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p53 expression in nitric oxide-induced apoptosis

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Abstract Nitric oxide (NO) is a diffusible messenger involved in several patho-physiological processes including immune-mediated cytotoxicity and neural cell killing. NO or the products of its redox chemistry can cause DNA damage and activate subsequent lethal reactions including energy depletion and cell necrosis. However, regardless of whether it is endogenously produced in response to cytokines, or generated by chemical breakdown of donor molecules, NO can also induce apoptosis in different systems. Here, we report that NO generation in response to a cytokine induced NO-synthase or by NO donors stimulates the expression of the tumor suppressor gene, p53, in RAW 264.7 macrophages or pancreatic RINm5F cells prior to apoptosis. NO-synthase inhibitors such as N^G-monomethyl-L-arginine prevent the inducible NO generation as well as p53 expression and apoptosis. Since p53 expression is linked to apoptosis in some cells exposed to DNA damaging agents, we suggest that NO-induced apoptosis in these cell systems is the consequence of DNA damage and subsequent expression of this tumor suppressor gene.

Key words: Nitric oxide; p53; Apoptosis; DNA fragmentation

1. Introduction

Nitric oxide (NO) is a messenger molecule involved in several processes including relaxation of smooth muscle, neurotransmission, and tumor cell as well as bacteria killing [1–3]. However, induction of a high output system for NO in response to cytokines or a massive production of NO following accumulation of the excitatory neurotransmitter glutamate [4,5] can result in cell killing. Neurons [6], pancreatic β-cells [7] or macrophages [8,9] seem to be particularly sensitive to NO toxicity. While in some systems NO can react with other radicals and effectively cause cell death by necrosis, in others the progressive intra- or extracellular generation of NO has been suggested to cause apoptosis [8,10,11]. Mechanisms proposed for NO toxicity include its interaction with protein thiol groups [3,12] or iron-sulfur proteins [13], or by direct DNA damage [14]. The latter, regardless of whether it is induced by radiation or by drugs such as etoposide, can result in apoptosis [15,16]. Expression of wild-type p53, a tumor suppressor gene, seems to be closely linked to apoptosis caused by most of the DNA-damaging agents [15,16]. The wild-type nuclear phosphoprotein p53, originally characterized as a tumor suppressor protein [17], acts as a checkpoint control in the cell cycle, permitting the repair of damaged DNA. The block in G_1/S transition which results from p53 activation has been suggested to cause apoptosis in the case of severe DNA damage [18,19]. More recently, it has become apparent that the p53 gene product can take part directly in the apoptotic process [20].

2. Materials and methods

2.1. Materials

The mouse macrophage-like cell line RAW 264.7 was provided by Prof. A. Wendel, Faculty of Biology, University of Konstanz, Germany. LPS (*Escherichia coli* serotype 0127:B8), NMMA, protein A-Sepharose, and SNP were purchased from Sigma, Deisenhofen, Germany. Recombinant murine interferon-γ was from Boehringer-Mannheim, Mannheim, Germany, while ^{125}I-labeled protein A (10 mCi/mg) was bought from DuPont-New England Nuclear, Dreieich, Germany. RPMI 1640 supplemented with 0.532 g/l *N*-acetyl-L-alanyl-L-glutamine was ordered from Biochrom, Berlin, Germany. Cell culture supplements, fetal calf serum and agarose were from Gibco, Berlin, Germany. All other chemicals were of the highest grade of purity commercially available.

2.2. GSNO synthesis

GSNO (*S*-nitroso derivative of glutathione) was freshly synthesized prior to use as described previously [21]. Briefly, glutathione was dissolved in 0.625 N HCl at 0°C to a final concentration of 625 mM. An equimolar amount of $NaNO_2$ was added and the mixture was stirred at 0°C for 40 min. After the addition of 2.5 vols. of acetone, stirring was continued for another 20 min, followed by filtration of the precipitate. GSNO was washed once with 80% acetone, two times with 100% acetone, and finally three times with diethylether, and then was dried under vacuum. GSNO was characterized by HPLC analysis and UV spectroscopy.

2.3. Immunoprecipitation and Western blotting

For each assay 2×10^7 cells were incubated in 10 cm Petri dishes with the appropiate substances for the times indicated, scraped off using a rubber policeman and lysed for 20 min in 700 μl lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0). Lysed cells were sonicated for 10 s using a Branson sonifier (duty cycle 100%, output control 1). After centrifugation for 5 min at $13,000 \times g$, non-specific adsorbants were removed from the resulting supernatant by an incubation with 40 μl 50% (v/v) protein A-Sepharose for 10 min at 4°C, followed by centrifugation for 15 min at $13,000 \times g$. p53 was immunoprecipitated overnight at 4°C by adding 200 μl hybridoma supernatant (clone PAb 122) and 50 μl 50% protein A-Sepharose. Immunecomplexes were spun down at $13,000 \times g$ for 60 s and washed 3 times with 500 μl SNNTE (5% sucrose, 1% Nonidet-40, 0.5 M NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4) and another time with 1 ml SNNTE. Finally, samples were resuspended in 40 μl sample buffer (125 mM Tris, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% Bromophenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 10% SDS-polyacrylamide gels, and blotted onto nitrocellulose sheets using the semi-dry blot system from Pharmacia (0.8 mA/cm^2, 1.25 h; 25 mM Tris, 192 mM glycine buffer system). The sheets 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 for 1 h at 20°C. 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 5 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)

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Abbreviations: IFN-γ, recombinant murine interferon-γ; LPS, lipopolysaccharide; NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide.

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for 2 h followed by quantitative determination of radioactivity using the phosphor image system (Molecular Dynamics) [22].

2.4. DNA agarose gel-electrophoresis

For the preparation of DNA for agarose gel-electrophoresis, cells were harvested, lysed, and centrifuged as described above to separate DNA fragments from intact chromatin. Supernatants were precipitated with 1 ml ice-cold ethanol and 50 μl 5 M NaCl at −20°C, centrifuged again at 13,000 × *g* for 15 min and each pellet was incubated in 500 μl 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/isoamylalcohol (25:24:1) and once again with an equal volume of chloroform/isoamylalcohol (24:1). DNA was precipitated overnight with 1 ml ethanol and 50 μl 5 M NaCl at −20°C. DNA pellets were recovered by centrifugation (13,000 × *g*, 15 min), air dried, resuspended in 10 μl TE buffer, supplemented with 2 μl 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.

2.5. Immunohistochemistry

Rat pancreatic RINm5F cells were seeded at a density of 5×10^3 cells/cm^2 onto poly-L-lysine-coated glass cover slips and grown for 4 days to a density of approximately 2×10^5 cells/cm^2. After exposure to IL-1β or SNP, cells were fixed in methanol/water (80:20), rinsed in phosphate-buffered saline with 0.1% Triton X-100 and incubated with 10% goat serum followed by overnight incubation with anti-p53 monoclonal antibody PAb421. Thereafter, cells were treated with FITC-conjugated anti-mouse IgG antibodies followed by staining with 20 μM ethidium homodimer 1. Cells mounted on glass slides were examined in a Bio-Rad MRC 600 confocal microscope system, where the excitation was provided by a Krypton-Argon laser line at 488 nm. Fluorescence was collected in the green and red region for anti-p53-Ig complexes and ethidium homodimer, respectively.

2.6. Nitrite determination

Nitrite, a stable NO oxidation product, was determined using the Griess reaction [23]. Cell-free culture supernatants were collected (200 μl), adjusted to 4°C, mixed with 20 μl sulphanilamide (dissolved in 1.2 M HCl) and 20 μl *N*-naphthylethylenediamine dihydrochloride. After 5 min at room temperature the absorbance was measured at 560 nm with a reference wavelength at 690 nm. Nitrite concentrations were calculated using a $NaNO_2$ standard.

3. Results and discussion

We have recently studied the characteristics of NO-mediated cell death in the mouse macrophage cell line RAW 264.7 as well as in the rat pancreatic β-cell line RINm5F. In both systems induction of the NO-synthase and concommitant NO generation results in cell death by apoptosis [10,11]. RAW 264.7 macrophages, stimulated with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) as agonists express high levels of the inducible form of the NO-synthase as determined by nitrite accumulation in the cell supernatant. Control cells produced virtually no nitrite over 24 h (<0.1 nmol $NO_2^-/10^6$ cells), while agonist addition led to massive nitrite accumulation (9.4 ± 2.5, 43.4 ± 1.7, and 63.6 ± 3.9 nmol $NO_2^-/10^6$ cells after 6, 14, and 24 h, respectively; mean ± S.D., $n = 6$). LPS/IFN-γ-induced nitrite production was significantly repressed in the presence of the NO-synthase inhibitor N^G-monomethyl-L-arginine (NMMA), applied together with the agonists at a concentration of 1 mM (1.1 ± 0.2, 7.2 ± 0.9, and 16.3 ± 4.2 nmol $NO_2^-/10^6$ cells after 6, 14, and 24 h, respectively; mean ± S.D., $n = 6$). Probing for

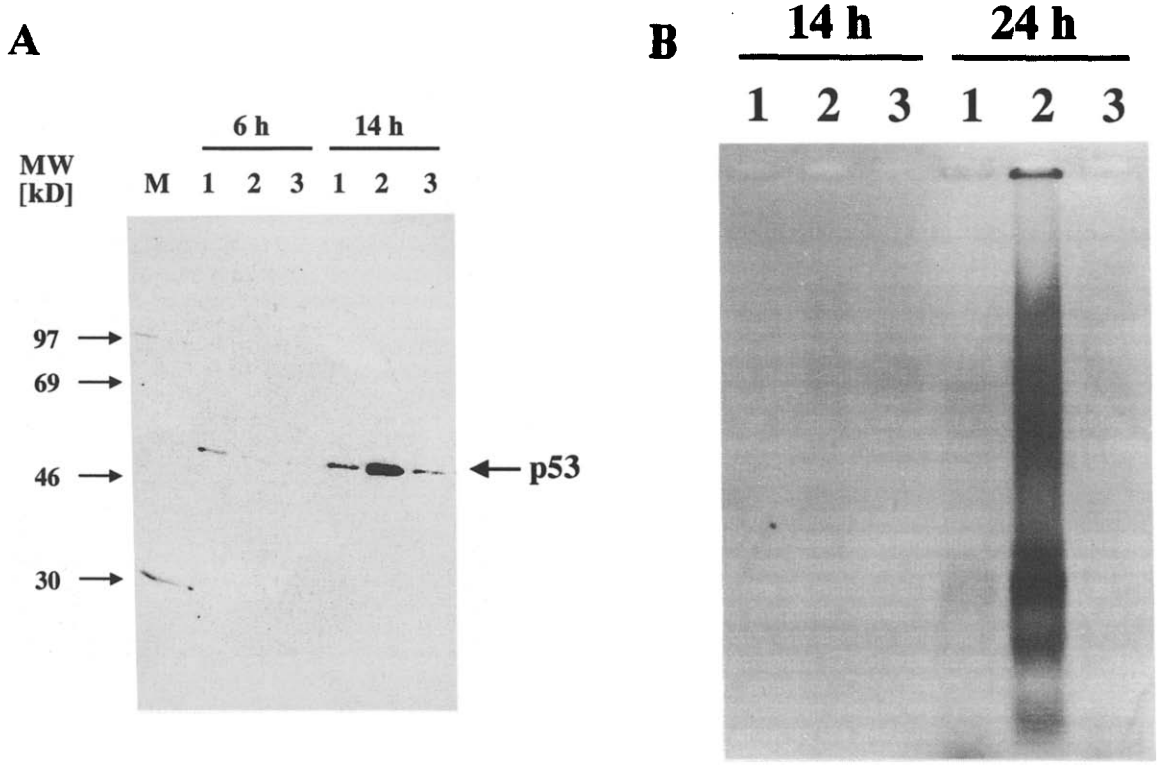

Fig. 1. (A) Immunoprecipitation and Western blotting of p53 from RAW 264.7 macrophages after endogenous NO-production. Cells (2×10^7) were treated for the times indicated with 10 μg/ml LPS and 100 U/ml IFN-γ (lane 2), 10 mg/ml LPS, 100 U/ml IFN-γ and 1 mM N^G-monomethyl-L-arginine (NMMA) (lane 3) or uintreated as a control (lane 1). M indicates ^{14}C-labelled molecular weight markers. The figure is representative of 3 similar experiments. (B) DNA fragmentation in RAW 264.7 macrophages induced by endogenous NO production. 2×10^7 cells were incubated as indicated in A. Cells were lysed and DNA fragments were separated from intact chromatin as described in section 2. The gel is typical of three independent experiments.

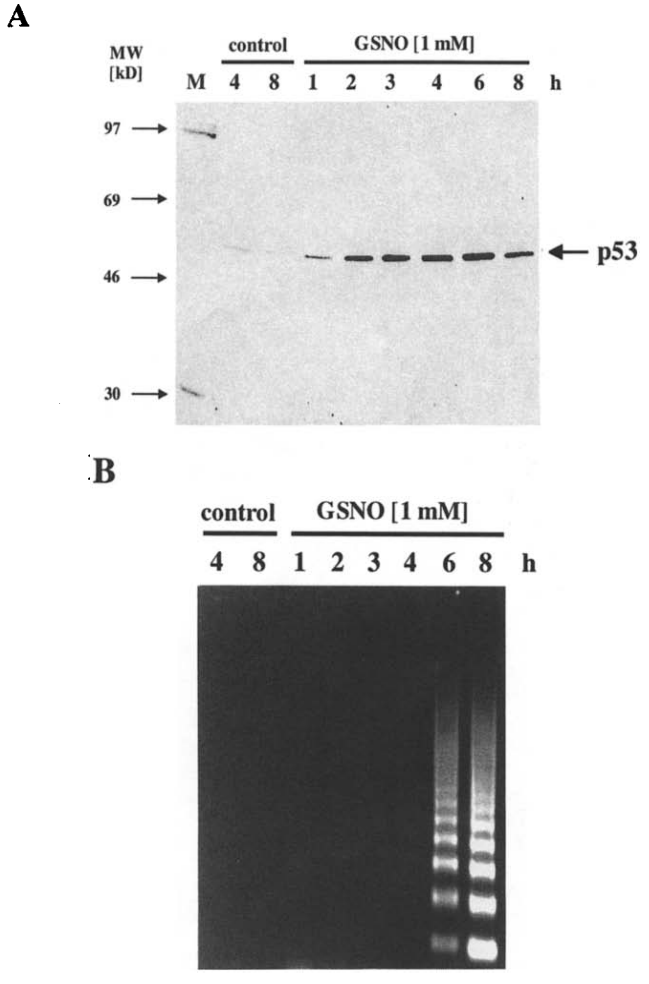

Fig. 2. (A) Time-dependent p53 up-regulation in RAW 264.7 macrophages induced by *S*-nitrosoglutathione (GSNO). 2×10^7 cells were incubated with 1 mM GSNO or vehicle (control) for the times indicated. Other details are as in Fig. 1A. M indicates ^{14}C-labelled molecular weight markers. The blot is typical of three similar experiments. (B) Time-dependent DNA fragmentation in RAW 264.7 macrophages induced by GSNO. 8×10^6 cells were incubated with 1 mM GSNO or vehicle (control) for the times indicated. DNA fragments separated from intact chromatin were visualized by UV transillumination after agarose gel-electrophoresis. Results are representative of three similar assays.

p53 levels in macrophage cell extracts employing immunoprecipitation followed by Western blotting revealed hardly detectable p53 protein levels under control conditions (Fig. 1A). Addition of LPS/IFN-γ caused a significant p53 accumulation within 14 h, which could be linked to NO production, as measured by nitrite generation. To directly prove a role for NO during p53 accumulation, cells were incubated with NMMA, an NO-synthase inhibitor, prior to the exposure to NO-synthase-inducing agents (Fig. 1A). NO production as well as p53 accumulation were inhibited. In LPS/IFN-γ exposed macrophages, death occurred then by apoptosis as juged by morphological parameters, i.e. chromatin condensation and as investigated by the formation of apoptotic DNA-laddering [10] (Fig. 1B).

To test the involvement of NO further, while at the same time excluding any interference of LPS/IFN-γ on p53 accumulation, macrophages were exposed to *S*-nitrosoglutathione (GSNO). The extracellularly applied NO donor decomposes non-enzymatically to release $NO^{\bullet}$ or NO^{+} (nitrosonium ion). Addition of GSNO resulted in marked DNA fragmentation, appearing after 6 h (Fig. 2B). p53 accumulation under these conditions was rapid, starting already 1 h after GSNO application, reaching maximum levels after 4–5 h. In line with experiments described in Fig. 1, p53 expression clearly preceded DNA ladder

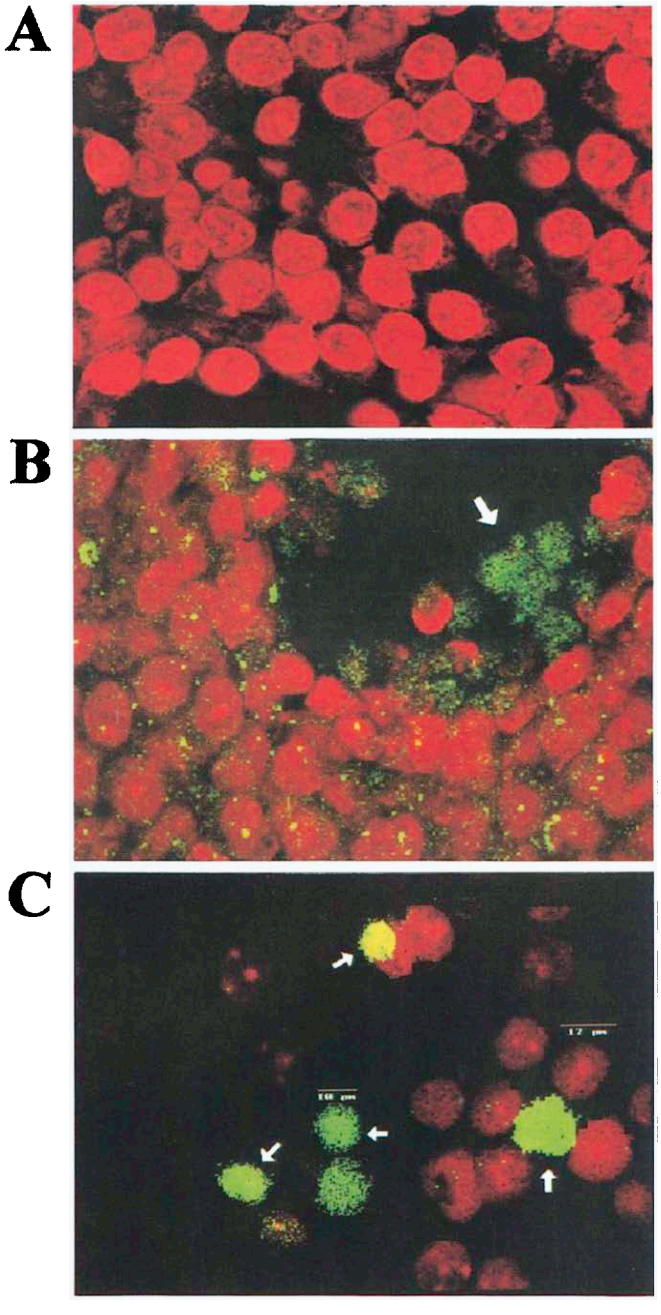

Fig. 3. Nitric oxide-induced p53 up-regulation in RINm5F cells. (A) Cells incubated with medium alone for 72 h, (B) cells exposed to recombinant human IL-1β (10 U/ml) for 72 h, (C) cells treated with 300 μM sodium nitroprusside (SNP) for 3 h. Images are representative of at least 10 separate images collected from different fields in 3 individual experiments. Following IL-1β treatment about 40% of the cells became positive to p53 staining between 48 and 72 h, consistent with our previous findings [11]. About 30% of cells treated with SNP were positive to p53 staining after 3 h. At 9 h 30–40% of all cells had apoptotic bodies.

formation, irrespective of whether NO is endogenously generated or exogenously applied.

IL-1β-induced NO generation in RINm5F cells causes the appearance of the typical apoptotic features, DNA fragmentation, nuclear condensation and apoptotic body formation [11]. Immunohistochemistry now reveals that IL-1β stimulates p53 expression in this cell line (Fig. 3B). p53 positive immunostaining was especially visible in cells located in the center of the colonies, which were in the process of shrinking and forming apoptotic nuclei (Fig. 3B). In untreated cells p53 was visibly absent (Fig. 3A). To ascertain whether NO itself elicited p53 accumulation, we administered the NO-generating compound sodium nitroprusside (SNP). The NO releaser caused increased p53 expression after 3 h (Fig. 3C) and subsequent apoptosis between 6 and 9 h (not shown).

Our observations using RAW 264.7 macrophages and RINm5F cells demonstrate that p53 protein accumulates in response to nitric oxide. An active, cytokine-inducible NO-synthase mediates both apoptosis and p53 accumulation under these conditions. The potential of NO to induce p53 accumulation is evident in different cells, regardless of whether it is formed endogenously after NO-synthase induction or generated by structurally different NO-releasing compounds. Thus, NO is directly responsible for the increased p53 expression. It is apparent that NO can cause DNA damage [24,25], and recent work has indicated that NMDA- and NO-mediated neurotoxicity is associated with the activation of poly(ADP-ribose) synthase [26], a nuclear enzyme activated by DNA strand breaks, in the presence of NAD^+. Following DNA damage, an increased demand for DNA repair is associated with p53 expression. The latter may have the dual role of causing cell cycle arrest via downstream genes [27] and to stimulate DNA repair directly [28]. However, intranuclear p53 accumulation following DNA damage can also be part of the signalling leading to apoptosis either directly acting on the DNA [20] or again by causing cell cycle block [29]. In view of these considerations, our findings are consistent with a mechanism whereby NO-induced DNA damage results in p53 expression and apoptosis. Accumulation of p53 in response to NO, together with previous evidence that NO can cause growth arrest, cell necrosis and has antitumor properties [1], supports the notion that NO may signal different forms of cell death, i.e. necrosis vs. apoptosis. This may depend on the cell type and/or the steady-state concentration of the NO redox species involved.

NO-induced p53 expression, with its potential role in regulating the cell cycle clock and initiating apoptosis, may have the dual role of signalling either growth arrest (supported by various reports that NO inhibits cell proliferation in different systems [30]) or death. Negative and positive modulators of apoptosis may then determine the susceptibility of cells to the toxic insult.

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