Nitric oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways

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Nitric oxide (NO) generation initiates apoptotic cell death in different experimental systems. In RAW 264.7 macrophages the appearance of typical apoptotic markers is linked to inducible NO synthase induction. Mechanistically, accumulation of tumour suppressor p53 precedes apoptotic DNA fragmentation. With the use of *S*-nitroglutathione (GSNO) we correlated a dose-dependent p53 up-regulation to DNA fragmentation measured after 4 h and 8 h, respectively. Our studies revealed a linear correlation between the potency of five different NO donors with respect to apoptosis induction and p53 accumulation. Furthermore, we probed for NO-induced apoptosis after stable transfection of RAW 264.7 macrophages with plasmids encoding p53 antisense RNA. Clones with down-regulated p53 levels in

response to GSNO exhibited a marked reduction in DNA fragmentation. Expression of the inducible NO synthase in response to lipopolysaccharide and interferon- γ caused apoptosis in RAW 264.7 macrophages and neomycin-vector controls within 24 h. In contrast, p53 antisense RNA-expressing clones appeared highly resistant towards endogenous NO, although inducible NO synthase induction with concomitant nitrite production remained unchanged. For RAW 264.7 macrophages our results established a functional role of the tumour suppressor p53 during NO-induced apoptotic cell death. However, p53 antisense experiments and the use of the p53-negative cell line U937 substantiated p53-independent signalling pathways operative during NO-mediated apoptosis.

INTRODUCTION

Nitric oxide (NO), enzymically generated from L-arginine by a family of NO synthases, takes part in diverse biological signalling. Cell-mediated immune responses, endothelium-dependent relaxation, and neurotransmission are considered as 'classical' NO-mediated actions (see [1] for a review). Physiological signal transduction is mainly attributed to soluble guanylate cyclase activation and concomitant cGMP formation. Subsequent activation of cGMP-dependent protein kinases, regulation of cGMP-gated ion channels, or cGMP-dependent cyclic nucleotide phosphodiesterases accounts for cellular responses [2]. In contrast, activation of the high-NO-output system mediates cytotoxicity as a first line of defence against invading pathogens or tumour cells and elicits apoptotic cell death in different cellular systems. Under pathophysiological conditions NO-producing cells themselves seem vulnerable towards NO toxicity. This is evident for NO-induced β -cell destruction during insulin-dependent diabetes mellitus type I and certain neurological disorders like Alzheimer's disease [3].

Previously, NO-induced apoptotic cell death in mouse macrophages has been described [4,5]. Apoptosis, or programmed cell death, was characterized morphologically by cell shrinkage and chromatin condensation, and biochemically by DNA laddering. Mechanistically, cytotoxic NO signalling, or the apoptogenic action of NO, is still poorly understood. Proposals centred around inhibition of iron-sulphur enzymes involved in cellular energy supply [1], the NAD(H)-dependent modification of protein thiols [6] or direct DNA damage [7,8]. In RAW 264.7 macrophages, NO-induced oxidative DNA damage and DNA

deamination were traced back to an active, inducible-type NO synthase [9]. Our studies established p53 protein accumulation prior to apoptotic DNA fragmentation in response to endogenously or exogenously generated NO in the same cell line [10].

The wild-type tumour suppressor protein, p53, is known as a critical regulator of the cellular response to DNA damage (see [11] for a review). Normally cells express low p53 levels due to an extremely short half-life of the protein. Irradiation or exposure to DNA-damaging agents such as mitomycin C or etoposide causes post-transcriptional p53 stabilization which prolongs the half-life of the protein, resulting in p53 accumulation. Up-regulated p53 either blocked cellular proliferation by a G₁ cell-cycle arrest or induced programmed cell death in the case of severe DNA damage. The role of p53 as a cell-cycle regulator is transduced by transcriptional activation of p21^{WAF1/CIP1} expression [12], an inhibitor of cyclin-dependent protein kinase 2 (Cdk 2) and Cdk 4. In contrast, p53-dependent apoptotic signalling is still unknown. Although p53 may induce apoptosis in the absence of transcriptional target gene activation [13], down-regulation of Bcl-2, which signals cell survival, and/or up-regulation of Bax, which promotes cell death, are proposed signalling mechanisms [14]. Generally, apoptotic cell death pathways can be ascribed as p53-dependent or p53-independent [15]. For example, immature mouse thymocytes lacking p53 underwent apoptosis when exposed to glucocorticoids but were resistant to otherwise lethal radiation [16].

In our study we elucidated the role of p53 during NO-induced apoptosis. To block p53 expression, DNA constructs encoding p53 antisense RNA were introduced into RAW 264.7 macrophages. Our experiments establish p53-dependent as well

Abbreviations used: DEA-NO, diethylamine-nitric oxide complex; DETA-NO, diethylenetriamine-nitric oxide complex; GSNO, S-nitrosoglutathione; ICE, interleukin-1 β -converting enzyme; IFN- γ , interferon- γ ; LPS, lipopolysaccharide; NO, nitric oxide; PKC, protein kinase C; SNP, sodium nitroprusside; spermine-NO, spermine-nitric oxide complex; SV 40, simian virus 40; TNF- α , tumour necrosis factor α .

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as p53-independent apoptotic signalling pathways initiated by NO.

MATERIALS AND METHODS

Materials

Spermine-NO, DEA-NO (diethylamine-NO complex) and DETA-NO (diethylenetriamine-NO complex) were delivered by Biotrend, Cologne, Germany. H33258, Protein A-Sepharose, diphenylamine, BSA and sodium nitroprusside (SNP) were purchased from Sigma, Deisenhofen, Germany, while [¹²⁵I]Protein A (10 mCi/mg) was bought from DuPont-New England Nuclear, Dreieich, Germany. RNase A from bovine pancreas was from Boehringer Mannheim, Mannheim, Germany. KAISER's glycerol gelatin was provided by Merck, Darmstadt, Germany. RPMI 1640 medium was ordered from Biochrom, Berlin, Germany. Cell culture supplements, fetal-calf serum, geneticin and agarose were from Gibco, Berlin, Germany. The plasmid pBK/CMV was from Stratagene, Heidelberg, Germany, and pSma-10 was generously provided by Professor M. Oren, Weizmann Institute, Israel. All other chemicals were of the highest grade of purity commercially available.

Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 and the human promyelocytic leukaemia cell line U937 were maintained in RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) heat-inactivated fetal-calf serum (complete RPMI). All experiments were performed using complete RPMI.

Transfections

The plasmid pBK/CMVΔp53asn was constructed by subcloning the coding sequences for amino acids 71–387 of the mouse p53 cDNA into the *Bam*HI and *Eco*RI sites of pBK/CMV in antisense orientation. RAW 264.7 macrophages (2×10^6) were grown in a 10-cm-diam. Petri dish. After overnight adhesion, cells were transfected either with 20 µg of the plasmid pBK/CMVΔp53asn or the control plasmid pBK/CMV lacking the p53 antisense gene (neomycin-vector control) by using the calcium phosphate precipitation method [17]. Alternatively, cells were co-transfected with 20 µg of the plasmid pSma-10, containing the whole p53 cDNA in antisense orientation under the control of the simian virus 40 (SV 40) promoter and 4 µg of the plasmid pBK/CMV containing the neomycin resistance gene. Stable transfected single clones were picked randomly after selection with 400 µg/ml geneticin and examined for p53 expression by immunoprecipitation and Western blot analysis.

Morphological investigations

Macrophages (4×10^5) were grown in 12-well culture plates. After adhesion, cells were stimulated, followed by fixation with 3% paraformaldehyde for 5 min on to glass slides. Samples were washed with PBS, stained with Hoechst dye H33258 (8 µg/ml) for 5 min, washed with distilled water, and mounted in KAISER'S glycerol gelatin. Nuclei were visualized using a Leitz fluorescence microscope. In each sample a minimum of 500 cells were counted and apoptotic nuclei were expressed as a percentage of total nuclei.

GSNO synthesis

GSNO (S-nitroso derivative of glutathione) was freshly synthesized every week as described previously [4,18]. Briefly,

glutathione was dissolved in HCl at 0 °C prior to the addition of NaNO₂. The mixture was stirred at 0 °C for 40 min followed by the addition of 2.5 vol. of acetone. The precipitate was filtered and washed once with 80% (v/v) acetone, two times with 100% acetone, and finally three times with diethyl ether and then it was dried under vacuum.

Quantification of DNA fragmentation

DNA fragmentation was mainly assayed as reported previously [19]. Cells (4×10^6) were incubated in 6-well culture plates for various times. After that, cells were scraped off the culture plates, centrifuged at 1500 rev./min for 10 min, resuspended in 250 µl of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE-buffer), and incubated with an additional volume of lysis-buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 4 °C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 13000 g. Pellets were resuspended in 500 µl of TE-buffer and samples were precipitated by adding 500 µl of 10% trichloroacetic acid at 4 °C. Samples were pelleted at 3000 g for 10 min and the supernatant was removed. After addition of 300 µl of 5% trichloroacetic acid, samples were boiled for 15 min. DNA contents were quantified using the diphenylamine reagent [20]. The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

DNA agarose gel electrophoresis

To obtain DNA for agarose gel electrophoresis, cells were cultured, harvested, lysed and centrifuged as described above to separate DNA fragments from intact chromatin. Supernatants were precipitated overnight with 2 vol. of ice-cold ethanol and 50 µl of 5 M NaCl at –20 °C, centrifuged again at 13000 g for 15 min and each pellet was incubated in 500 µl of TE-buffer supplemented with 100 µg/ml RNase A at 37 °C for 30 min. Samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and once again with an equal volume chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated again and pellets were recovered by centrifugation (13000 g, 15 min), air dried, resuspended in 10 µl of TE-buffer, supplemented with 2 µl of sample buffer (0.25% Bromophenol Blue, 30% glyceric acid), and electrophoretically separated on a 1% agarose gel containing 1 µg/ml ethidium bromide for 2.5 h at 100 V. Pictures were taken by UV transillumination.

p53 quantification

p53 levels were quantified by immunoprecipitation followed by Western blot analysis as described previously [10]. Briefly, 2×10^7 cells were incubated for the times indicated, scraped off and lysed in 700 µl of lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0). Cells were sonicated with a Branson sonifier (10 s, duty cycle 100%, output control 1). After centrifugation non-specific adsorbents were removed from the resulting supernatant with 40 µl of 50% (v/v) Protein A-Sepharose. p53 was immunoprecipitated by adding 200 µl of hybridoma supernatant (clone PAb 122) and 50 µl of 50% Protein A-Sepharose. Immune complexes were washed three times with 500 µl of SNNT (5% sucrose, 1% Nonidet-40, 0.5 M NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4) and once with 1 ml of SNNT. Finally, samples were resuspended in 40 µl of sample buffer (125 mM Tris, 2% SDS, 10% glycerol, 1 mM dithiothreitol, 0.002% Bromophenol Blue,

Figure 1 Correlation between GSNO-induced p53 up-regulation and DNA fragmentation

(A) RAW 264.7 macrophages were incubated for 4 h with increasing GSNO concentrations. p53 protein was determined by immunoprecipitation and Western blot analysis as described in the Materials and methods section. The blot is typical of three similar experiments. p53-associated radioactivity was quantified and plotted against fragmented DNA. Fragmentation analysis was quantified with the diphenylamine reaction after an 8 h incubation (B). The p53 image quant data of unstimulated RAW 264.7 macrophages served as a control (relative level of 1) and all other values were calculated proportionally. Values are means $\pm$ S.D. of at least four separate determinations.

Figure 2 Correlative NO donor-induced p53 accumulation and DNA fragmentation

(A) RAW 264.7 macrophages (2×10^7) were incubated for 4 h with the compounds indicated. p53 protein was analysed as described in Figure 1. M indicates ^{14}C -labelled molecular-mass markers. p53-bound radioactivity was quantified using the phosphor image system. Data were plotted against fragmented DNA measured after 8 h with the diphenylamine reaction (B). Values are means $\pm$ S.D. of four to six individual experiments.

pH 6.9), and proteins were resolved on SDS/10%-polyacrylamide gels and blotted on to nitrocellulose sheets using the semi-dry blot system from Pharmacia (0.8 mA/cm², 1.25 h, 25 mM Tris/192 mM glycine). Blots were washed twice with TBS (140 mM NaCl, 50 mM Tris, pH 7.2) containing 0.1% Tween-20 before blocking unspecific binding with TBS/2% BSA. The p53 antibody was added (hybridoma supernatant against p53; clone PAb122; 1:6 in TBS/0.2% BSA) and incubated overnight at 4°C. Nitrocellulose sheets were washed five times and unspecific binding was blocked as described. For

detection, blots were incubated with [^{125}I]Protein A (2 ng/ml Protein A, 1 μCi in TBS/0.06% Tween-20/0.1% BSA) for 2 h followed by quantitative determination of radioactivity using the phosphor image system (Molecular Dynamics) [21].

Statistical analyses

Each experiment was performed at least three times and statistical analysis was done using the two-tailed Student's *t*-test.

RESULTS

Correlative p53 up-regulation and DNA fragmentation

We searched for a correlation between the amount of p53 protein and the extent of fragmented DNA in RAW 264.7 macrophages. Maximal p53 accumulation was observed 4 h after NO application, while fragmentation was assayed after an 8 h period. Cells were exposed to increasing concentrations of GSNO followed by p53 determination and DNA fragmentation analysis. p53 protein was nearly undetectable under control conditions (Figure 1A). GSNO up to 1 mM caused a dose-dependent p53 up-regulation. Figure 1(B) shows a quantitative analysis of both DNA fragmentation and p53 accumulation in response to GSNO. In further experiments RAW 264.7 macrophages were incubated for 4 h with five different NO donors (GSNO, SNP, spermine-NO, DEA-NO and DETA-NO). Probing for p53 levels in cell extracts, employing immunoprecipitation followed by Western blotting, revealed significant p53 accumulation for all NO donors (Figure 2A). Subsequently, all NO-releasing compounds caused apoptotic DNA fragmentation as determined

Figure 3 p53-antisense-RNA-expressing RAW 264.7 macrophages

RAW 264.7 cells, a neomycin-vector control transfected clone (Rneo-2) and three p53 antisense RNA transfectants (RΔp53asn-1, RΔp53asn-11 and Rp53asn-1) were incubated for 4 h with 1 mM GSNO (G) or remained untreated (control, C) and were analysed for p53 protein expression (2×10^7 cells/lane). M indicates ^{14}C -labelled molecular-mass markers. In (A) a representative Western blot is shown. (B) Quantitative p53 protein determination was done by phosphor image analysis. The p53 image quant data of unstimulated RAW 264.7 macrophages served as a control (relative level of 1) and all other values were calculated proportionally. Values are means $\pm$ S.D. of three independent experiments.

Figure 4 p53 antisense RNA expression attenuates apoptotic DNA fragmentation in response to GSNO exposure

Cells, cultured as outlined in the Materials and methods section, were incubated for 8 h with increasing GSNO concentrations. DNA fragmentation was measured with the diphenylamine reaction. Data are means $\pm$ S.E.M., $n \geq 4$.

after an 8-h incubation period. Furthermore the p53 levels determined by phosphor image analysis and apoptotic DNA fragmentation quantified by the diphenylamine assay were mathematically correlated. Figure 2(B) displays a linear correlation between the ability of these NO donors in stimulating p53 up-regulation and apoptotic cell death in response to the NO donors employed. These results therefore led to the assumption that NO-induced apoptosis depends on p53 expression.

Stable p53 antisense RNA expression in RAW 264.7 macrophages

To elucidate the role of p53 during NO-induced apoptosis, NO-susceptible RAW 264.7 macrophages were stably transfected with the plasmid pBK/CMVΔp53asn. The plasmid contains the N-terminal part of mouse p53 cDNA in antisense orientation under the control of the cytomegalovirus (CMV) promoter, and a neomycin resistance gene. Individual clones are termed RΔp53asn followed by their clone-numbers. Additionally, cells were co-transfected with the plasmids pΔSma-10 carrying the full p53 cDNA in antisense orientation under the control of the SV 40 promoter and pBK/CMV encoding a neomycin resistance gene, serving as a selection marker (clones termed Rp53asn). Three different clones, RΔp53asn-1, RΔp53asn-11 and Rp53asn-1, exhibited substantially reduced p53 accumulation following GSNO stimulation (Figure 3). Clone RΔp53asn-1 most potently suppressed p53 accumulation in response to NO. Compared with parental RAW 264.7 macrophages, as well as the neomycin-vector control-transfected clone (Rneo-2), p53-antisense-containing clones lacked basal p53 protein expression. Cell morphology and proliferative behaviour of transfectants were comparable with parent RAW 264.7 macrophages (results not shown).

p53 antisense RNA expression suppressed NO-induced apoptosis

Following GSNO addition parent RAW 264.7 macrophages as well as neomycin-vector control cells (Rneo-2) dose-dependently cleaved DNA (Figure 4). Quantitative analysis revealed roughly 30% fragmented DNA in response to 500 μM GSNO while

Table 1 p53 antisense RNA expression blocks apoptosis after inducible NO synthase induction

RAW 264.7 macrophages, a neomycin-vector control clone (Rneo-2) and p53 antisense RNA transfectants were incubated for 24 h with vehicle (control) or 10 μ g/ml LPS and 100 units/ml IFN- γ , fixed and stained with the DNA-specific fluorochrome Hoechst dye 33258. Nitrite was determined in the culture supernatant with the Griess reaction and apoptotic cells exhibiting characteristic chromatin condensation were counted by fluorescence microscopy. Values are means $\pm$ S.D. of four independent experiments. Statistical significance: * $P < 0.02$ versus Rneo-2 and ** $P < 0.01$ versus Rneo-2.

	Nitrite (μ M)		Apoptotic cells (%)	
	Control	LPS + IFN- γ	Control	LPS + IFN- γ
RAW 264.7	0.9 $\pm$ 0.4	36.8 $\pm$ 5.4	1.4 $\pm$ 0.4	28.7 $\pm$ 5.9
Rneo-2	0.0 $\pm$ 0.1	38.6 $\pm$ 11.0	1.1 $\pm$ 0.7	21.7 $\pm$ 3.2
R Δ p53asn-1	0.0 $\pm$ 0.1	43.5 $\pm$ 5.6	1.0 $\pm$ 0.3	10.4 $\pm$ 2.7*
R Δ p53asn-11	0.1 $\pm$ 0.1	42.9 $\pm$ 11.3	0.7 $\pm$ 0.2	8.3 $\pm$ 1.5**
Rp53asn-1	0.0 $\pm$ 0.1	43.6 $\pm$ 6.8	0.9 $\pm$ 0.2	9.5 $\pm$ 1.5**

1 mM GSNO produced about 35 % DNA degradation. In contrast, in p53-antisense-RNA-expressing clones DNA cleavage was substantially reduced. After application of 1 mM GSNO clone R Δ p53asn-1 not only expressed the lowest amount of p53 but also revealed the best protection. Inhibition of DNA fragmentation in clones R Δ p53asn-11 and Rp53asn-1 was less pronounced but significantly lower compared with non-transfected cells.

Next, we probed for p53 antisense RNA transfection related to inducible NO synthase induction. Combinatory lipopoly-saccharide (LPS) and interferon- γ (IFN- γ) application promoted NO synthase induction and apoptosis in RAW 264.7 macrophages. Parent RAW cells, neomycin-vector control cells, and p53 antisense RNA transfectants respond to LPS/IFN- γ with increased nitrite production. Nitrite amounted to values around 40 μ M (Table 1). Variations between the different cells were not significant. Apoptotic cell death was determined by counting cells exhibiting condensed chromatin using the Hoechst dye 33258 and fluorescence microscopy. Despite unaltered nitrite production, p53-antisense-transfected clones showed significant protection compared with neomycin-vector control cells and parent RAW macrophages.

NO-induced apoptosis in U937 cells

Previous experiments implied RAW 264.7 macrophage apoptosis to be, at least in part, p53-dependent. However, in p53-antisense-RNA transfectants NO still produced DNA cleavage (Figure 4) and apoptotic cell death (Table 1) to a certain degree. Therefore we intended to study p53-independent signalling related to NO-induced apoptosis. Experimentally, the p53 negative human promyelocytic leukaemia cell line U937 was chosen for further experiments. Exposure to 1 mM GSNO resulted in DNA ladder formation within 8 h, whereas fragmentation in controls was absent (Figure 5). GSNO (1 mM)-induced DNA cleavage amounted to 37.5 $\pm$ 6.9 % (mean $\pm$ S.D., $n = 4$) which is comparable with values obtained with RAW 264.7 macrophages (Figures 1 or 4 for comparison). To characterize NO-independent apoptosis, U937 cells were treated with 25 ng/ml human tumour necrosis factor α (TNF- α). Within 8 h TNF- α elicited internucleosomal DNA fragmentation (17.0 $\pm$ 0.6 %; mean $\pm$ S.D., $n = 3$) leading to an apoptotic DNA ladder (Figure 5). These experiments re-establish DNA fragmentation following TNF- α

Figure 5 TNF- α and GSNO-induced apoptosis in U937 cells

The human promyelocytic leukaemia cell line U937 (4×10^6 cells) was exposed for 8 h to 25 ng/ml human TNF- α or to 1 mM GSNO. DNA fragments separated from intact chromatin were visualized by UV transillumination after agarose gel electrophoresis. The gel is representative of three independent experiments.

addition in U937 cells and prove NO-initiated DNA cleavage in a p53-negative system.

DISCUSSION

NO cytotoxicity may be viewed as a double-edged sword. On one hand NO kills invading pathogens or tumour cells and therefore can be regarded as an essential component of the non-specific immune defence. On the other hand a massive NO production relates to severe medical symptoms like sepsis or type-I diabetes mellitus and thus seems cell destructive and detrimental to the whole organism. In general there is some information regarding toxic NO mechanisms but less information is available on molecular alterations underlying NO-mediated apoptosis. Previously we demonstrated p53 accumulation prior to endonuclease-mediated DNA laddering in RAW 264.7 macrophages. This led to the assumption of a tumour suppressor involvement during NO-induced apoptosis [10]. Supporting evidence came from a close correlation between p53 up-regulation and apoptotic DNA fragmentation (Figures 1 and 2).

Furthermore our assumption was experimentally tested by stable RAW cell transfection with plasmids encoding p53 antisense RNA in order to block p53 expression [22]. Antisense experiments establish p53-dependent as well as p53-independent apoptotic signalling pathways. Transfected RAW 264.7 macrophages exhibit markedly reduced p53 expression which is paralleled by suppressed DNA fragmentation and attenuated programmed cell death. This holds for endogenously generated as well as exogenously supplied NO. However, residual fragmentation and apoptotic cell death remained in antisense transfectants. Additionally, NO clearly initiated the apoptotic suicide program in p53-negative U937 cells.

An important question needs to address the molecular mechanism resulting in NO-induced p53 accumulation. Some reports established DNA damage evoked by UV-irradiation or DNA-damaging agents to be sufficient for p53 up-regulation, subsequent G₁ growth arrest, and/or apoptosis [11]. Recently endogenously generated NO in RAW 264.7 macrophages has

been demonstrated to cause oxidative DNA damage and DNA deaminations [9]. Additionally, studies *in vitro* revealed DNA deamination reactions and DNA strand breaks in response to NO [7,8]. Generally, one associates p53 involvement during DNA damage as being linked to strand breaks only [23]. Most likely we assume NO-induced DNA damage, i.e. strand breaks, as one early NO signal, responsible for p53 accumulation in RAW 264.7 macrophages. It remains unclear so far, whether NO directly hits DNA or if intermediate signalling is required. Additionally, the chemical nature of the damaging NO-species remains unknown. In this respect, peroxynitrite (ONOO⁻), generated by the chemical interaction of NO and superoxide, was identified to induce apoptosis in HL-60 cells. At the same time peroxynitrite failed to apoptotically affect normal endothelial cells and peripheral blood mononuclear cells [24]. To test a possible involvement of peroxynitrite in RAW 264.7 macrophage apoptosis, we used the NO donor SIN-1 which is known to generate significant amounts of O₂⁻ and NO [25], thus resulting in diffusion-controlled peroxynitrite generation [26]. As SIN-1 proved to be a weak apogen and SIN-1-induced DNA fragmentation was not altered in the presence of up to 2000 units/ml superoxide dismutase (SOD) [4], our results would deny the participation of peroxynitrite during NO-initiated apoptosis in RAW 264.7 macrophages.

However, besides DNA-damage-induced p53 accumulation and apoptosis, several lines of evidence suggest additional, p53-dependent but damage-unrelated signals during apoptosis. For example, wild-type p53 is required for apoptosis induced by growth factor deprivation in leukaemic cells [27], or haematopoietic cell death initiated by interleukin-3 or interleukin-6 deprivation [28]. In the presence of these survival factors, cells stay alive even when p53 is overexpressed. Obviously, p53-dependent apoptotic signalling can be compensated by cytokine-induced growth stimulation. Furthermore, in rat brain oligodendrocytes, interleukin-2-dependent induction of apoptosis can be attributed to p53 [29]. In particular, neuronal cells emerged as being prone to apoptotic cell death elicited by various stimuli. In this respect, administration of the glutamate analogue kainic acid induced p53 expression and neurotoxicity in adult rat brain [30]. Interestingly, glutamate- or kainate-induced cytotoxicity involving *N*-methyl-D-aspartate receptor stimulation is linked to NO formation [31]. Although in the latter case p53 accumulation may be attributable to NO-induced DNA damage, additional pathways leading to NO-mediated p53 up-regulation may apply. One possible mechanism to be considered is a direct modulatory action of NO on p53 activity or protein stability by targeting cysteine residues normally implicated in zinc binding [32].

Our methodology using p53-antisense-RNA-expressing RAW 264.7 transfectants and the p53-negative cell line U937 refers to a p53-independent, NO-initiated signalling pathway during apoptosis. This is in analogy to p53-independent apoptosis elicited by other genotoxic stressors. For example, cycling T lymphocytes from p53^{-/-} mice underwent apoptosis after irradiation or genotoxic drug treatment [33], similar to the application of DNA-damaging agents to p53-negative cell lines such as HL-60 and U937 [34]. Hence, these results were suggestive of a p53-independent DNA damage G₁ checkpoint, and p53-independent cell death signalling pathways. This also applies, at least in part, to NO-mediated apoptosis. For NO multiple signalling pathways exist, some of which may be relevant for apoptosis. One possibility is the inhibition of protein kinase C (PKC) by S-nitrosylation [35]. Previously we reported on modulation of NO-induced apoptosis by altering PKC activity. Whereas phorbol esters like phorbol 12-myristate 13-acetate blocked apoptotic cell death in RAW 264.7 macrophages,

inhibition of PKC promoted cell death [4]. However, other modulatory activities of NO, like inactivation of low-molecular-mass phosphotyrosine protein phosphatases [36] or glutathione peroxidase [37], modulation of iron metabolism by regulation of the iron-responsive-element binding protein (IRE-BP) [38], and inhibitory actions on transcription factors [39], may be considered as well. Further investigations need to address these possible functions in relation to apoptotic signalling.

The simplest model to combine p53-dependent and p53-independent NO-signalling events is their convergence upon a final damaging pathway. Features of this pathway have been evolutionarily conserved and mostly comprise activation of cysteine proteases, with homology to the mammalian interleukin-1 β -converting enzyme (ICE) [40]. One substrate for ICE enzymes is the nuclear enzyme poly(ADP-ribose) polymerase (PARP) that is degraded during NO-induced apoptosis. Our own observations revealed NO-induced apoptosis as well as PARP cleavage to be completely prevented by *bcl-2* gene transfer [41]. *Bcl-2* acts, at least in part, by nullifying its counterpart Bax. As Bax was identified to be constitutively expressed in RAW 264.7 macrophages [42], it might be part of the proposed final death pathway. Bax-mediated apoptosis requires cells to be apoptosis prone. Therefore, our further experimental interest is centred around factors like c-myc [43], E2F-1 [44], and pRB [45] which allow cells to enter the apoptotic pathway.

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