence and contribute to cell death positively or negatively (40, 43). For all these reasons, we chose to concentrate on maspin and PAI-1 as candidate mediators of NO-induced, p53-dependent apoptosis.

We next examined NO-induced apoptosis as well as maspin and PAI-1 expression in stable SH-Sy5y cell lines expressing a small COOH-terminal fragment of p53 that forms transcriptionally inactive heteromers with wild-type p53, thereby, selectively inhibiting its transactivation potential in a dominant-negative manner (44). DN-p53 showed a moderate increase upon NO donor treatment (Fig. 5A) and was localized in the nuclei of SH-Sy5y cells (Fig. 5B). DN-p53 stable cells expressed higher basal and NO-inducible levels of wild-type p53, which remained localized in the nucleus together with DN-p53 (data not shown). The enhanced accumulation of p53 in the presence of DN-p53 has been described (44). DN-p53 stable cells were relatively resistant to NO as judged by independent apoptosis and caspase-3 cleavage assays (Fig. 5C and D). As a control for the effectiveness of the DN-p53 protein, we did Q-PCR with an established p53-regulated gene $p21^{waf1}$, which codes for a cyclin-dependent kinase inhibitor. In a time course, $p21^{waf1}$ mRNA was induced by DETA-NO at ~2.5- to 5-fold lower levels in the DN-p53 cells compared with the vector control cells (data not shown). Q-PCR analyses for *maspin* and *PAI-1*



	SERPINB5 (Maspin), SERPINE1 (PAI-1)
	PTGS2, FAS, SLC7A11
	HBEGF, NDRG1, PRODH, CASP1, FHL2, FDXR, BIK, RND3, BBC3, FLJ11259, GADD45A, SCN3B, NQO1, PML, TNFRSF10A
	BCL2, BIRC5, SNAI1
	SESN2, RRM2B, BAD, C12orf5, IKIP, GLIPR1, SCOTIN, DUSP4, SESN1, BAX, PEG3, PPM1D, LRDD, RGS16, LGALS3, TP53INP1, GPX1, TNFRSF10B, P53CSV, IGFBP3, BID, DAPK1, PSEN1, CABC1, CFLAR, MYO6, AMID, PIK3R1, DDR1, TP53TG3, SNAI2, CASP6, APAF1, ALDH4A1, PTEN, EI24, CLIC4, SUMO1, P2RXL1, STEAP3, HTRA2, IGF1R, SCO2, SOD2, SIVA, CARD12, UNC5B
	TRAF4, TNFRSF10C, TNFRSF10D, P53AIP1, TP53I3, UBD, GML

FIGURE 3. Pie chart illustrating the proportions of putative p53-regulated genes that are up- or down-regulated by 1 mmol/L DETA-NO. The 77 putative p53-regulated genes are shown grouped arbitrarily according to the maximum degrees of regulation observed at 16 or 24 h after NO treatment. See Supplementary Table S1 for full details of the normalized values for regulation by NO at 2, 4, 8, 16, and 24 h after NO treatment, together with the gene names and Unigene numbers. The 47 genes not regulated by NO are defined as those that exhibit <2-fold difference from the untreated control at any time after NO treatment.

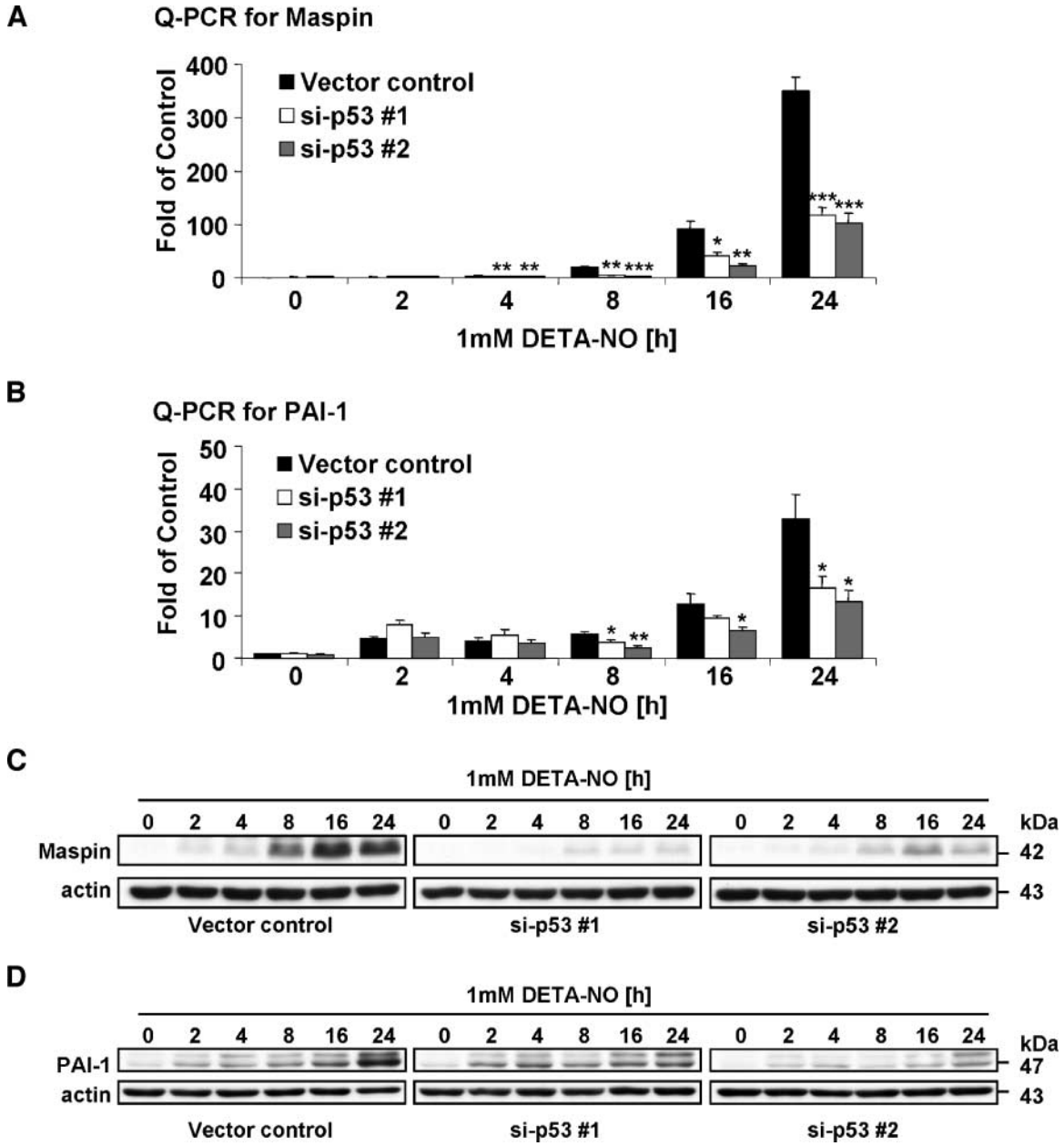


FIGURE 4. p53 mediates the NO-induced up-regulation of maspin and PAI-1 in SH-Sy5y cells. **A** and **B.** Quantitative real-time PCR analysis. SH-Sy5y vector control cells and si-p53 clones #1 and #2 were treated with 1 mmol/L DETA-NO for various times and total RNA was extracted and reverse transcribed. The levels of maspin (**A**) or PAI-1 (**B**) mRNA transcripts were determined by Q-PCR. Columns, mean fold of untreated wild-type cells ($n = 5$) after normalization to β-actin expression; bars, SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with vector control cells treated with DETA-NO for the same length of time. **C** to **D.** Western blot analysis. SH-Sy5y cells were treated with 1 mmol/L DETA-NO for various times. Total cell lysates were separated in a 10% polyacrylamide gel, and the membrane was probed with anti-maspin (**C**) or anti–PAI-1 (**D**) antibodies.

showed that the up-regulation of both mRNAs was strongly inhibited in the DN-p53 cells, with the mRNA encoding maspin showing the greatest percentage reduction (Fig. 6A). Western blotting showed that the NO-induced synthesis of maspin was delayed and PAI-1 was strongly reduced in the DN-p53 stable cells (Fig. 6B and C). Together, these results again confirm that NO-induced up-regulation of maspin and PAI-1 is largely p53 dependent in SH-Sy5y cells.

p53 siRNA cells showed incomplete knockdown of p53, and it cannot be excluded that the DN-p53 cells exhibit leaky p53 function. To investigate NO-induced p53-dependent up-regulation of maspin and PAI-1 in a p53 null background, and to determine the generality of NO-mediated up-regulation of these serpins, we treated human HCT116 colon carcinoma cells and their p53-deficient ($p53^{-/-}$) counterparts with DETA-NO for up to 40 hours. Similar to SH-Sy5y cells, p53 was

up-regulated by NO in a time-dependent manner in the wild-type HCT116 cells beginning around 8 hours (Fig. 7A), which underwent significant apoptosis starting between 16 and 24 hours (Fig. 7B). Apoptosis was strongly suppressed in the $p53^{-/-}$ cells as judged both by fluorescence-activated cell sorting analysis and the substantial reductions in the cleavages of caspase-3 and caspase-9 (Fig. 7B-D). Q-PCR analyses showed massive NO-induced, time-dependent up-regulation of the mRNAs for *maspin* and *PAI-1* by up to ~11- and ~50-fold, respectively, in the p53 wild-type HCT116 cells (Fig. 8A). In contrast, the up-regulation of these serpin mRNAs was almost completely prevented in the $p53^{-/-}$ HCT116 cells (Fig. 8A). The failure of up-regulation of these mRNAs was reflected at the protein level because both maspin and PAI-1 were only up-regulated in a time-dependent manner in the p53 wild-type cells (Fig. 8B and C). Thus, NO induces the p53-dependent expression of maspin and PAI-1 in cell lines representing diverse tumor cell types (neuroblastoma and colon carcinoma).

Discussion

Excessive NO can induce DNA/oxidative/nitrosative damage as well as S-nitrosylation of selective proteins (4-6, 12). NO-mediated DNA/oxidative/nitrosative damage can also activate p53 and potentially induce the transcription of p53-dependent targets, some of which may be essential for apoptosis. A link between S-nitrosylation and nuclear p53-dependent apoptosis of neurons has recently emerged. NO causes S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase, which is required for its nuclear translocation, activation of nuclear acetyltransferase p300/CBP, and subsequent induction of p53 acetylation by p300/CBP required for cell death (45). Consistent with our results, these data strongly support a role for p53 transcriptional targets in NO-induced apoptosis. c-Jun-NH_2-kinase and p38 mitogen-activated protein kinases, as well as c-Jun have also been associated with the promotion of NO-induced apoptosis via the transcription of specific target mRNAs; whereas AP-1, cyclic AMP response element-binding protein and Nrf2 have been identified as

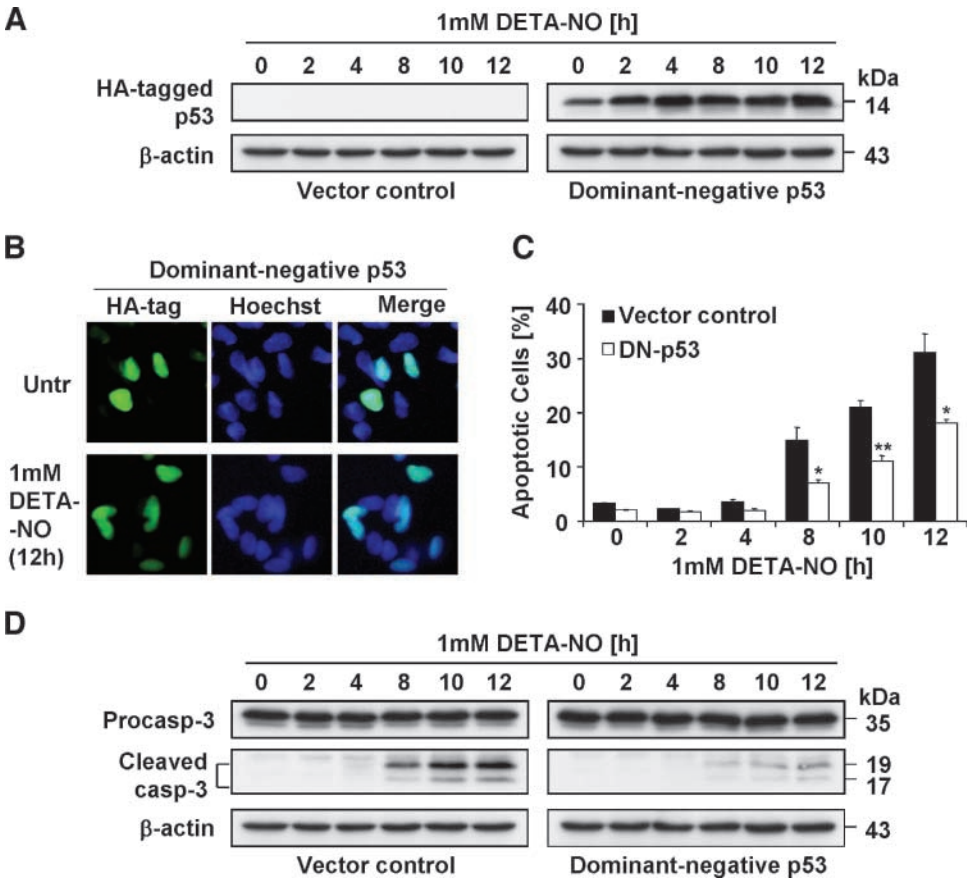


FIGURE 5. Stable SH-Sy5y cells expressing DN-p53 are resistant to NO-induced apoptosis. **A.** SH-Sy5y vector control and DN-p53 cells were treated with 1 mmol/L DETA-NO for various times. Total cell lysates were separated in a 10% polyacrylamide gel, which was probed with an anti–HA-tag antibody to detect DN-p53. **B.** Immunofluorescence. SH-Sy5y vector control and DN-p53 cells were treated with 1 mmol/L DETA-NO for 12 h. The cells were probed with anti–HA-tag antibodies. Bound antibodies were probed with Alexa Fluor 488 goat anti-mouse IgG, incubated with Hoechst 33342 nuclear stain, washed, and mounted. Green, HA-tag staining; blue, nuclear staining. Pale greenish-blue, overlap between HA-tag and the nucleus in the merged images. **C.** Fluorescence-activated cell sorting analysis after PI staining. SH-Sy5y vector control and dominant-negative p53 cells were treated with 1 mmol/L DETA-NO and fixed in 70% ethanol. After rehydration, intracellular DNA was stained with PI, and the percentage of apoptotic cells was determined by flow cytometry. Columns, mean ($n \geq 3$); bars, SE. *, $P < 0.05$; **, $P < 0.01$ compared with vector control cells treated with DETA-NO for the same length of time. **D.** As in **A** except the membrane was probed with an anti–caspase-3 antibody.

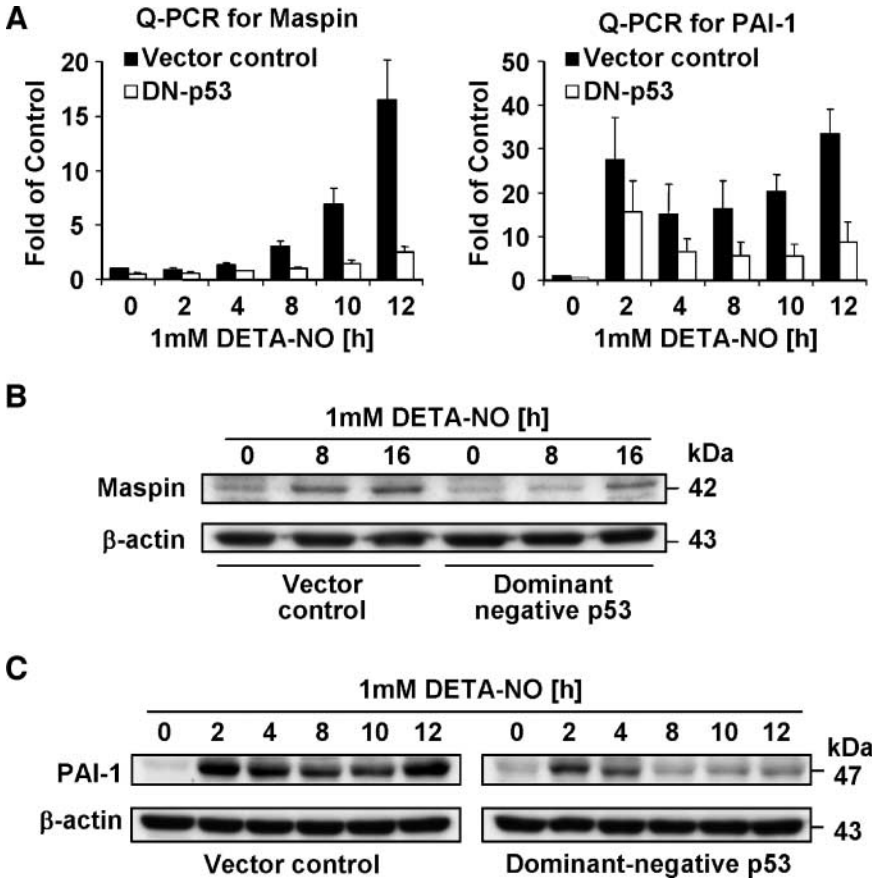


FIGURE 6. Failure of maspin and PAI-1 up-regulation by NO in stable SH-Sy5y cells expressing dominant-negative p53. **A.** Quantitative real-time PCR analysis. SH-Sy5y vector control and dominant-negative p53 cells were treated with 1 mmol/L DETA-NO for various times, and total RNA was reverse transcribed. The levels of mRNA transcripts for maspin (*left*) and PAI-1 (*right*) were determined by Q-PCR. Columns, mean fold of untreated vector control cells ($n = 3$) after normalization to β-actin expression; bars, SE. **B** to **C.** Western blot analysis. SH-Sy5y vector control and dominant-negative p53 cells were treated with 1 mmol/L DETA-NO for various times. Total cell lysates were separated in a 10% polyacrylamide gel, and the membrane was probed with antibodies against maspin (**B**) or PAI-1 (**C**).

transcriptional regulators involved in counteracting excess NO (7-11, 46, 47). In all cases, the crucial target genes linked to these pathways remain to be established. Several studies have addressed this problem by using classic RNA microarrays, but no consistent candidate NO-regulated death or survival genes have emerged (2, 19, 20).

In our present study, we established that p53 plays a dominant role in NO-induced apoptosis using three different approaches in two distinct tumor cell types (neuroblastoma and colon carcinoma). The greater p53 dependence of apoptosis in HCT116 cells is probably due to the fact that p53 is absent in these cells but is not fully suppressed in the stable SH-Sy5y cells expressing siRNA to p53 or dominant-negative p53. We restricted our RT-PCR survey of mRNAs that might respond to NO to genes known (or suspected) to be regulated by p53—a total of 77 genes, primarily those involved in cell death, survival, senescence, signaling, redox regulation, cancer, metabolism and cell division. We then reasoned that NO-induced/repressed genes that are regulated in a p53-dependent fashion are the stronger candidate death-regulating genes. We found that over two thirds of the 77 genes (70%) were either not significantly or not consistently regulated by NO, or not detected. Surprisingly, these include several p53 target genes strongly implicated in the regulation of apoptosis or tumor suppression (or known components of apoptosis pathways) in other systems, such as *NOXA, PIG3,* and *p53AIP1* (not detected); and *HTRA2, PTEN, APAF1, CASP-6, DAPK1, BID, BAX,* and *BAD* (regulated <2-fold; refs. 23-25, 27). Of the remainder, the great majority of genes were up-regulated by NO by ≥2- to 10-fold (total of 20), whereas only 3 genes were down-regulated by NO. Several genes associated with cell death or survival were up-regulated by >2-fold, such as *TNFRSF10A* (*death receptor 4*; *DR4*), *NQO1, GADD45A, BBC3* (*PUMA*), *BIK*, and *FAS*, but they were similarly up-regulated in NO-resistant p53 knockdown cells. Although this might argue against a role for these genes in NO-induced apoptosis, it will be important to examine the NO-mediated regulation of their encoded proteins as well as perform functional studies before any judgment about their effect on cell death can be made.

Only four genes were consistently up-regulated by NO in a strongly p53-dependent manner—these were *maspin, PAI-1, CASP-1*, and *SLC7A11*, of which *maspin, PAI-1*, and *CASP-1* are well-known p53-induced genes (42, 48, 49). However, NO-mediated procaspase-1 protein up-regulation and cleavage to generate the active caspase was not observed, suggesting that the massive (~45- to 50-fold) up-regulation of *CASP-1* mRNA is not functionally significant. In any event, caspase-1 probably plays no significant role in apoptosis but rather functions in the activation of inflammatory cytokines and in pyroptosis, a specialized form of cell death in infected macrophages (50, 51). Similarly, SLC7A11 protein was found not to be up-regulated. We found that *maspin* and *PAI-1* were the most highly up-regulated at the mRNA level of all the genes tested in SH-Sy5y

cells. The kinetics of increased maspin and PAI-1 protein synthesis correlated fairly well with the up-regulation of their mRNAs. We obtained very similar results for HCT116 colon carcinoma cells, where apoptosis and the up-regulation of maspin and PAI-1 proteins were almost completely p53 dependent. It is unclear why previous microarray studies in lymphoblastoid, monocytic, and fibroblast cells did not detect maspin and PAI-1 regulation by NO (2, 19, 20); although one study did find that NO up-regulates maspin in normal mammary epithelial and breast cancer cells (52).

The majority of studies suggest that p53-dependent apoptosis proceeds via the transactivation of p53 target genes (23-25, 27). However, there are some reports that cytoplasmic p53 might mediate apoptosis independently of (or in association with) transcription by binding to mitochondria or neutralizing certain Bcl-2 family proteins bound to mitochondria (26, 53, 54). This raises the question whether NO-induced, p53-dependent apoptosis might proceed independently of the transcription of p53 target genes, such as *maspin* and *PAI-1*. We showed that NO-induced p53 is localized mainly in the nuclei of neuroblastoma cells, a result in accord with previous studies showing that NO caused retention of p53 in the nuclei of neuroblastoma cells by inhibiting its nuclear export (15, 16). Moreover, the DN-p53 construct we used, which selectively inhibits p53-dependent transcriptional activation, also blocks NO-induced cell death. Although our evidence is consistent with a role for nuclear p53 in NO-induced apoptosis, we cannot rule out an additional cytoplasmic nontranscriptional function for p53 in apoptosis of neuroblastoma cells. Nevertheless, it will now be

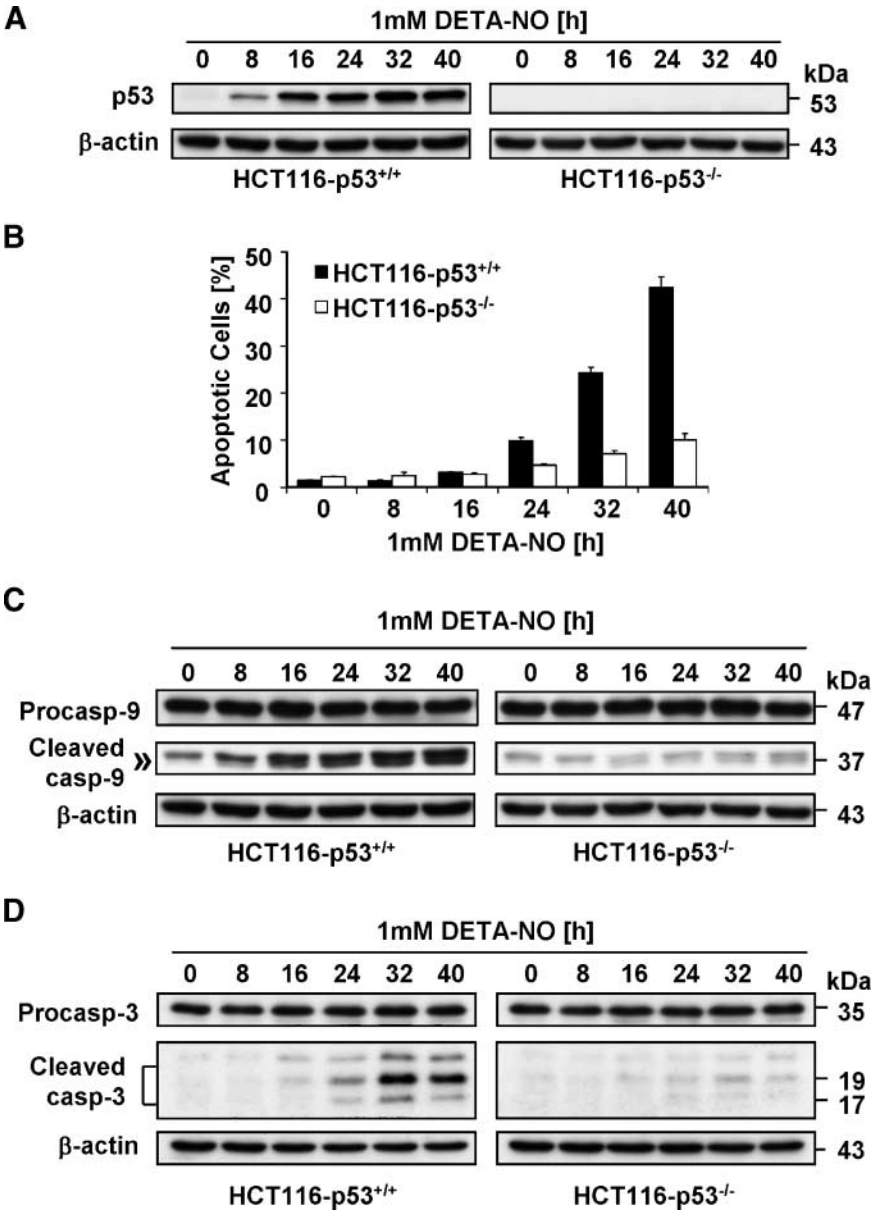


FIGURE 7. $p53^{-/-}$ HCT116 cells are resistant to NO-induced apoptosis. **A.** Western blot analysis. HCT116-$p53^{+/+}$ and HCT116-$p53^{-/-}$ cells were treated with 1 mmol/L DETA-NO for the indicated times. Total cell lysates were separated in a 10% polyacrylamide gel, and the membrane was probed with anti-p53 antibodies. **B.** Fluorescence-activated cell sorting analysis after PI staining. HCT116-$p53^{+/+}$ and HCT116-$p53^{-/-}$ cells were treated with 1 mmol/L DETA-NO and fixed in 70% ethanol. After rehydration, intracellular DNA was stained with PI, and the percentage of apoptotic cells was determined by flow cytometry. Columns, mean ($n \geq 3$); bars, SE. **C** to **D.** Western blot analyses. HCT116-$p53^{+/+}$ and HCT116-$p53^{-/-}$ cells were treated as in **A** above, except that total proteins were probed with anti–caspase-9 (**C**) or anti–caspase-3 (**D**) antibodies.

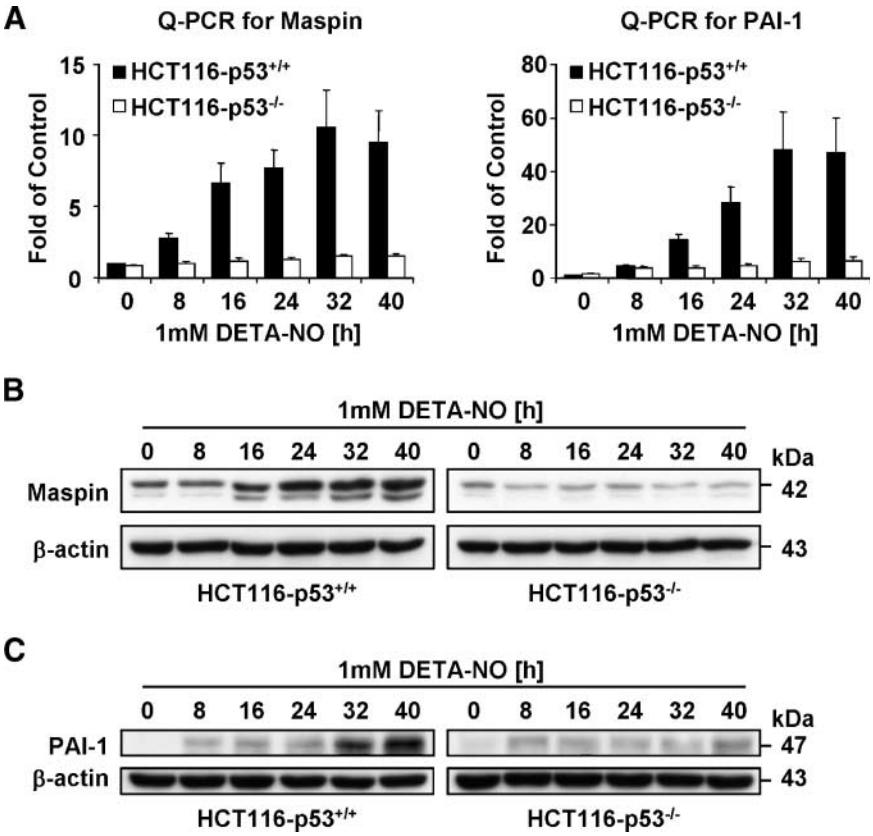


FIGURE 8. Failure of maspin and PAI-1 up-regulation by NO in $p53^{-/-}$ HCT116 cells. **A.** Quantitative real-time PCR analysis. HCT116-$p53^{+/+}$ and HCT116-$p53^{-/-}$ cells were treated with 1 mmol/L DETA-NO for the indicated times, and total RNA was extracted and reverse transcribed. The levels of mRNA transcripts for maspin (*left*) or PAI-1 (*right*) were determined by Q-PCR. Columns, mean fold of untreated HCT116-$p53^{+/+}$ cells ($n = 3$) after normalization to β-actin expression; bars, SE. **B** to **C.** Western blot analysis. HCT116-$p53^{+/+}$ and HCT116-$p53^{-/-}$ cells were treated with 1 mmol/L DETA-NO for various times. Total cell lysates were separated in a 10% polyacrylamide gel, and the membrane was probed with anti-maspin (**B**) or anti–PAI-1 (**C**) antibodies.

worth investigating whether maspin and/or PAI-1 might play roles in apoptosis after their transcriptional activation by p53 based on our findings as follows.

First, we showed that *maspin* and *PAI-1* were two of only four NO-induced genes we tested (of 77) that were regulated at the mRNA level in a strongly p53-dependent manner. Second, in both HCT116 and SH-SY5Y cells, the up-regulation of maspin and PAI-1 occurred before or concomitant with NO-induced apoptosis. Third, maspin and PAI-1 have p53-binding sites in their promoters and are established p53 target genes (39, 40, 42, 49). Fourth, maspin is strongly down-regulated in mammary carcinomas and in some cancer cell lines compared with normal untransformed cells (55). In the same vein, we observed extremely low basal levels of maspin in the untreated neuroblastoma cell line. Maspin is a bone fide tumor suppresser, acting in a complex pleiotropic manner to inhibit angiogenesis, tumor cell invasion and metastasis. Maspin also contributes to apoptosis, but the mechanism is unclear (39). It is improbable that maspin overexpression alone is sufficient to induce cell death; rather, it is more likely that maspin sensitizes tumor cells to cell death induced by other apoptosis mediators (56-58). In contrast to the other serpin family members, maspin has no detectable protease inhibitor activity, and the immediate substrate(s) is unknown (39). However, the pro-apoptotic Bcl-2 family member Bax may mediate the apoptosis-sensitizing effect of maspin in breast and prostate tumor cell lines, thereby acting through the mitochondrial pathway (57). This is consistent with many studies showing that NO mediates apoptosis through this pathway (4, 20, 34). Therefore, the question may be asked: does the massive maspin up-regulation we observed contribute to apoptosis in conjunction with one or more additional signals generated by NO?

The role of PAI-1 in NO-induced apoptosis needs to be assessed in the light of its multiple, complex, and apparently contradictory modes of action (41). PAI-1 has intracellular and secreted cell-autonomous or secreted non–cell autonomous functions. Secreted PAI-1 is a specific inhibitor and therefore regulator of the serine proteases urokinase-type and tissue-type plasminogen activator, which function prominently in fibrinolysis and tissue remodeling. PAI-1 can, independently of its action as a serine protease inhibitor and with high affinity, bind to and shield vibronectin from proteolysis, and bind endocytosis receptors of the LDL family (41). The frequently raised levels of PAI-1 in tumors correlate with poor prognosis in some cancers, which together with the capability of PAI-1 to regulate the degradation of extracellular proteins like fibrin and laminin, has led to the controversial conclusion that PAI-1 plays a causal role in tumor growth and spread (40, 41). PAI-1 has been reported to exhibit proapoptotic/antiproliferative or antiapoptotic/proproliferative properties, a contradiction that is not understood (41). PAI-1 is an essential target of p53, and both are necessary for the induction of replicative senescence of normal fibroblasts, which is associated with down-regulation of the growth-promoting Akt kinase pathway (43, 49). The restoration of the Akt pathway in PAI-1–deficient fibroblast and endothelial cells may allow these cells to escape growth arrest or senescence (43, 59, 60).

In conclusion, through a rigorous semiquantitative RT-PCR screening approach, we showed that both NO-induced apoptosis and the up-regulation of the serpins maspin and PAI-1 are dependent on p53. However, further studies are required to establish if these two serpins act as mediators of NO-induced apoptosis of tumor cells; and we leave open the possibility that one or more of the 75 other known or putative p53-regulated genes we investigated may have a role in NO-mediated killing. It will now be interesting to investigate whether maspin and PAI-1 are up-regulated by other cell death–inducing stimuli, and if their deliberate induction in tumor cells may aid in tumor regression.

Materials and Methods

Materials

DETA-NO, propidium iodide (PI), RNase A, Tween 20, and other chemicals were from Sigma-Aldrich. GeneSuppressor plasmids containing si-p53 inserts (IMG-803) were from Imgenex. Lipofectamine 2000 transfection reagent and opti-MEM were from Invitrogen. The nuclear extraction kit was from Panomics. The BCA protein assay kit was from Pierce. Polyvinylidene difluoride membranes were from Millipore. Nonfat dry milk was purchased from Bio-Rad. The enhanced chemiluminescence detection system was from PerkinElmer. The RNeasy mini kit and QuantiTect SYBR Green PCR Mastermix were from Qiagen. Primers used in screening and HotStart *Taq* DNA polymerase were from Superarrray. All other primers and M-MLV reverse transcriptase were from Promega. Paraformaldehyde and Triton X-100 were from BDH Laboratory Supplies. Normal goat serum and Vectashield mounting medium were from Vector Laboratories. Hoechst 33342 was from Molecular Probes. The antibodies against SERPINB5/maspin (#554292) and SERPINE1/PAI-1 (#612025) were from BD Pharmingen and BD Transduction Laboratories, respectively. The antibodies against actin (#sc-1616) and p53 (#sc-126) were from Santa Cruz Biotechnology. The cell lysis buffer and antibodies against caspase-3 (#9662), caspase-9 (#9508), PARP (#9542), p53 (#2524), Ser15-p53 (#9284), β-tubulin (#2128), topoisomerase II (#4733), lamin A/C (#2032), and HA-tag (#2367) were from Cell Signaling Technology. The secondary horse anti-mouse (#7076) and goat anti-rabbit (#7074) IgG antibodies conjugated to horseradish peroxidase were also from Cell Signaling Technology. The Alexa Fluor 488 goat anti-mouse IgG antibody was from Molecular Probes (#A-11001).

Cell Culture and Transfection

The human neuroblastoma cell line SH-Sy5y was obtained from Eva Feldman (University of Michigan, Ann Arbor) and maintained in Dulbecco's modified medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. To knockdown p53, SH-Sy5y cells were transfected with GeneSuppressor plasmids containing si-p53 inserts using Lipofectamine 2000. Transfected cells were selected in medium containing 500 μg/mL of G418. After selection, stable clones were maintained in medium containing 100 μg/mL of G418. The SH-Sy5y dominant-negative p53 and vector control cell lines were maintained in Dulbecco's modified medium containing 10% fetal bovine serum and 100 μg/mL hygromycin B. The human colon carcinoma cell line HCT116-p53$^{+/+}$ was obtained from American Type Culture Collection and maintained in McCoy's 5A medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The HCT116-p53$^{-/-}$ cell line was obtained from B. Vogelstein.

Semiquantitative RT-PCR and Quantitative Real-time PCR Analysis

Cells were treated with DETA-NO for various times, and total RNA was extracted using the Qiagen RNeasy mini kit according to the manufacturer's protocol. For each PCR reaction, first-strand cDNA was synthesized from 0.25 μg total RNA using random primers and M-MLV reverse transcriptase, and amplified using HotStart *Taq* DNA polymerase. All primers for the 77 putative p53-regulated genes were purchased from Superarray. The PCR cycling conditions were as follows: 95°C for 15 min, followed by 25 or 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 1 min. The PCR-amplified products were electrophoresed in a 2% agarose gel at 85 V for 1 h. Band densities were quantified using the Quantity One software (Bio-Rad) and normalized to β-actin expression levels. For Quantitative Real-time PCR Analysis, first-strand cDNA was prepared from 0.4 μg total RNA using oligo(dT) primers and M-MLV reverse transcriptase. PCR amplification was carried out using QuantiTect SYBR Green PCR Mastermix and reactions were monitored continuously in a Rotor-Gene thermal cycler (Corbett Research) with the following program: 95°C for 15 min, followed by 50 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. All data were normalized to β-actin expression levels.

Western Blot Analysis

Cytosolic and nuclear extracts were prepared using the Panomics nuclear extraction kit according to the manufacturer's protocol. Whole-cell extracts were obtained using cell lysis buffer supplemented with 0.1 mmol/L phenylmethylsulfonyl fluoride. Fifty micrograms of proteins were separated on 10% polyacrylamide gels, transferred onto polyvinylidene difluoride membranes, and blocked in tris-buffered saline with 0.1% Tween 20 and 5% nonfat dry milk. Blots were probed with the appropriate primary antibodies overnight at 4°C, followed by secondary horse anti-mouse or goat anti-rabbit IgG conjugated to horseradish peroxidase. The bands were developed using enhanced chemiluminescence detection and quantified with the Quantity One software (Bio-Rad).

PI Staining and Flow Cytometric Analysis

Cells, both attached and floating, were collected by centrifugation and washed with ice-cold PBS. The cell pellet was thoroughly resuspended in 100 μL of PBS to achieve a single cell suspension before being introduced dropwise into 70% ethanol at −20°C. After overnight fixation at 4°C, the permeabilized cells were rehydrated with PBS for 20 min at 24°C. Double-stranded RNA was eliminated with 150 μg/mL of RNase A and intracellular DNA was stained with 15 μg/mL of PI for 40 min at 37°C. To dissociate any cell aggregates, the suspension was passed through a 40 μm cell strainer (BD

Biosciences) before analysis on a FACSCalibur flow cytometer (BD Biosciences). Using the CellQuest pro software, 10,000 cells were sampled and the proportion of cells in the sub-G_1 phase (apoptotic cells) was determined.

Indirect Immunofluorescence Staining

Cells were seeded overnight in 8-well chamber slides (Lab-Tek) and treated with DETA-NO for various times. Cells were fixed in 4% paraformaldehyde for 20 min at 24°C and permeabilized in PBST (0.1% Triton X-100 in PBS) with 10% normal goat serum for 1 h at 24°C. Primary antibodies were diluted in PBST with 1% normal goat serum (1:50 dilution for p53 and 1:100 dilution for HA-tag) and incubated with the cells for 1 h at 24°C or 16 h at 4°C. Bound antibodies were then probed with Alexa Fluor 488 goat anti-mouse IgG for 1 h at 24°C. To stain for nuclei, 1 μg/mL of Hoechst 33342 was added during the first wash with PBST for 5 min. After another four washes with PBST, the chambers were removed and the slides were mounted with Vectashield. Cells were observed and photographed under an Olympus BX61 microscope using the appropriate filters.

Statistical Analysis

Data are expressed as mean ± SE. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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