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Nitric oxide induced poly(ADP-ribose) polymerase cleavage in RAW 264.7 macrophage apoptosis is blocked by Bcl-2

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Abstract Endogenously generated or exogenously supplied nitric oxide causes cleavage of poly(ADP-ribose) polymerase (PARP) and apoptotic cell death in RAW 264.7 macrophages. With the use of NO donors such as *S*-nitrosoglutathione or spermine-NO we established that PARP digestion occurs in parallel with DNA fragmentation, and is preceded by accumulation of the tumor suppressor gene product p53. PARP cleavage in response to lipopolysaccharide and interferon-γ treatment is prevented by N^G-monomethyl-L-arginine, thus proving a NO requirement. Endogenous NO generation, p53 accumulation, and PARP degradation occurred prior to the detection of significant chromatin condensation. In contrast, in stable Bcl-2 transfected cells, NO-initiated PARP cleavage was almost completely blocked. Our data implicate PARP as a proteolytic substrate during NO-mediated apoptotic cell death in RAW 264.7 macrophages and establish Bcl-2 as an efficient signal terminator in this process.

Key words: Nitric oxide; Apoptosis; PARP cleavage; DNA fragmentation; p53 accumulation; Bcl-2

1. Introduction

Nitric oxide is endogenously produced by constitutive and inducible NO-synthase isoenzymes [1,2]. Biological actions of the molecule, classified by cGMP-dependent and cGMP-independent pathways, are attributed to physiology and pathophysiology. Recent evidence has established that endogenously generated or exogenously supplied NO is a potentially toxic compound. Toxicity of NO can often be described by morphological and biochemical features, characteristic of apoptosis. NO-induced apoptosis has been established for macrophages [3,4], β-cells [5,6], chondrocytes [7], thymocytes [8], and several other systems. Accompanying apoptotic features one finds accumulation of the tumor suppressor gene product p53 as an early indicator of cell damage [9]. Although the apoptogenic action of NO is established, the NO species or the product of its redox chemistry as well as signaling mechanisms accounting for lethal reactions largely remain unknown. Recently, however, we have demonstrated that stable Bcl-2 gene transfer effectively suppressed the cell damaging activity of endogenously generated or exogenously supplied NO in RAW 264.7 macrophages, suggesting that the cell death pathway initiated by NO or its derivatives can be regulated by Bcl-2 family proteins.

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Abbreviations: GSNO, *S*-nitrosoglutathione; LPS, lipopolysaccharide; IFN-γ, interferon-γ; Rbcl2-14, Bcl-2 transfected RAW 264.7 macrophages clone 14; PARP, poly(ADP-ribose) polymerase.

Despite the lack of knowledge about specific NO targets, some features of the death program appear highly conserved among experimental systems and different apoptogens. For example specific proteolytic events have been found to be involved in the process. In this regard interleukin-1β converting enzyme (ICE protease) or other members of the group of proteolytic enzymes, related to the *ced-3* gene product from *Caenorhabditis elegans*, become active during the early stages of apoptosis [10]. Apoptosis-related proteases, when activated, degrade proteins including histones [11], lamins [12], DNA topoisomerases [11], protein kinase C β1, U1 small nuclear ribonucleoprotein [13], and poly(ADP-ribose) polymerase (PARP) [14,15]. Protease inhibitors of different specificities, including serine and cysteine protease inhibitors, have been shown to inhibit apoptosis which illustrates that apoptosis may require the activation of several classes of proteolytic enzymes [16]. An early biochemical event that accompanies apoptosis in many cell types is the proteolytic cleavage of PARP from a 116 kDa polypeptide to a 31 kDa fragment containing the N-terminal DNA binding domain and a 85 kDa polypeptide containing the automodification domain and the NAD^+ binding domain of the protein [14].

It is believed that DNA damage elicits a rapid stress response in mammalian cells, which involves attachment of PARP to the strand breaks and extensive synthesis of short-lived polymers by the bound enzyme. Although PARP has no direct role in DNA excision repair, the enzyme binds tightly to DNA breaks and sometimes interferes with repair if poly (ADP-ribose) synthesis is prevented [17]. However, with massive PARP activation following extensive DNA damage, NAD^+, the ADP-ribose donor, is depleted [18]. In an effort to resynthesize NAD^+, ATP becomes depleted which ultimately leads to cell death due to energy deprivation. With regard to NO signaling, PARP activation followed by energy depletion has been associated with neurotoxicity [19] and NO-mediated islet cell death [20].

In our studies we became interested to define the role of PARP as a potential substrate of ICE-like proteases during NO-mediated apoptotic cell death. We show here that NO-induced apoptosis is accompanied by extensive PARP cleavage, p53 accumulation, and apoptotic cell morphology. Moreover, we provide evidence that Bcl-2 overexpression blocks PARP cleavage in response to NO in RAW 264.7 macrophages.

2. Materials and methods

2.1. Materials

Hoechst dye 33258, protein A sepharose, diphenylamine, and LPS (*E. coli* serotype 0127:B8) were purchased from Sigma (Deisenhofen,

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Germany). [^{125}I]Protein A (10 mCi/mg) was from DuPont New England Nuclear (Dreieich, Germany). The ECL detection reagents were ordered from Amersham (Braunschweig, Germany). Recombinant murine IFN-γ was delivered by Boehringer Mannheim (Mannheim, Germany). The mouse monoclonal anti-PARP antibody (clone C-II-10) was used with the kind permission of Guy Poirier, Department of Molecular Endocrinology, Centre Hospitalier de l'Université Laval Research Center and Laval University (Quebec, Canada) provided by Dr. Alexander Bürkle, German Cancer Research Center (Heidelberg, Germany). RPMI 1640 was ordered from Biochrom (Berlin, Germany). Cell culture supplements and fetal calf serum were from Gibco (Berlin, Germany). All other chemicals were of the highest grade of purity commercially available.

2.2. Cell culture and cell treatment

The mouse monocyte/macrophage cell line RAW 264.7 was maintained in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (complete RPMI). All experiments were performed using complete RPMI. For DNA fragmentation experiments, 4×10^6 cells were seeded into 6-well culture plates (10^6 cells/ml), allowed to adhere overnight, and incubated with the selected substances after the medium was changed. To investigate p53 expression, 2×10^7 cells were cultured and treated in the same way (10 cm Petri dishes, 20 ml/plate). Stimulation of endogenous NO production was achieved with a combination of LPS (10 μg/ml) and IFN-γ (100 U/ml).

2.3. Bcl-2 transfection

For transfection 2×10^6 RAW 264.7 macrophages were seeded into a 10 cm Petri dish. After overnight adhesion, cells were transfected with 20 μg of the plasmid pRC/CMVbcl2 encoding the full-length human *bcl-2* gene under the CMV promoter by using the calcium phosphate precipitation method [21]. Transfected single clones were picked randomly after selection with 400 μg/ml geneticin. Stable Bcl-2 protein expression was assessed by Western blot analysis with an anti-human Bcl-2 specific antibody (clone 124, DAKO, Hamburg, Germany). One clone termed Rbcl2-14 showed the highest level of the Bcl-2 protein. Alternatively, RAW cells were transfected with a control plasmid lacking the *bcl-2* gene (termed Rneo-2).

2.4. GSNO synthesis

GSNO (*S*-nitroso derivative of glutathione) was synthesized, characterized, and used as described previously [9].

2.5. Quantitation of DNA fragmentation

DNA fragmentation was essentially assayed as reported previously [3]. Briefly, after incubation cells were scraped off the culture plates, resuspended in 250 μl 10 mM Tris, 1 mM EDTA, pH 8.0 (TE buffer), and incubated with an additional volume 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 $13\,000\times g$. Pellets were resuspended in 500 μl TE buffer and samples were precipitated by adding 500 μl 10% trichloroacetic acid at 4°C. Samples were pelleted at 4000 rpm for 10 min and the supernatant was removed. After addition of 300 μl 5% trichloroacetic acid, samples were boiled for 15 min. DNA contents were quantitated using the diphenylamine reagent [22]. The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

2.6. Quantitation of apoptotic cells

Macrophages (4×10^5) were grown in 12-well plates. After adhesion, cells were stimulated, followed by fixation onto glass slides with 3% paraformaldehyde for 5 min. Samples were washed with phosphate-buffered saline, 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 percent of total nuclei.

2.7. Nitrite determination

Nitrite, a stable NO oxidation product, was determined using the Griess reaction [23] following a standard procedure [9].

2.8. p53 quantification

p53 was quantified by immunoprecipitation followed by Western blot analysis as described previously [9]. Briefly, cells were scraped off and lysed 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 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 50% (v/v) protein A-sepharose. p53 was immunoprecipitated by adding 200 μl hybridoma supernatant (clone PAb 122, kindly provided by Hans Stahl, Universität des Saarlandes, Homburg, Germany) and 50 μl 50% protein A-sepharose. Immune complexes were 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 once more with 1 ml SNNTE. Finally, samples were resuspended in sample buffer, resolved on SDS-polyacrylamide gels, and blotted onto nitrocellulose sheets. Sheets were washed twice with TBS (140 mM NaCl, 50 mM Tris, pH 7.2) containing 0.1% Tween-20 before blocking non-specific 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 5 times and non-specific 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) [24].

2.9. PARP Western blot analysis

Cell lysis was achieved with lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0) and sonication (Branson sonifier; 10 s, duty cycle 100%, output control 40%), followed by centrifugation ($4000\times g$, 5 min), and Bradford protein determination [25]. Proteins were normalized to 100 μg/lane, resolved on 7.5% polyacrylamide gels and blotted onto nitrocellulose sheets basically following the method as described for p53. Filters were incubated with the mouse anti-PARP antibody (clone C-II-10, hybridoma supernatant, 1:5 in TBS+0.5% milk powder) overnight at 4°C. Detection was by horseradish peroxidase-conjugated goat anti-mouse monoclonal antibodies (1:5000) for 1 h at room temperature using the ECL method (Amersham).

2.10. Statistical analyses

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

3. Results and discussion

We have recently studied the characteristics of NO-mediated cell death in several cellular systems including mouse macrophages [3,9]. In this respect the RAW 264.7 cell system serves as a model system in analogy to mouse peritoneal macrophages [4]. NO-initiated signaling pathways may be, at least in part, extrapolated to other systems like β-cells [9] or thymocytes [26]. Generally, exogenous NO donor application or induction of the NO synthase followed by concomitant NO generation resulted in cell death by apoptosis. RAW 264.7 macrophages respond to *S*-nitrosoglutathione or spermine-NO with a time-dependent increase in p53 protein accumulation and DNA fragmentation, quantitated with the diphenylamine assay (Fig. 1A). Following the addition of NO donors, p53 accumulation clearly preceded DNA fragmentation by several hours. Probing for PARP cleavage in macrophage cell extracts employing Western blotting revealed proteolytic digestion of the 116 kDa holoenzyme into the 85 kDa subunit in response to exogenously supplied NO (Fig. 1B). PARP cleavage was more pronounced with spermine-NO which is in line with a slightly enhanced p53 response and apoptotic DNA fragmentation of raw cells treated with spermine-NO compared to GSNO. The 85 kDa PARP protein, which is indicative of proteolytic holoenzyme digestion, is

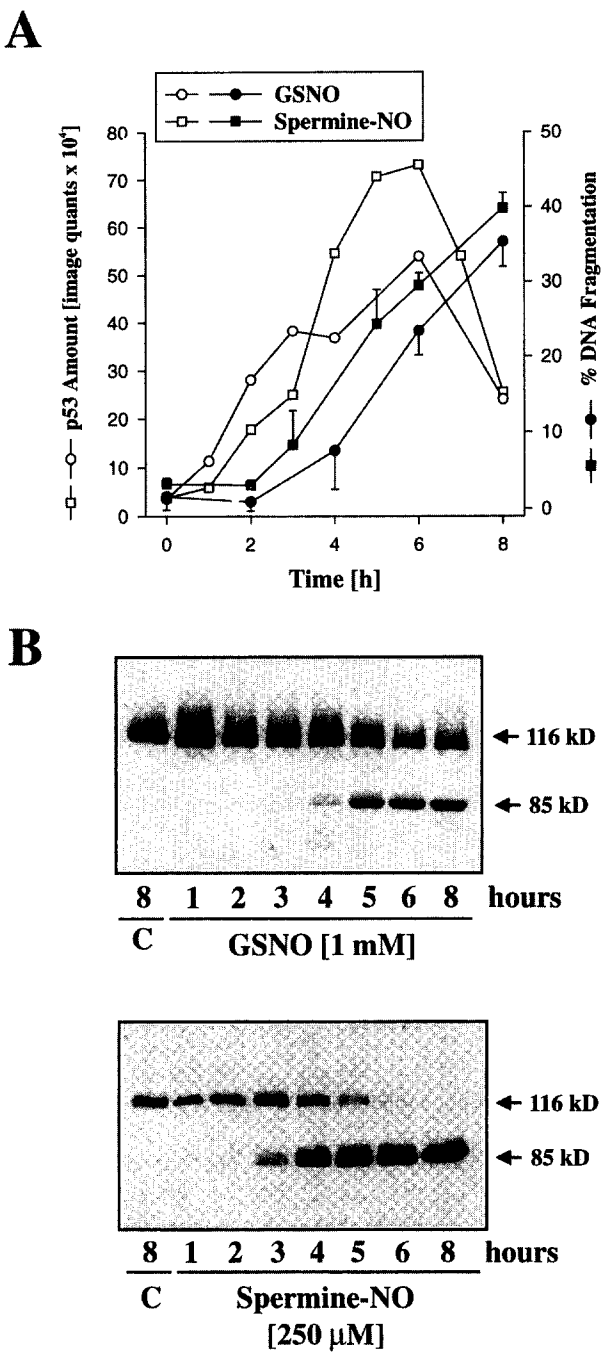

Fig. 1. PARP cleavage, p53 accumulation, and DNA fragmentation in response to NO-releasing compounds. (A) RAW 264.7 macrophages were exposed to 1 mM GSNO or 250 μM spermine-NO for the times indicated. p53 was immunoprecipitated and analyzed by Western blotting as outlined in section 2. The data are representative of three independent experiments. DNA fragmentation was quantitated with the diphenylamine assay (mean values ± standard deviation, $n \geq 3$). (B) Cells (5×10^6) were incubated with 1 mM GSNO or 250 μM spermine-NO for various times. Controls (C) were left untreated for 8 h. PARP (116 kDa holoenzyme or 85 kDa fragment) was analyzed by Western blotting using the monoclonal anti-PARP antibody C-II-10, followed by ECL detection. Blots are representative of three similar experiments.

visible after a 3–4-h incubation period with spermine-NO and a 4–5-h exposure period to GSNO. For both NO-releasing compounds PARP digestion coincided with DNA fragmentation rather than p53 accumulation.

RAW macrophages, stimulated with LPS and IFN-γ as agonists, express high levels of the inducible form of NO-synthase as determined by nitrite accumulation in the cell supernatant (Fig. 2). Nitrite generation is significant after a lag period of 9 h and showed a plateau phase after 20–24 h following agonist addition. LPS/IFN-γ-induced nitrite production was drastically repressed in the presence of the NO-synthase inhibitor N^G-monomethyl-L-arginine (NMMA) (Fig. 2A), applied together with agonists at a concentration of 1 mM. Probing for p53 levels in control macrophages revealed hardly detectable levels of the tumor suppressor (Fig. 2B). Addition of LPS/IFN-γ caused visible p53 accumulation after 12–15 h with a peak of protein detection around 22 h. The NO-synthase inhibitor NMMA completely suppressed the p53 response. In LPS/IFN-γ-exposed macrophages, death occurred by apoptosis as judged by morphological parameters, i.e. chromatin condensation (Fig. 2C). Again cellular apoptosis is prevented by NMMA addition. Furthermore, we tested the involvement of endogenously produced NO in PARP cleavage (Fig. 2D). At 15 h post LPS/IFN-γ addition PARP was partially cleaved as demonstrated by detection of the 85 kDa protein fragment. In contrast, PARP cleavage was suppressed in NMMA-treated cells, thus substantiating the action of endogenously generated NO for PARP digestion in LPS/IFN-γ-treated cells. These data therefore show that analogous to exogenously supplied NO donors, endogenous NO formation caused PARP cleavage which became detectable in parallel with the onset of DNA fragmentation or the appearance of characteristic apoptotic cell morphology.

Taken together, these observations suggest a link between NO-mediated apoptotic signaling and an increase in the cellular ICE or ICE-like protease activities which ultimately result in the proteolytic cleavage of PARP. In the nematode *C. elegans* programmed cell death is dependent on *ced-3*, an ICE-like cysteine protease, and suppressed by *ced-9*, a homolog of the anti-apoptotic mammalian protein Bcl-2 [27]. We therefore focused our investigations around Bcl-2. Stable transfection of Bcl-2 provided macrophages with protection against NO intoxication and apoptotic cell death. To assay the role of Bcl-2 in terms of PARP cleavage, we incubated control-transfected (Rneo-2) and bcl-2 transfected (Rbcl2-14) RAW 264.7 macrophages with GSNO, and then determined the relative levels of the 116 kDa versus the 85 kDa form of the PARP protein. After 4 and 8 h, respectively, PARP becomes degraded in parent and neomycin-control-transfected RAW 264.7 cells (Fig. 3). In contrast, Bcl-2 gene transfer completely abrogated NO-initiated PARP cleavage. In addition to inhibition of PARP proteolysis, Bcl-2 also prevented NO-mediated induction of apoptosis in transformed RAW 264.7 macrophages (Meßmer, Reed, and Brüne, manuscript submitted).

Our data on NO-mediated PARP cleavage extend previous reports demonstrating proteolytic digestion of PARP induced by agonists such as TNF-α and Fas [28]. PARP cleavage to a 85 kDa fragment is an early event in apoptosis and is observed in virtually every form of programmed cell death. Proteolysis results in separation of its DNA binding versus poly (ADP-ribose) polymerase activities. The endonuclease implicated in apoptotic DNA cleavage is negatively regulated by poly-ADP-ribosylation. Therefore, PARP cleavage could result in endonuclease activation in DNA-damaged cells. Our

observations suggest an increase in the intracellular ICE-like protease activities either by elevated expression or by activation as a result of NO-signaling. Although it has been difficult

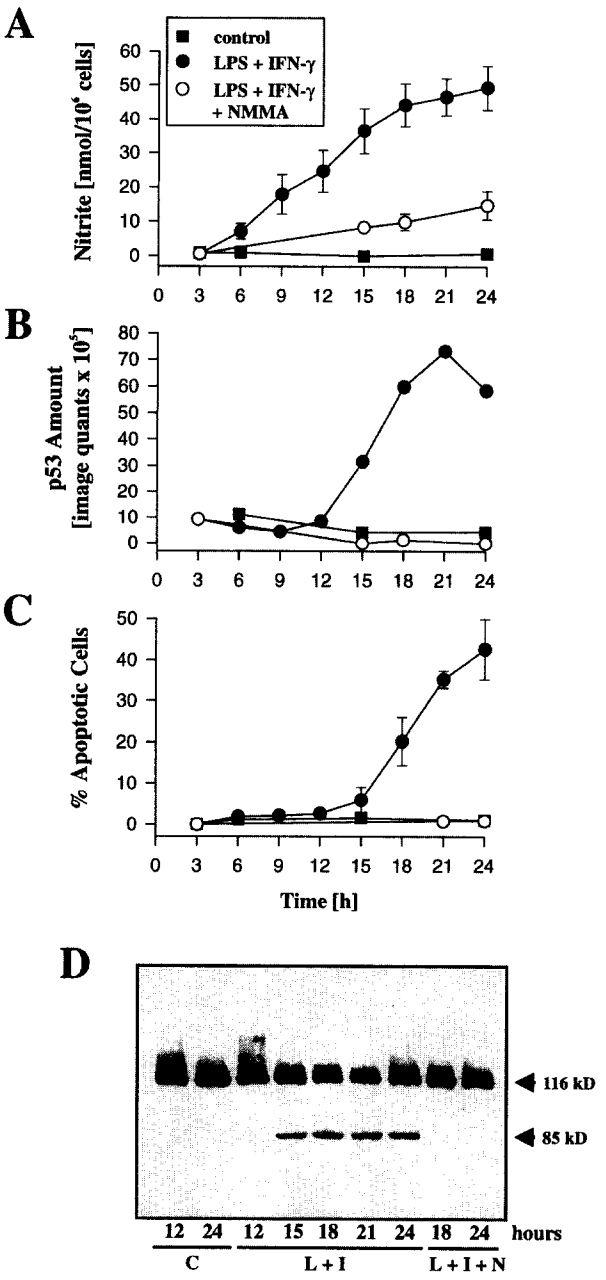

Fig. 2. Endogenous NO production induces PARP cleavage, p53 accumulation, and apoptotic cell death in RAW 264.7 macrophages. (A, C) Macrophages (4×10^5) were seeded into 12-well culture plates and stimulated for various times with vehicle (filled squares), 10 μg/ml LPS and 100 units/ml IFN-γ (filled circles), or 10 μg/ml LPS, 100 units/ml IFN-γ and 1 mM NMMA (open circles). Nitrite was determined with the Griess reaction. Apoptotic cells exhibiting characteristic chromatin condensation were counted by fluorescence microscopy. Values represent means ± standard deviation of four independent experiments. (B) Cells (2×10^7) were stimulated as outlined in (A) and p53 protein was determined as described in Fig. 1. (D) Cells (5×10^6) were exposed to 10 μg/ml LPS and 100 units/ml IFN-γ (L + I), 10 μg/ml LPS, 100 units/ml IFN-γ and 1 mM NMMA (L + I + N), or remained as controls (C) for the times indicated. PARP cleavage was monitored by Western blot analysis. For other details see Fig. 1B.

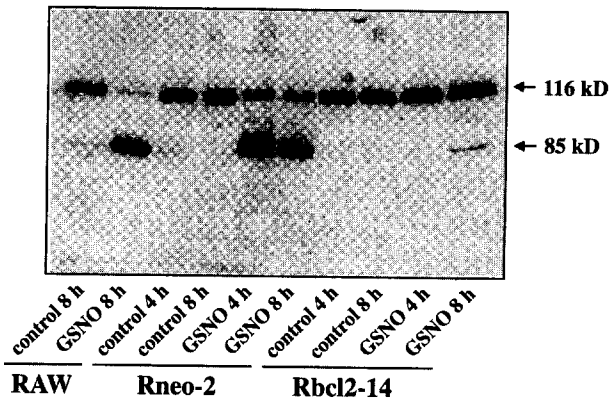

Fig. 3. Bcl-2 blocks PARP cleavage in response to NO donor exposure. RAW 264.7 macrophages (5×10^6), a neomycin-vector control clone (Rneo-2), and a Bcl-2 transfectant clone (Rbcl2-14) were incubated for 4 or 8 h with vehicle (control) or 1 mM GSNO as indicated. Proteins were normalized to 100 μg/lane and PARP protein (116 kDa holoenzyme and 85 kDa fragment) was examined by Western blotting. Details are as in Fig. 1B.

to define exactly the pathophysiological role(s) of PARP, the enzyme appears to be associated with DNA damage-evoked stress responses, as well as with apoptosis [29]. In special cases, such as NO-induced neurotoxicity [19] or pancreatic islet cell lysis [20], massive activation of PARP, and subsequent NAD^+ depletion followed by ATP deprivation may represent a major cause of cell death. Protection against cell killing by the rather non-specific PARP inhibitor 3-aminobenzamide [30] has provided some supportive evidence. In contrast, in most other systems, PARP inhibitors sensitize cells to DNA damage [31]. Thus PARP activation and toxicity is not directly correlative [32]. In RAW 264.7 macrophages, NO-mediated apoptosis neither evokes NAD^+ depletion nor results in ATP deprivation [33]. Moreover, PARP inhibitors are non-protective. In macrophages the death pathway characterized as apoptotic seems to involve PARP cleavage rather than causing its activation. This seems consistent with the idea that apoptosis initiated by NO is an energy requiring process. Although PARP cleavage may be a concomitant of apoptosis, cleaved and inactivated PARP is probably not the cause of apoptosis in this circumstance. Generally, we like to conclude that NO-induced apoptosis activates a common proteolytic pathway that induced PARP digestion in analogy to agonists like TNF-α or Fas. The process of apoptotic PARP cleavage has to be discriminated from death signals which cause PARP activation and NAD^+ depletion.

Considering that PARP cleavage inhibits most of its DNA repair activity [14], we assume that NO-mediated activation of ICE or related proteases may contribute to the demise of the cell. It has been proposed that proteases of the ICE enzyme family are involved in initiating the active phase of apoptosis with inhibition of activated proteases abolishing all manifestations of apoptosis [34]. For NO-mediated apoptosis, PARP cleavage occurs in parallel with the relatively late phase characterized by DNA fragmentation. In contrast, accumulation of p53 appears to be a much earlier marker for the apoptogenic signaling of NO.

NO-initiated apoptosis is effectively blocked by Bcl-2 transfection. Bcl-2 transfection into macrophages also provided protection against NO-evoked DNA fragmentation, although leaving NO-mediated p53 accumulation unaltered (Meßmer,

Reed and Brüne, manuscript submitted). Here we demonstrate that Bcl-2 transfection blocks NO-induced PARP breakdown. It must be concluded that Bcl-2 blocks apoptotic signaling at a step distal to the p53 response and proximal to PARP cleavage or activation of the ICE-related proteases.

Our results encompass the involvement of an ICE-like protease in PARP proteolysis during NO-mediated apoptotic cell death in RAW 264.7 macrophages. In the RAW cell line, and presumably as a general mechanism apoptotic signaling initiated by NO promotes PARP breakdown in parallel with DNA cleavage. NO-initiated signal transducing mechanisms resulting in proteolytic digestion of proteins such as PARP are efficiently antagonized by the anti-apoptotic protein Bcl-2.

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