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Specific Contribution of p19ARF to Nitric Oxide-Dependent Apoptosis

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NO is an important bioactive molecule involved in a variety of physio- and pathological processes, including apoptosis induction. The proapoptotic activity of NO involves the rise in the tumor suppressor p53 and the accumulation and targeting of proapoptotic members of the Bcl-2 family, in particular Bax and the release of cytochrome c from the mitochondria. However, the exact mechanism by which NO induces p53 activation has not been fully elucidated. In this study, we describe that NO induces p19ARF through a transcriptional mechanism. This up-regulation of p19ARF activates p53, leading to apoptosis. The importance of p19ARF on NO-dependent apoptosis was revealed by the finding that various cell types from alternate reading frame-knockout mice exhibit a diminished response to NO-mediated apoptosis when compared with normal mice. Moreover, the biological relevance of alternative reading frame to p53 apoptosis was confirmed in vivo models of apoptosis. Together, these results demonstrate that NO-dependent apoptosis requires, in part, the activation of p19ARF. The Journal of Immunology, 2006, 177: 3327–3336.

Apoptosis is a form of physiological cell death involved in development and homeostasis as well as in pathological processes such as neurodegenerative diseases and tumors (1, 2). The regulatory signals of the apoptosis cascade are extremely complex. In addition to receptor-mediated apoptosis, highly reactive molecules, such as NO, influence cell viability either by protecting against apoptotic stimuli, or by inducing apoptosis when produced at elevated concentrations (3–6). NO has been shown to up-regulate and stabilize p53, and this increase in p53 expression has been associated with increased apoptosis in several systems (4, 5, 7). Indeed, p53 transcriptionally activates a number of proapoptotic proteins, including Bax, Noxa, Puma, and Fas (8, 9). The signaling cascade induced by p53 is regulated at different levels, depending on the type of cell examined. In normal cells, p53 is expressed at low constitutive level. The latent form of p53 is stabilized and activated by posttranslational modifications, mainly multiphosphorylations and acetylation (10). Protein turnover is achieved via degradation by the 26S proteasome system, and this is mediated through the association of p53 with Mdm2 (11, 12).

An important regulator of p53 stability and activation is p19ARF. The inhibitor of cyclin-dependent kinase 4a (INK4a)3/alternative reading frame (ARF) locus encodes two unrelated proteins, p16INK4a and p19ARF, which regulate the activity of two tumor suppressors, Rb and p53, respectively (13, 14). The ARF protein (p19ARF in the mouse and p14ARF in humans) exerts its tumor suppressor action by activating the p53 pathway (15, 16). ARF controls the levels of the p53 protein due to its interaction with Mdm2, thereby interfering with Mdm2-mediated degradation of the p53 protein by the proteasome (17, 18). p19ARF exists at low or undetectable levels in most normal cells and tissue types (19). However, its expression is specifically activated by abnormal proliferative signals. These include the continued in vitro culturing of mouse embryonic fibroblasts (MEFs) (20) and the inappropriate expression of proliferative oncopgenes, including activated Ras, c-myc, E2F, E1A, and v-Abl (13, 21–23). p19ARF has activities that do not depend on Mdm2 and p53. At least some of the p53-independent effects of p19ARF might be mediated by its ability to inhibit ribosomal RNA processing (24) and transcriptional factors that induce proliferation such as E2F1 (25), Myc (26), and Foxm1b (27–29). The ability of NO to induce p53 accumulation has been largely studied; however, the molecular mechanisms underlying the induction of p53 by NO have not been fully elucidated and a direct connection between NO and p19ARF has not been established. In this work, we studied the interplay between NO and p19ARF in the context of apoptosis.

Materials and Methods

Cell culture

Primary cultures of MEFs derived from wild-type (WT), p53⁻/⁻, and ARF⁻/⁻ mice were obtained, as previously described (30, 31). Cells were maintained in DMEM supplemented with 10% FBS (Invitrogen Life Technologies) and antibiotics. MEFs were always used within their first in vitro passages (passages 1–3).

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Preparation of elicited peritoneal macrophages

WT, p53−/−, and ARF−/− mice were maintained free of pathogens, and 4 days before use were i.p. injected with 1 ml of sterile 10% thiglycollate broth. Peritoneal macrophages were prepared as follows: CO2-anesthetized animals were injected i.p. with 10 ml of sterile DMEM. The peritoneal fluid was carefully aspirated to avoid hemorrhage and kept at 4°C to prevent the adhesion of the macrophages to the plastic. After centrifugation at 20,000 × g for 10 min at 4°C, the cell pellet was washed twice with 45 ml of ice-cold PBS. Cells were seeded at 1 × 10^6/cm^2 in DMEM containing 10% FCS. Nonadherent cells were removed 2 h after seeding by extensive washing with medium.

Transfection assays

Cells were transiently transfected with the ARF promoter (3.4-kb genomic DNA fragment) by using Lipofectamine 2000, according to the manufacturer’s instructions. Cells were cotransfected with a Renilla luciferase expression vector to control transfection efficiency.

Flow cytometric analysis of apoptosis

Analysis of apoptotic cells was performed after incubation of the cells for 30 min at 37°C with Hoechst 33224 (5 μg/ml), a DNA-staining dye, and 0.002% propidium iodide (PI). Cells were carefully resuspended and run in a Cyan MLE-R flow cytometer (DakoCytomation), equipped with three excitation wavelengths (488, 635, and 365 nm). Quantification of the percentage of apoptotic cells was performed using a dot plot of the Hoechst 33242 fluorescence against the PI fluorescence. Apoptotic and viable cells percentage of apoptotic cells was performed using a dot plot of the Hoechst 33242 fluorescence against the PI fluorescence. Apoptotic and viable cells were counted, and the integrity of the DNA was analyzed in agarose gels to confirm the criteria of gating (32).

Preparation of cytosolic and total protein extracts

Cells were washed twice with ice-cold buffer A (10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMFS, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml N-tosyl-lys-chloromethyl ketone, 5 mM NaF, 1 mM NaVO₄, and 10 mM Na₂MoO₄) containing 120 mM NaCl and scraped off the plate. Cells were lysed at 4°C with 0.2 ml of buffer A supplemented with 0.5% Nonidet P-40 and under continuous shaking. After centrifugation of the cell lysate, the supernatant was stored at −80°C (cytosolic extract). The presence of cytochrome c in the cytosol was determined by Western blotting cell extracts obtained by controlled lysis of the plasma membrane, as previously described (32). Total cell extracts were prepared after homogenization of the cells with buffer A supplemented with 0.5% 3-(3-cholamidopropyl)dimethylammonio)-1-propane sulfonate. Protein content was assayed using the Bio-Rad protein reagent. The DEVDase (corresponding mainly to caspases 3 and 9) activities were linear over a 30-min reaction period.

Western blot analysis of proteins

Protein extracts were size separated in 10–15% SDS-PAGE. The gels were blotted onto a Hybond-P membrane (Amer sham Biosciences) and incubated with the following Abs: anti-p19ARF (R&D Systems); anti-p53, anti-Bax, anti-Bcl-2, anti-Bcl-xl, anti-p16INK4a, and anti-caspases 3 and 9 (Santa Cruz Biotechnology); anti-p19ARF (Abcam ab80); and anti-cytochrome c (BD Pharmingen).

Immunocytochemistry of p19ARF

Cells grown on coverslips were fixed with methanol:acetone (1:1) at −20°C for 10 min. After washing and blocking, the coverslips were incubated with an anti-p19ARF (1 μg/ml) diluted in PBS 0.1% BSA for 1 h at room temperature. The Ab was visualized after incubation with a Cy3-labeled anti-rabbit Ig. Fluorescence was analyzed and quantified on a Bio-Rad Radiance 2100 confocal microscope, with the LaserPix program. For the histological examination, 8-μm-thick sections were stained with H&E.

Results

NO induces apoptosis through the p19ARF-p53 pathway

NO initiates apoptosis in part by a p53-dependent pathway. Because p19ARF is among the most important regulators of p53, we studied the involvement of the p19ARF-p53 pathway in NO-dependent apoptosis. For this, primary MEFs WT, p53−/−, and ARF−/− were exposed to NO donors (S-nitrosoglutethimide, GSN0), 500 μM) for 18 h, and the percentage of apoptotic cells was determined by flow cytometry. We observed that NO induced apoptosis in WT MEFs, but not in p53−/− cells, suggesting a dependence of NO apoptosis on p53 in these cells (Fig. 1A). Interestingly, when ARF−/− MEFS were treated with GSN0, the percentage of apoptotic cells was significantly reduced (Fig. 1A).

The central component of apoptosis is a proteolytic system involving a family of proteases called caspases (35). Analysis of caspase activation in these cells showed an important increase of caspase 3 after 6 h of GSNO treatment in WT MEFs (Fig. 1C). As a consequence of these events, cytochrome c was released into the cytosol of these cells, suggesting a dependence of NO on the mitochondria (36). Activation of caspase 8 was not observed in any of the experimental conditions (data not shown). In addition to these parameters, the processing of procaspase 9 and 3 was evaluated by Western blot and confirmed the previous results (Fig. 1D). NO-dependent apoptosis involves the release of mitochondrial apoptogenic factors, such as cytochrome c. To detect whether cytochrome c release was involved in ARF-induced apoptosis, we examined its distribution in cells after treatment with GSN0 for 2 h. A significant

Real-time PCR analysis

Total RNA was isolated from cells cultures with TRIZol reagent (Invitrogen Life Technologies), and cDNA was synthesized using 50 U of Expand Reverse Transcriptase (Roche) essentially according to the recommendations of the manufacturer.

Real-time PCR was conducted with AmpliTaq Gold polymerase on an ABI Prism 7900 HT Sequence Detection system using the SyBr Green method with the following primers: 36B4 (forward primer, 5'-AGATG CACGAGATCCGCAT-3'; reverse primer, 5'-GTTCGCTGCCCATGC CACC-3') and p19ARF (forward primer, 5'-CATGTGTGGTAGGCTA GAGAGG-3'; reverse primer, 5'-TCGAGATCCGGCACTGTG-3').
amount of cytochrome c was detected in the cytosol of WT MEFs; however, cytochrome c was not released from mitochondria in p53/H11002/H11002 cells (Fig. 1D). In the case of ARF/H11002/H11002 MEFs, cytochrome c was detected, although at lower levels than in WT cells. In view of these data, we conclude that p19ARF is involved in the apoptosis induced by NO in MEFs.

Pathways involved in ARF-dependent apoptosis

Cells were analyzed for expression of proapoptotic (Bax) and antiapoptotic (Bcl-2 and Bcl-xL) proteins of the Bcl-2 family. The amount of Bax increased after GSNO treatment in WT MEFS; however, the levels were notably lower in p53/H11002/H11002 cells (Fig. 2). In contrast, Bax remained undetectable in p53/H11002/H11002 cells. When we studied the antiapoptotic members of the Bcl-2 family (Bcl-2 and Bcl-xL), NO decreased the levels of Bcl-2 and Bcl-xL in WT and ARF/H11002/H11002 MEFs, whereas these antiapoptotic proteins did not change in p53/H11002/H11002 cells (Fig. 2).

Among the most important regulators of caspases are the IAPs (36). The expression levels of the antiapoptotic proteins cIAP (cellular inhibitors of apoptosis protein)-1, cIAP-2, and X-linked IAP were determined in the different cells. cIAP-1 and cIAP-2 decreased in the presence of GSNO in WT and ARF/H11002/H11002 cells, although the decrease was notably lower in ARF/H11002/H11002 cells (Fig. 2). In contrast, X-linked IAP remained without changes in all cases. From these data we conclude that NO-induced apoptosis is mediated by decreased levels of antiapoptotic proteins, namely Bcl-2,
Bcl-xL, cIAP-1, and cIAP-2, and increased levels of the proapoptotic protein Bax. All these changes are completely dependent on the function of p53 and are partially dependent on the function of p19ARF.

**NO activates p53 in MEFs**

To study the p53 response, the different MEFs (WT, p53−/−, and ARF−/−) were exposed to NO donors (GSNO, 500 μM) and protein levels were monitored by Western blot analysis. A significant induction of endogenous p53 was seen in WT cells. Importantly, the ablation of ARF compromised the ability of NO to promote an increase in the levels of p53, and ARF−/− cells showed a slight increase in p53 accumulation that was delayed and of lesser magnitude compared with WT cells (Fig. 2). These data imply that ARF is required, at least in part, for the activation of p53 by NO. Consistent with the above-described up-regulation of p53, the protein levels of its transcriptional target Bax varied in parallel to the changes in p53 (Fig. 2). Together, these data indicate that p19ARF plays an important role in the activation of p53 triggered by NO.

**Involvement of p19ARF in NO-dependent apoptosis in macrophages**

To investigate whether the involvement of p19ARF in NO-dependent apoptosis is a common pathway in other cells or is restricted to fibroblasts, we examined the effect of NO on macrophages. Primary cultures of macrophages obtained from WT, p53−/−, and ARF−/− mice were stimulated with 500 μM GSNO for 18 h, and the percentage of apoptotic cells and caspase activation were determined (Fig. 3, A, C, and D). Incubation of WT and ARF−/− macrophages with GSNO induced apoptosis, although the percentage of apoptotic cells and caspase activity in ARF−/− macrophages were significantly reduced with respect to WT macrophages. In addition to this, NO had no effect on p53−/− macrophages (Fig. 3A). Moreover, macrophages were stimulated with LPS/IFN-γ to induce NOS-2 expression and NO release (6) in presence or absence of 1400W, a specific NOS-2 inhibitor. In these conditions, similar results to those obtained with GSNO were observed in WT and ARF−/− macrophages after treatment with LPS/IFN-γ. Incubation of cells with LPS/IFN-γ + 1400W showed that apoptosis and caspase activity remained dependent on the synthesis of NO, because they were totally suppressed by 1400W (Fig. 3, A, C, and D). We also studied the involvement of the mitochondrial pathway and the Bcl-2 family on NO-dependent apoptosis in macrophages. WT macrophages showed an important rise on cytochrome c, p53, and Bax after NO stimulation, and a decrease of Bcl-2, Bcl-xL, and IAPs (Fig. 3B). However, ARF−/− macrophages showed a diminished response. In contrast to the effect on WT macrophages, p53−/− macrophages did not change the expression of apoptotic proteins. These data suggest that the involvement of p19ARF in NO-dependent apoptosis not only occurs in fibroblasts, but also in macrophages.

**NO induces p19ARF expression**

To assess whether NO up-regulates p19ARF, WT, ARF−/−, and p53−/−, MEFs were incubated with 500 μM GSNO for different times and p19ARF levels were measured by immunoblotting. As Fig. 4A shows, NO induced the accumulation of p19ARF in WT and p53−/−. As a negative control, p19ARF protein was not detected on ARF−/− cells. The induction was confirmed in intact cells by immunofluorescence (Fig. 4B). Previous reports described that MEFs derived from p53−/− embryos expressed relatively high basal levels of p19ARF (14, 37). This can be appreciated at the protein level (Fig. 4, A and B); however, treatment with GSNO further augmented the levels of p19ARF protein on p53−/− cells. Similar results were obtained in macrophages. Incubation of macrophages obtained from WT animals with 500 μM GSNO for 6 h and with LPS and IFN-γ for 18 h induced p19ARF accumulation. In addition, treatment with 1400W, a specific NOS-2 inhibitor,
inhibited p19ARF expression after stimulation with LPS and IFN-γ, indicating that p19ARF expression is dependent on NO synthesis (Fig. 4C).

Transcriptional up-regulation of p19ARF by NO

The changes in p19ARF expression might be due to either increased levels of p19ARF mRNA or translational and posttranslational events. To test whether the accumulation of p19ARF was attributed to increased protein stability, cells were treated with cycloheximide in the presence or absence of GSNO. As shown in Fig. 5A, exposure to GSNO had no effect on the t1/2 of p19ARF, indicating that p19ARF might be regulated at the transcription level. To evaluate this possible mechanism, we analyzed p19ARF mRNA levels by quantitative PCR. Stimulation with GSNO increased p19ARF mRNA levels in MEFs and macrophages (Fig. 5B). To confirm these results, we next examined whether other NO donors can mimic the effect of GSNO in both types of cells. We observed that Deta-NO and 3-morpholinosydnonimine (SIN-1) increased p19ARF mRNA levels in WT and p53−/−/− cells (Fig. 5B). These results indicate that NO is able to up-regulate p19ARF at the transcriptional level. In addition, we examined the response of ARF promoter to NO stimulation. ARF−/− MEFs were transiently transfected with ARF promoter and stimulated with NO donors (GSNO and Deta-NO). We found that NO donors activated ARF promoter, demonstrating that ARF expression is transcriptionally regulated by NO (Fig. 5E).

Among the various mechanisms that activate ARF, a role for AP-1 has been recently described (38, 39). Activation of AP-1 is achieved via the MAPK pathway (40) and NO is known to activate MAPK and JNK pathways as well as AP-1 (40, 41). In this context, we explored the possible implication of MAPK pathway in the transcriptional regulation of ARF by NO. WT cells were incubated with GSNO and specific MAPK inhibitors, and levels of ARF protein were detected by Western blot. As Fig. 5C shows, MAPKs seem to play an important role on the regulation of ARF, as we can observe by the inhibition of ARF levels after treatment with p38MAPK and ERK inhibitors (SB 202190 and PD 098059, respectively).

p16INK4a is not involved in the NO-dependent apoptosis

The INK4a-ARF locus encodes two unrelated proteins, p16INK4a and p19ARF. To exclude the possible implication of p16INK4a on NO effects, we analyzed p16INK4a protein levels after treatment with NO. As Fig. 6A shows, no changes on p16INK4a protein levels were detected upon treatment with GSNO. A similar
increase of p19ARF was obtained on INK4a/ARF^+/+ and INK4a/ARF^-/-^Atp after GSNO stimulation. Indeed, when INK4a/ARF^-/-^ cells were incubated with GSNO for 18 h, NO did not exert any effect on apoptosis, as we have described before; however, apoptosis increased in INK4a/ARF^+/+ and INK4a/ARF^-/-^ cells in a similar way (Fig. 6C). Besides, INK4a/ARF^-/-^ cells were more sensitive to apoptosis according to the previous reports that described that additional INK4a/ARF activity confers a generalized increased resistance to cancer (43). To confirm these results, caspase 3 activity was determined in the same conditions (Fig. 6D). As expected, DEDvase activity increased in INK4a/ARF^+/+ and INK4a/ARF^-/-^ cells and GSNO enhanced DEVvase activity in INK4a/ARF^-/-^ cells. These data indicate that p19ARF is involved in the NO-dependent apoptosis, without participation of p16INK4a.

**Lack of p19ARF inhibits d-GalN/LPS-induced apoptosis in an in vivo model of liver injury**

To validate in vivo the involvement of p19ARF on NO-dependent apoptosis, we used an in vivo experimental model of apoptosis mediated by NO. In this model, LPS, with the additional help of d-GalN, produces a systemic activation of macrophages, which in turn secrete massive amounts of proinflammatory cytokines that activate apoptotic pathways (44). This apoptosis is mainly mediated by TNF-α and NO released by up-regulation of NOS-2 expression (45). Accordingly, mice were injected i.p. with a mixture of d-GalN and LPS, and 5 h later liver sections were examined by TUNEL staining. TUNEL-positive hepatocytes were abundantly observed in the livers of WT mice injected with d-GalN and LPS (Fig. 7A); in contrast, positively stained nuclei were rarely detected in treated ARF^-/-^ mice (Fig. 7A). To demonstrate the involvement of NO in this model as well as ARF induction, the expression of NOS-2 was studied immunohistochemically in similar sections of treated livers and p19ARF levels were determined by Western blot. The administration of D-GalN/LPS resulted in induction of iNOS in WT and ARF^-/-^ mice, as we can observe in Fig. 7A, whereas p19ARF expression was only detected in WT animals after d-GalN/LPS administration (Fig. 7B). Histological changes in liver tissues were investigated after H&E staining (Fig. 7A). After 5 h of d-GalN/LPS administration, hepatocyte destruction was observed on WT animals, whereas no significant hepatic lesions were produced in livers of ARF^-/-^ mice.

To evaluate the hepatocyte damage induced by d-GalN/LPS, the enzymatic activities of AST and ALT were determined 5 h after i.p. administration of d-GalN/LPS. Both AST and ALT were increased in WT animals, but not in ARF^-/-^ mice (Fig. 7C). Finally, to demonstrate that induction of apoptosis by d-GalN/LPS involves caspase activation, DEVvase activity was determined in these animals, showing an increase of activity only in WT animals (Fig. 7D). These results reinforce the functional importance of ARF on NO-dependent apoptosis.

**Discussion**

The mechanisms underlying the activation of p53 have been extensively studied (10, 46). NO-mediated apoptosis is controlled by the mitochondrial pathway with the implication that p53 accumulation is upstream of cytochrome c release.
FIGURE 6. p16INK4a is not involved in NO-dependent apoptosis. WT, p53−/−, and p19ARF−/− MEFS were incubated with GSNO (500 μM) for 4 h. The protein levels of p16INK4a were determined by Western blot (A). INK4a/ARF−/−, INK4a/ARF−/−tg, INK4a/ARF−/−tg, or INK4a/ARF−/−tg MEFS were tested for the expression of p19ARF by Western blot after treatment with 500 μM GSNO for 4 h (B). INK4a/ARF−/−, INK4a/ARF−/−tg, INK4a/ARF−/−tg, or INK4a/ARF−/−tg MEFS were incubated with 500 μM GSNO, and apoptosis and caspase 3 activity were determined after 18 and 8 h of treatment, respectively (C and D). **, p < 0.01 with respect to the control condition.

FIGURE 7. Lack of p19ARF inhibits o-GalN/LPS-induced apoptosis in an in vivo model of liver injury. WT and p19ARF−/− animals were injected i.p. with LPS (10 μg/kg) and o-GalN (800 mg/kg) dissolved in 1 ml of saline solution. Animals with no treatment were injected with 1 ml of saline. Livers were removed at 5 h, and nonconsecutive liver sections were stained with Hoescht (blue) and analyzed by TUNEL (green) following the instruction of supplier. iNOS (green) was detected after staining with specific Ab by immunocytochemistry, and values of intensity fluorescence were indicated in figures (A). p19ARF levels were determined by Western blot in liver cytosolic extracts (B). Tissues were stained with H&E (A), and AST and ALT were measured in plasma (C). The caspase 3, 8, and 9 activity was determined by fluorometry in cell extracts (D). *, p < 0.05; **, p < 0.01 with respect to the control condition; and a, p < 0.05 and b, p < 0.01 with respect to the WT condition.
A key regulator of p53 levels is the tumor suppressor protein ARF. ARF inhibits the p53 ubiquitin ligase, Mdm2, allowing activation of the p53 tumor suppressor (17, 18). Although the ability of NO to up-regulate p53 is well documented, much less is known about the role of p19ARF in NO-dependent apoptosis. In this study, we demonstrate that lack of ARF causes a significant decrease in the rise of apoptosis. We have found that WT MEFs and macrophages display a normal behavior after NO treatment. Thus, NO induces apoptosis in these cells through p53 up-regulation and involvement of mitochondrial mediators (changes in the expression of proteins of the Bcl-2 family, cytochrome c release, and caspase activation). However, in the absence of ARF, cells are more resistant to apoptosis, and changes on the mitochondrial proteins and caspase activation are partially abrogated, suggesting that ARF is an important contributor to the induction of apoptosis by NO. Finally, apoptosis was considerably impaired in p53−/− fibroblasts and macrophages, stressing the relevance of p53 activation in NO-induced apoptosis. In support to all these data, experiments in an in vivo model of hepatic apoptosis due to the NO generation show that lack of ARF prevents this process, providing additional evidence to the notion that induction of apoptosis by NO is mediated by ARF. Besides, all these effects are specific for ARF because the other protein encoded by the locus INK4a/ARF, p16INK4a, did not show any modification after treatment with NO. Taken together, these data indicate that NO elicits an apoptotic response mediated by the up-regulation of p19ARF, but not p16INK4a.

There is no evidence of a direct activation of p19ARF by NO. In the present study, we have shown a remarkable increase in ARF expression when cells (fibroblasts and macrophages) are incubated with NO, and we have demonstrated that p19ARF accumulation in response to NO occurs through direct transcriptional activation (mRNA increase and promoter activation), without involvement of protein stabilization. Unlike a prior report that shows ARF activation in response to SIN-1, a peroxynitrite donor (47), we have established that not only exogenous NO induces ARF, but proinflammatory stimuli such as LPS and IFN-γ promote ARF activation via NO. Studies in macrophages obtained from WT mice clearly demonstrate the involvement of NO in ARF activation as reflected by its inhibition after treatment with 1400W, a NOS inhibitor. Moreover, NO-dependent apoptosis was lower in ARF−/− macrophages than in WT macrophages, and was totally abrogated when cells were incubated with 1400W, the NOS inhibitor, in the presence of LPS and IFN-γ. These data clearly establish a relationship between NO and ARF expression. In addition to this, our data show a reduced activation of p53 by NO in the absence of ARF at early times (4–8 h). This reduction was even observed with low concentrations of GSNO (100–200 μM) (data not shown). These results contradict those published by Wang et al. (48), whose studies reported that NO neither uses the ARF tumor suppressor protein nor ataxia telangiectasia-mutated (ATM) to accumulate p53. However, it is important to take into account that these experiments were conducted with a high concentration of GSNO (1 mM vs 100–500 μM) and for a long period of time (10 h), and at this time we have also observed p53 activation (Fig. 2). Moreover, high concentrations of NO (in millimolar range) can increase mitochondrial reactive oxygen species production, and oxidative stress is known to be a potent activator of p53. In addition to our results, the involvement of ATM on p53 accumulation was recently questioned by demonstrating that phosphorylation of p53 at serine 15 after NO treatment is ATM and ATM- and Rad3-related (ATR) dependent (49, 50).

In view of these results, it is not possible to exclude p19ARF as an important contributor to NO-induced p53 accumulation, although it is worthwhile to mention that there are ARF-independent pathways that activate p53. Between them, the inhibition of NF-κB through a mechanism that does not require either p53 or Mdm2 has recently been described. ARF represses the transcriptional activation domain of the NF-κB family member RelA by inducing its association with the histone deacetylase, HDAC1. Moreover, ARF activates the ATR/Chk1 pathway, and this is required for its ability to both repress RelA and induce p53. Therefore, the presence of NF-κB might determine the sensitization of the cells to apoptosis through the inhibition of NF-κB trans activation (51, 52). Indeed, we cannot rule out that NF-κB might play a role in the NO-dependent apoptosis induced through ARF, because the involvement of ATM/ATR in

**FIGURE 8.** Proposed model for the mechanism involved in ARF activation by NO. Exposure to NO induces p53 up-regulation leading to apoptosis. In normal cells, MDM2 maintains low levels of p53 by targeting the protein for degradation by the proteasome (dashed lines). MDM2 activity is counteracted by ARF, which prevents the MDM2-mediated degradation of p53. NO increases ARF transcription and protein levels through a mechanism that seems to involve MAPK pathways and possibly AP-1 activation.
the phosphorylation of p53 after NO treatment has been described recently (49, 50). Nevertheless, NO stimulation provides a more complex scenery, because NO has direct effects on Mdm2. Indeed, p53 accumulation upon treatment with NO is preceded by a decrease in Mdm2 protein levels; however, extended exposure to NO augmented the levels of Mdm2 due to the activation of the mdm2 gene by p53 (48, 53). In this context, experiments with ARF-deficient cells to determine NO-induced changes in Mdm2 might establish the significance of Mdm2 in this process; however, no significant differences on Mdm2 protein levels were observed in ARF-deficient cells vs WT cells NO treatment (data not shown), indicating that at least in our model, additional experiments must be required to elucidate the contribution of p53-dependent and independent mechanisms to the NO-induced apoptosis.

To gain insight on mechanism by which NO leads to ARF activation, we are analyzing the transcriptional activity of the ARF promoter that seems to be subjected to a rather complex regulation, probably requiring the combined action of several transcription factors. A large number of nuclear factors have been implicated in this process, such as Sp-1, DMP-1, E2F, and AP-1. Preliminary results demonstrated that treatment with MAPK inhibitors, especially p38MAPK and ERK inhibitors, prevented the up-regulation of p19ARF by NO, indicating a possible involvement of this pathway in the ARF activation (Fig. 8). However, the relevance of this mechanism requires further investigation.

In conclusion, the results presented in this work describe for the first time the involvement of ARF in NO-dependent apoptosis as a universal mechanism observed in various cell types and model animals. Moreover, we have provided evidence for the transcriptional regulation of ARF by NO. Therefore, the novel pathway we describe in this study is an important component of NO-dependent apoptosis and contributes to unraveling the apoptotic activity of NO.

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Disclosures
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References