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Protease activation during nitric oxide-induced apoptosis: comparison between poly(ADP-ribose) polymerase and U1-70kDa cleavage

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Abstract

Nitric oxide (NO) promotes apoptotic cell death in the mouse macrophage cell line RAW 264.7 and in the human promyelocytic leukaemia cell line U937, which exemplifies p53-dependent and p53-independent executive death pathways. Here, we followed the cleavage of two caspase substrates during NO-intoxication, assaying poly(ADP-ribose) polymerase and U1-70kDa small ribonucleoprotein (U1-70kDa) degradation. By using pharmacological inhibitors, we found that *Z*-aspartyl-2,6-dichlorobenzoyloxymethylketone (*Z*-Asp-CH_2-DCB; 100 μM), a caspase-like protease inhibitor, completely blocked *S*-nitrosoglutathione (GSNO)-induced apoptosis in both RAW 264.7 and U937 cells ($IC_{50} = 50$ μM for RAW 264.7 macrophages vs. $IC_{50} = 33$ μM for U937 cells). Notably, a characterized caspase-3 (Ac-DEVD-CHO) inhibitor left NO-induced DNA fragmentation and the appearance of an apoptotic morphology unaltered, although completely blocking caspase-3 activity. However, *Z*-Asp-CH_2-DCB suppressed protease-mediated U1-70kDa cleavage and DNA fragmentation in parallel. In contrast, poly(ADP-ribose) polymerase cleavage in U937 cells was only delayed by *Z*-Asp-CH_2-DCB, while poly(ADP-ribose) polymerase digestion in RAW 264.7 macrophages proceeded unaltered. We further compared U1-70kDa and poly(ADP-ribose) polymerase cleavage in stably Bcl-2 transfected RAW 264.7 macrophages. Rbcl2-2, a Bcl-2 overexpressing clone, suppressed DNA fragmentation and U1-70kDa digestion in response to GSNO, although allowing delayed but complete poly(ADP-ribose) polymerase degradation. Conclusively, poly(ADP-ribose) polymerase cleavage not causatively coincided with the appearance of other apoptotic parameters. Our results suggest that NO-induced apoptosis demands a *Z*-Asp-CH_2-DCB inhibitable caspase activity, most likely distinct from caspase-3 and caspase-1. NO-mediated executive apoptotic signaling results in U1-70kDa and poly(ADP-ribose) polymerase cleavage. Whereas U1-70kDa digestion closely correlates to the occurrence of apoptotic parameters such as DNA fragmentation or an apoptotic morphology, poly(ADP-ribose) polymerase-breakdown does not.

Keywords: Apoptosis; Bcl-2; ICE-like protease; Nitric oxide (NO); Poly(ADP-ribose) polymerase

1. Introduction

The molecule nitric oxide (NO) received wide attention as an essential ubiquitous biological messenger in vertebrates (Nathan, 1992). NO is synthesized from L-arginine by a family of constitutive or cytokine-inducible NO synthase isoenzymes. The inducible-type NO synthase isoform is expressed in macrophages, astrocytes, mesangial cells, and many other cellular system. Its activation represents a long-lasting, high-output NO generating system (Nathan and Xie, 1994). Severe medical-related symptoms like sepsis or type I diabetes mellitus are causative-linked to a massive NO production (Änggard, 1994; Moncada and Palmer, 1991). In turn, cell destructive mechanisms and NO cytotoxicity can be ascribed at least in part by apoptotic parameters (Meßmer et al., 1995). Apoptosis, in contrast to necrosis, meets a well-controlled executive signaling cascade, thus considering cell death as a highly ordered biological process (Hale et al., 1996). A hallmark of apoptosis is endonuclease-mediated internucleosomal cleavage of nuclear DNA. Although endonuclease activation is initiated by many apoptotic inducers and DNA laddering is widely used as an indicator of apoptotic cell death, DNA fragmentation is not compulsorily linked to the apoptotic process. This is obvious in experiments

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demonstrating apoptotic morphology in the absence of internucleosomal DNA cleavage (Oberhammer et al., 1993) or in cytoplasts lacking nuclei (Schulze-Osthoff et al., 1994).

Besides DNA cleavage, recent experimental interests focused on intracellular protease activation as the final executive step during the death programme. Protease activation seems required for the cellular reorganization that occurs during apoptosis and represents a universal, irreversible, and final end point (Patel et al., 1996). Potential proteases implicated in the degradation of various substrates in apoptotic cells embrass serine proteases such as granzymes and above all caspase enzymes, commonly known as Ced-3/interleukin 1-β converting enzyme (ICE)-like isoenzymes (Fraser and Evan, 1996; for review). The mammalian cysteine protease caspase-1/ICE is the prototype apoptotic protease identified by its homology to the *Caenorhabditis elegans* cell death gene Ced-3. In the last two years, several Ced-3/ICE homologues have been identified. The Ced-3/ICE subfamilies subsumes the ICE subfamily consisting of caspase-1 (ICE), caspase-4 (TX/ICH-2/ICE_{rel}II), and caspase-5 (ICE_{rel}III/TY); the Nedd-2 subfamily, Nedd-2 and caspase-2 (ICH-1); and the CPP32 subfamily which consists of caspase-3 (CPP32/YAMA/apopain), caspase-6 (Mch2), caspase-7 (ICE-LAP3/Mch3/CMH1), caspase-9 (ICE-LAP6/Mch6), and Ced3 (Hale et al., 1996, for references). All caspase-1/caspase-3 protease family members are synthesized as pro-proteins that require proteolytic processing to generate active, heterodimeric enzymes. Several recent reports suggested that the proteolytic cleavage of caspase-3/CPP32 may be functionally important in TNF-α, Fas, staurosporine, and other agonist-induced apoptosis (Schlegel et al., 1996). Moreover, protease cascades have been identified in apoptotic signaling, suggesting sequential activation of caspase-1/ICE-like and caspase-3/CPP32-like proteases (Enari et al., 1996).

The identification of cellular substrates for caspase-1/ICE-related proteases is an important step in understanding the role of proteolytic digestion during apoptosis. The best characterized substrate reported for many caspases, except caspase-1/ICE itself, is the nuclear enzyme poly(ADP-ribose) polymerase. Proteolytic cleavage of poly(ADP-ribose) polymerase 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 accompanies apoptosis in many cells (Kaufmann et al., 1993). Other proteolytic targets, among others, are lamin A (cleaved by caspase-6/Mch2) (Orth et al., 1996a), U1-70kDa small ribonucleoprotein (U1-70kDa) (cleaved by caspase-3/CPP32) (Casciola-Rosen et al., 1996), and the catalytic subunit of DNA-dependent protein kinase (cleaved by caspase-3/CPP32) (Song et al., 1996).

In previous works, we investigated NO-induced apoptotic cell death in RAW 264.7 macrophages (Meßmer et al., 1995; Meßmer and Brüne, 1996a) and underscored the role of p53 for apoptotic signaling (Meßmer et al., 1994; Meßmer and Brüne, 1996b). Focusing on protease activation which occurred concomitant to apoptotic DNA fragmentation, NO-signaling caused cleavage of the 116 kDa nuclear enzyme poly(ADP-ribose) polymerase (Meßmer et al., 1996b), thereby implying the involvement of caspases in NO-mediated cell death. We further established that Bcl-2 overexpression efficiently abrogated NO-induced apoptosis (Meßmer et al., 1996a), although leaving the p53-response unaltered.

In our present study we further examined the role of ICE/Ced-3 proteases in NO-mediated cell death. Our data make the involvement of cysteine proteases most likely. The proteases seem to be distinct from caspase-3 and caspase-1 but reveal a high sensitivity to Z-Asp-CH_2-DCB, a caspase-like protease inhibitor (Mashima et al., 1995b). One likely substrate for the proteases is the U1-70kDa protein. We further discuss that poly(ADP-ribose) polymerase digestion may not causatively linked to the initiation and propagation of NO-induced apoptosis, because poly(ADP-ribose) polymerase cleavage and the occurrence of other apoptotic parameters are separately regulated.

2. Materials and methods

2.1. Materials

Hoechst dye 33258, diphenylamine, *N*-α-tosyl-L-arginine methyl ester (TLCK), *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma (Deisenhofen, Germany). *N*-acetyl-aspartyl-glutamyl-valinyl-aspartyl-aldehyde (Ac-DEVD-CHO) was from Biomol (Hamburg, Germany). *N*-acetyl-tyrosynyl-valinyl-alanyl-aspartyl-aldehyde (Ac-YVAD-CHO), *Z*-aspartyl-2,6-dichlorobenzoyloxymethylketone (*Z*-Asp-CH_2-DCB), and *N*-acetyl-aspartyl-glutamyl-valinyl-aspartyl-7-amino-4-coumarin (Ac-DEVD-AMC) were delivered by Bachem (Heidelberg, Germany). ECL detection reagents were ordered from Amersham (Braunschweig, Germany). The mouse monoclonal anti-poly(ADP-ribose) polymerase antibody (clone C-II-10) was used with the kindly 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). The mouse monoclonal anti-U1 small nuclear ribonucleoprotein antibody was kindly donated by Antony Rosen, Department of Medicine, John Hopkins University School of Medicine (Baltimore, MD, USA). 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 and the human promyelocytic leukaemia cell line U937 were 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), and incubated with the selected substances the following day. For Western blot analysis, 5×10^6 cells were cultured and treated in the same way (10 cm Petri dishes, 10 ml/plate).

2.3. Bcl-2 transfection

RAW 264.7 macrophages were transfected with the plasmid pRc/CMVbcl2 encoding the full-length human *bcl-2* gene under the CMV promoter essentially as described (Meßmer et al., 1996a). Clone Rbcl2-14 showed the highest and clone Rbcl2-2 the lowest 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 as described previously (Meßmer et al., 1994; Hart, 1985). Briefly, glutathione was dissolved in HCl at 4°C prior to the addition of $NaNO_2$. The mixture was stirred at 4°C for 40 min followed by the addition of 2.5 volumes of acetone. Precipitates were filtered, washed with acetone and diethylether, and dried under vacuum. GSNO was characterized by HPLC (high-pressure liquid chromatography) analysis and UV spectroscopy.

2.5. Quantitation of DNA fragmentation

DNA fragmentation was essentially assayed as reported previously (Meßmer et al., 1995). Briefly, after incubation cells were scraped off the culture plates, resuspended in 250 μl 10 mM Tris, 1 mM EDTA, pH 8.0 (Tris/EDTA [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 (Burton, 1956). 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. DNA agarose gel electrophoresis

For the preparation of 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 volumes ice-cold ethanol and 50 μl 5 M NaCl at −20°C, centrifuged at $13\,000 \times g$ for 15 min followed by an incubation of the pellet in 500 μl TE-buffer supplemented with 100 μg/ml RNase A at 37°C for 30 min. Samples were extracted with phenol:chloroform:isoamylalcohol (25:24:1) and once again with chloroform:isoamylalcohol (24:1). DNA was precipitated, and pellets were recovered by centrifugation ($13\,000 \times g$, 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.

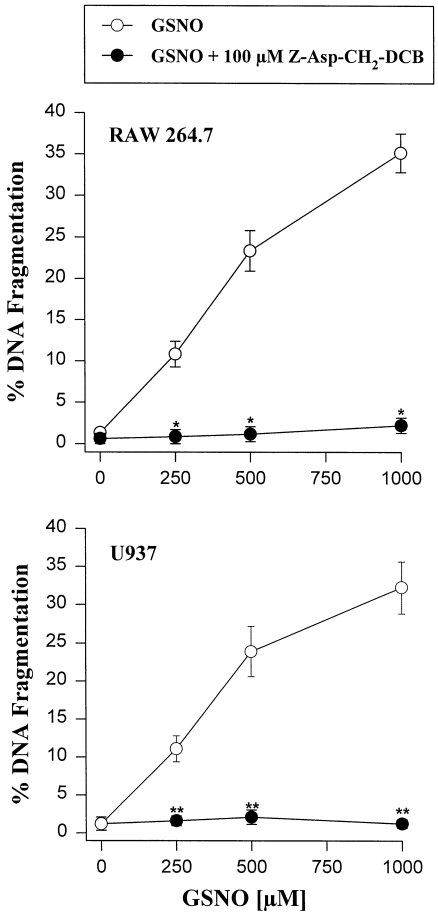

Fig. 1. Caspase involvement in NO-induced apoptotic DNA fragmentation. RAW 264.7 macrophages (4×10^6; upper panel) or U937 cells (4×10^6, lower panel) were incubated for 8 h with increasing GSNO concentrations in the presence of the caspase-like protease inhibitor *Z*-Asp-CH_2-DCB (100 μM), which was added 1 h prior to the addition of GSNO. DNA fragmentation was quantitated by the diphenylamine reaction. Values are means ± S.E.M. of four individual experiments. $* P \leq 0.01$ and $** P \leq 0.001$ vs. GSNO.

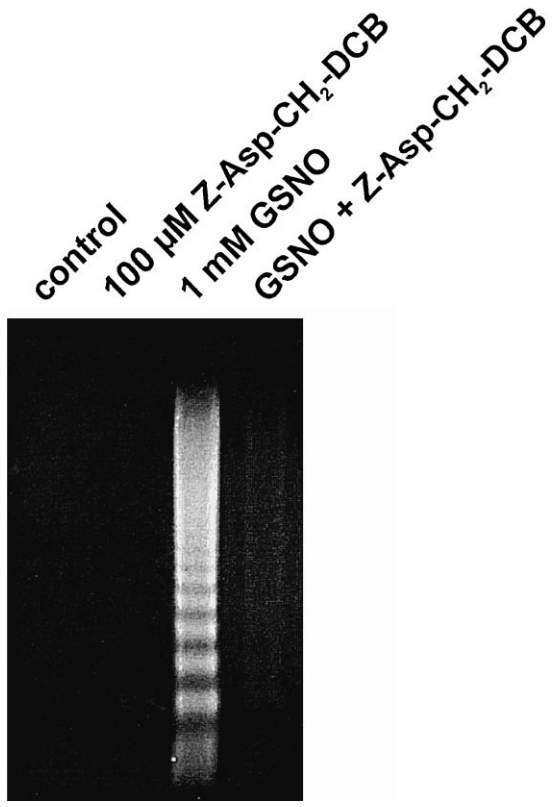

Fig. 2. Modulation of oligonucleosomal DNA fragmentation by various protease inhibitors. U937 cells (8×10^6 cells) were cultured as outlined in Section 2 and treated for 8 h with 1 mM GSNO or vehicle (control) in the presence of 100 μM *Z*-Asp-CH_2-DCB as indicated. Protease inhibitor were preincubated for 1 h. DNA fragments were isolated, resolved on 1% agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

2.7. Morphological investigations

RAW 264.7 macrophages and U937 cells (4×10^5) were grown in 12-well culture plates. Cells were stimulated, followed by fixation with 3% paraformaldehyde for 5 min onto glass slides. 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.

2.8. Caspase-3 enzyme activity

For detection of caspase-3 activity, RAW 264.7 macrophages (3×10^6 cells) were incubated for 5 h with 500 μM GSNO in the absence or presence of caspase inhibitors and lysed in lysis buffer (10 mM Tris/HCl, 0.32 M sucrose, 5 mM EDTA, 1% Triton X-100, 1 mM phenymethanesulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM DTT, pH 8.0) for 30 min. Following sonication (10 s, output control 1) lysates were centrifuged ($10\,000\times g$, 5 min, 4°C) and stored at −80°C. Protein determinations were performed with the Bradford method. Caspase-3 activity was detected by measuring the proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC. Cell lysates (50 μg protein) were incubated in 100 mM HEPES, 10% sucrose, 0.1% CHAPS, pH 7.5, 1 mM phenymethanesulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM DTT at 37°C with 12 μM DEVD-AMC in a total volume of 700 μl. Substrate cleavage and AMC accumulation was followed fluorometrically with excitation at 380 nm and emission at 460 nm.

2.9. Poly(ADP-ribose) polymerase and U1 small nuclear ribonucleoprotein Western blot analysis

5×10^6 cells were cultured and incubated as described. Cell lysis was achieved with lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM phenymethanesulfonyl fluoride, 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 (Bradford, 1976). Proteins were normalized to 100 μg/lane, resolved on 7.5% 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 unspecific binding with TBS/2% BSA. Filters were incubated with the mouse anti-poly(ADP-ribose) polymerase antibody (clone C-II-10, hybridoma supernatant, 1:5 in TBS + 0.5% milk powder) overnight at 4°C. Nitrocellulose sheets were washed 5 times and unspecific binding was blocked as described. Detection was by horseradish peroxidase-conjugated goat anti-mouse monoclonal antibodies (1:5000) for 1 h at room temperature using the enhanced chemiluminescence (ECL) method (Amersham).

2.10. p53 Western blot

Cell lysis was achieved as described for poly(ADP-ribose) polymerase and U1-70kDa, followed by centrifuga-

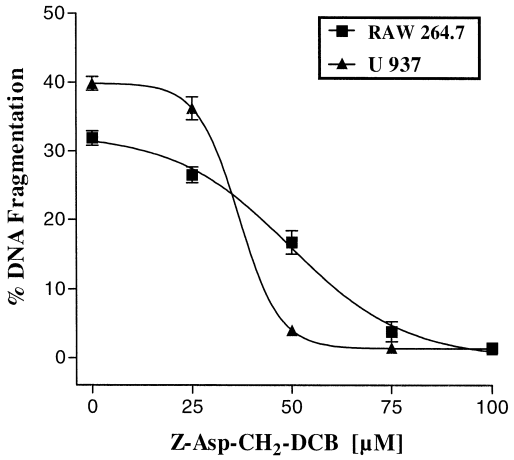

Fig. 3. Concentration-dependent inhibition of apoptotic DNA fragmentation by *Z*-Asp-CH_2-DCB. RAW 264.7 macrophages (4×10^6) and U937 cells (4×10^6) were stimulated for 8 h with 1 mM GSNO in the absence or presence of increasing *Z*-Asp-CH_2-DCB concentrations. *Z*-Asp-CH_2-DCB was added 1 h before GSNO. DNA fragmentation was quantitated using the diphenylamine reaction. Values are means ± S.E.M. of three similar experiments.

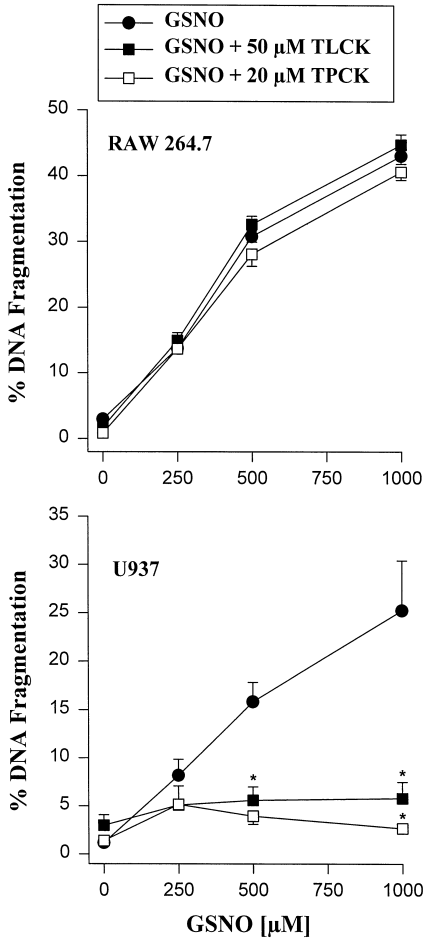

Fig. 4. Variable protection by TLCK and TPCK on GSNO-induced apoptosis in RAW 264.7 macrophages and U937 cells. RAW 264.7 (4×10^6) or U937 (4×10^6) cells were cultured as outlined in Section 2 and incubated for 8 h with increasing GSNO concentrations in the presence of TLCK (50 μM) or TPCK (20 μM). Protease inhibitors were supplemented 1 h before GSNO. Other details are as in Fig. 1. Results are means ± S.E.M. of three to four individual experiments. $* P \leq 0.05$ vs. GSNO.

tion ($4000 \times g$, 5 min), and protein determination with the Bradford method. Proteins were normalized to 300 μg/lane and resolved on 10% polyacrylamide gels and blotted onto nitrocellulose sheets basically following the method as described for poly(ADP-ribose) polymerase. Filters were incubated with the monoclonal anti-p53 antibody PAb122 (hybridoma supernatant; 1:6 in TBS/0.5% milk powder) overnight at 4°C. Detection was by horseradish peroxidase-conjugated goat anti-mouse monoclonal antibodies as described. Video densitometry quantification was performed with a Vilber Lourmat BIO1D V 6.02c.

2.11. 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

3.1. Protease inhibitors during GSNO-induced apoptosis

In the present study, we elucidated the role of ICE-like and trypsin/chymotrypsin-like proteases during NO-induced apoptotic signaling by pharmacological intervention. To investigate NO signaling irrespective of NO synthase induction, NO donors such as the *S*-nitroso-derivative of glutathione (GSNO) were used to convey the apoptotic signal in the mouse macrophage cell line RAW 264.7 and the human promyelocytic cell line U937. Apoptosis was assessed by quantitative, diphenylamine-based, oligonucleosome-length DNA fragmentation and conventional agarose gel electrophoresis. First, we examined caspase inhibitors such as Ac-YVAD-CHO, which is highly specific for caspase-1/ICE ($K_i = 0.76 \pm 0.16$ nM; Thornberry et al., 1992), Ac-DEVD-CHO which blocks caspase-3/CPP32 ($IC_{50} = 0.2$ nM; Nicholson et al., 1995), and *Z*-Asp-CH_2-DCB which was characterized as a caspase-like protease inhibitor (Mashima et al., 1995b). GSNO dose-dependently elicited oligonucleosomal DNA cleavage within 8 h in both, RAW 264.7 and U937 cells. Following GSNO (1 mM) exposure, DNA fragmentation amounted to values around 35% (Figs. 1 and 2). As demonstrated in Figs. 1 and 2, 100 μM *Z*-Asp-CH_2-DCB totally blocked GSNO-induced apoptosis. Based on morphological criteria, similar results were obtained (data not shown). In contrast,

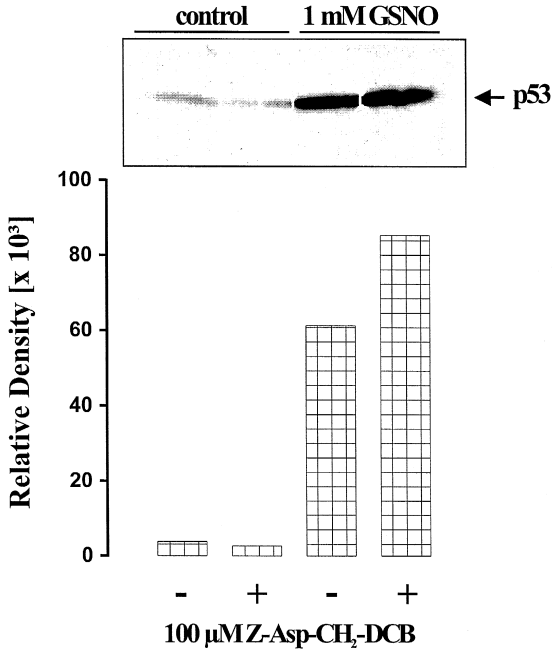

Fig. 5. Influence of *Z*-Asp-CH_2-DCB on NO-induced p53 accumulation in RAW 264.7 macrophages. RAW 264.7 macrophages (5×10^6) were incubated for 4 h with 1 mM GSNO in the absence or presence of 100 μM *Z*-Asp-CH_2-DCB as indicated. Thereafter, cells were lysed, proteins were normalized to 300 μg/lane and separated by SDS-PAGE. p53 protein was determined by Western blot using the monoclonal p53 antibody PAb122 and ECL detection followed by video densitometry. The blot is representative of three similar experiments.

RAW 264.7 macrophages and U937 cells, preincubated for 1 h with 200 μM of the caspase-1 or caspase-3-specific inhibitors Ac-YVAD-CHO/Ac-DEVD-CHO followed by an 8-h exposure to 1 mM GSNO, revealed an unaltered nuclear morphology and DNA fragmentation response. Neither Ac-YVAD-CHO nor Ac-DEVD-CHO suppressed NO-mediated apoptosis (data not shown). In order to verify whether Ac-DEVD-CHO blocked caspase-3 activity in intact cells, we incubated RAW 264.7 macrophages for 5 h with 500 μM GNSO in the absence or presence of the inhibitor and determined caspase-3 activity in the cytosol afterwards. Control cytosols revealed a basal caspase-3 activity of 0.1 nM AMC/min. Treatment with 500 μM GSNO promoted the release of 3 nM AMC/min, whereas a 1-h preincubation with 50 μM Ac-DEVD-CHO totally abrogated GSNO-stimulated caspase-3 activity (0.1 nM AMC/min). Also, a 1-h preincubation with 100 μM *Z*-Asp-CH_2-DCB prevented activation of caspase-3.

We further examined the action of *Z*-Asp-CH_2-DCB and determined its inhibitory dose–response effects. Apoptotic DNA cleavage in response to 1 mM GSNO was suppressed by *Z*-Asp-CH_2-DCB in RAW 264.7 macrophages and in U937 cells in a comparable concentration range ($IC_{50} = 50$ μM for RAW 264.7 macrophages vs. $IC_{50} = 33$ μM for U937 cells) (Fig. 3).

In U937 cells, several lines of evidence implicated the involvement of serine proteases in the initiation of apoptosis (Kumar and Harvey, 1995; Ghibelli et al., 1995). In order to test whether this applies to NO-induced apoptosis as well, we used TLCK, a trypsin-like protease inhibitor, and TPCK, which preferentially inhibits chymotrypsin-like proteases. In control experiments, TLCK and TPCK proved to be non-toxic at their effective concentrations. A 1-h preexposure with 50 μM TLCK or 20 μM TPCK significantly prevented GSNO-induced DNA cleavage in U937 cells (Fig. 4). In contrast, NO-initiated apoptosis in RAW 264.7 macrophages was unaffected by TLCK or TPCK (Fig. 4, upper panel).

3.2. Z-Asp-CH_2-DCB leaves NO-induced p53 accumulation unaltered

In previous studies, we demonstrated NO-mediated accumulation of the nuclear phosphoprotein p53 in RAW 264.7 macrophages (Meßmer et al., 1994). Most characteristic, p53 up-regulation clearly preceded subsequent apoptotic DNA laddering. To exclude a potential *Z*-Asp-CH_2-DCB sensitive target in RAW 264.7 cells upstream of p53 protein accumulation, we evaluated GSNO-induced p53 expression in the absence and presence of the protease

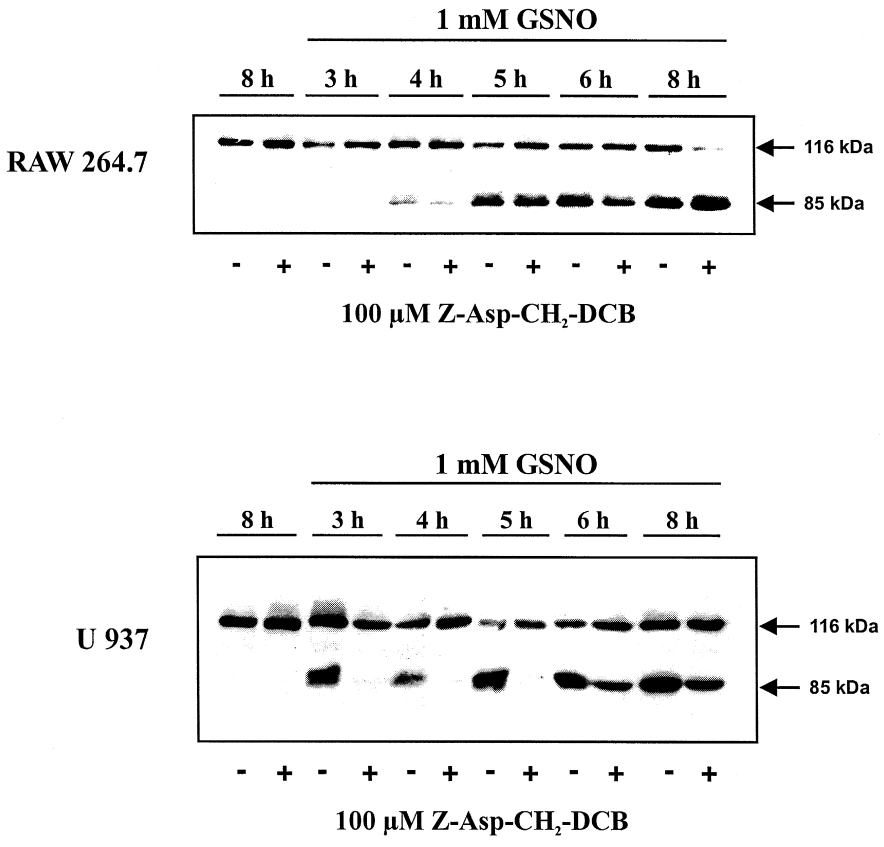

Fig. 6. Modulation of GSNO-induced poly(ADP-ribose) polymerase cleavage in RAW 264.7 and U937 cells by *Z*-Asp-CH_2-DCB addition. RAW cells (upper panel) and U937 cells (lower panel) were exposed to 1 mM GSNO in the absence (−) or presence (+) of 100 μM *Z*-Asp-CH_2-DCB for times indicated. Poly(ADP-ribose) polymerase cleavage (116 kDa holoenzyme and 85 kDa cleavage fragment) was monitored by Western blot analysis using the monoclonal anti-poly(ADP-ribose) polymerase antibody C-II-10 followed by ECL detection. The blots are representative of three similar experiments.

inhibitor. GSNO elicited a rapid p53 response within 4 h (Fig. 5), which was unaltered in the presence of *Z*-Asp-CH_2-DCB. Therefore, *Z*-Asp-CH_2-DCB inhibits NO-induced apoptosis in RAW 264.7 macrophages downstream of pathways promoting p53 accumulation.

3.3. Interference of Z-Asp-CH_2-DCB during poly(ADP-ribose) polymerase and U1-70kDa cleavage

Z-Asp-CH_2-DCB was supposed to show selectivity towards caspase-1 or other caspases (Mashima et al., 1995b). Previous examinations from our own laboratory revealed poly(ADP-ribose) polymerase digestion in RAW 264.7 macrophages to occur in parallel to DNA fragmentation following GSNO treatment (Meßmer et al., 1996b). As poly(ADP-ribose) polymerase represents the most prominent caspase-3-like protease substrate, we questioned a possible *Z*-Asp-CH_2-DCB interference with the protease activity. Western blot analysis in RAW 264.7 macrophage cell extracts revealed proteolytic cleavage of the 116-kDa poly(ADP-ribose) polymerase holoenzyme into the 85-kDa degradation fragment in response to GSNO (Fig. 6). Poly(ADP-ribose) polymerase digestion starts 4 h after GSNO addition and timely parallels apoptotic DNA fragmentation (Meßmer et al., 1996b). Similar results were obtained in U937 cells, with the exception that poly(ADP-ribose) polymerase proteolysis was more rapid than in RAW 264.7 macrophages. Surprisingly, GSNO-mediated poly(ADP-ribose) polymerase digestion in the presence of *Z*-Asp-CH_2-DCB was unaltered in RAW 264.7 macrophages, whereas poly(ADP-ribose) polymerase cleavage in U937 cells was delayed, but not blocked. In U937 cells, poly(ADP-ribose) polymerase digestion in the presence of *Z*-Asp-CH_2-DCB starts 6 h post-GSNO addition (Fig. 6, lower panel) although DNA fragmentation and morphological alterations were completely blocked during an entire 8-h incubation period.

With the notion that poly(ADP-ribose) polymerase cleavage and DNA fragmentation were differently affected by *Z*-Asp-CH_2-DCB, we searched for another putative apoptotic caspase substrate. Casciola-Rosen et. al. (Casciola-Rosen et al., 1994) recently reported on the

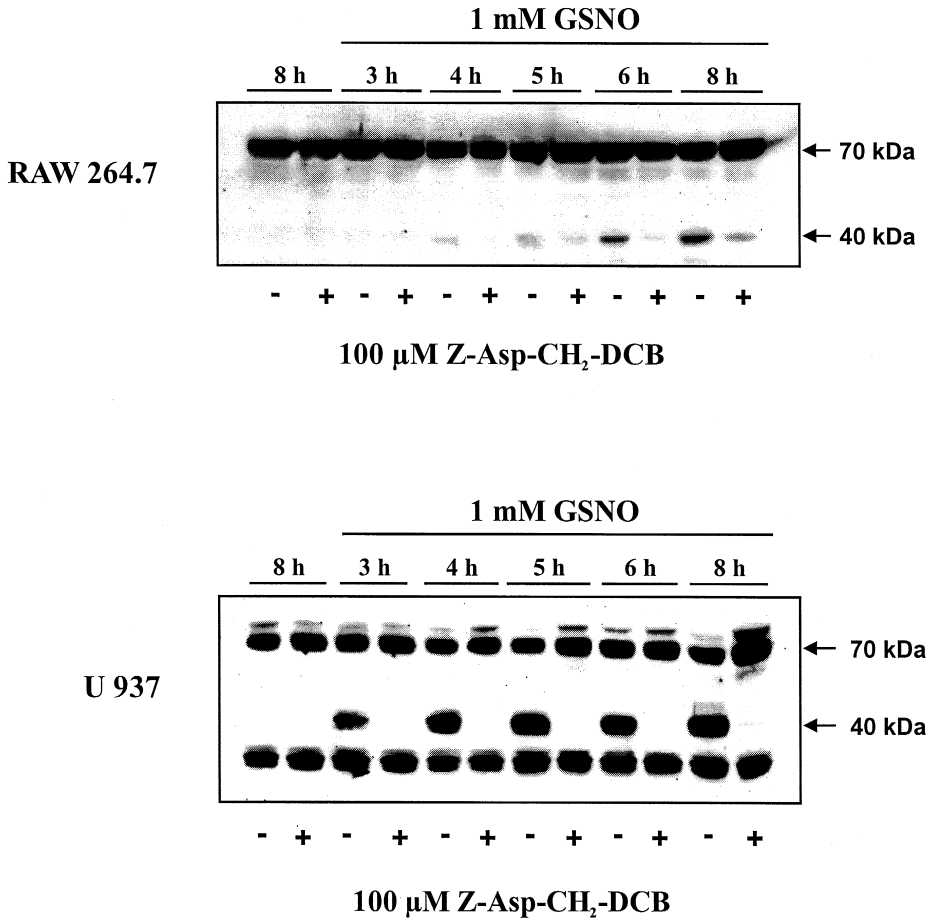

Fig. 7. Prevention of NO-induced U1-70kDa cleavage by *Z*-Asp-CH_2-DCB. RAW 264.7 macrophages (upper panel) and U937 cells (lower panel) were treated with 1 mM GSNO in the absence (−) or presence (+) of 100 μM *Z*-Asp-CH_2-DCB for times indicated. Cell lysates (equal amounts of proteins, 100 μg/lane) were separated by SDS-PAGE and analyzed by Western blotting using a U1-70kDa-reactive antiserum as described under Section 2. The arrow designated '40 kDa' indicates the 40 kDa cleavage product of the U1-70kDa component of the U1 small ribonucleoprotein. The blots are representative of three similar experiments.

proteolytic digestion of the 70-kDa protein of the U1 small ribonucleoprotein (U1-70kDa) during apoptosis. Suggestively, cleavage is mediated by a protease with a chemical caspase-like inhibitor profile. To search for U1-70kDa protein cleavage, we treated RAW 264.7 macrophages and U937 cells with GSNO for up to 8 h, prepared cell lysates, and analyzed for the integrity of the U1-70kDa protein by Western blotting. The time course of U1-70kDa cleavage paralleled poly(ADP-ribose) polymerase digestion in both RAW 264.7 macrophages and U937 cells (Fig. 7). As documented in Fig. 7, U1-70kDa protein cleavage was potently suppressed by *Z*-Asp-CH_2-DCB in both cell lines. U1-70kDa degradation was blocked during the entire 8-h incubation period.

3.4. Bcl-2 blocks U1-70kDa cleavage in RAW 264.7 macrophages

Bcl-2 is a prototype programmed cell death regulator in mammalian cells. We and others demonstrated that Bcl-2 effectively antagonized NO-mediated apoptosis in macrophages (Meßmer et al., 1996a; Albina et al., 1996), preferentially antagonizing apoptotic death upstream of caspase activation (Meßmer et al., 1996b). To gain further insights into protease activation during NO-induced apoptosis, and to evaluate the susceptibility of poly(ADP-ribose) polymerase and U1-70kDa protease substrates, we studied their cleavage in Bcl-2-transfected RAW 264.7 macrophages. Two RAW 264.7 macrophage cell clones stably transfected with human Bcl-2 termed Rbcl2-2 and Rbcl2-14 were used for the following experiments. Clone Rbcl2-14 expressed the highest level of Bcl-2 (relative density = 132), whereas expression of Bcl-2 in clone Rbcl2-2 was significantly lower (relative density = 78) (see Meßmer et al., 1996a). Stimulation of RAW 264.7 macrophages and Rneo-2, a neomycin-vector control transfected clone (relative density = 0), with 1 mM GSNO for 8 h, gave 37.0 ± 3.0% and 32.2 ± 4.7% (means ± S.D., n = 5) DNA fragmentation, respectively. In contrast, Rbcl2-14 and Rbcl2-2 clones exhibited marked protection. Fragmentation values in response to GSNO were lowered to 1.3 ± 1.1% and 11.8 ± 3.5% (means ± S.D., n = 5), respectively. Poly(ADP-ribose) polymerase and U1-70kDa cleavage in both RAW 264.7 parent and Rneo-2 control transfected cells was positive, 4 h and 8 h following GSNO (1 mM) stimulation (Fig. 8). In contrast, poly(ADP-ribose) polymerase proteolysis in clone Rbcl2-2 was potently suppressed after a 4-h incubation, but not 8 h after GSNO (1 mM) challenge. Clone Rbcl2-14 remained unaffected with only a minor evidence of poly(ADP-ribose) polymerase cleavage after the 8-h incubation period. Opposed to poly(ADP-ribose) polymerase cleavage, U1-70kDa diges-

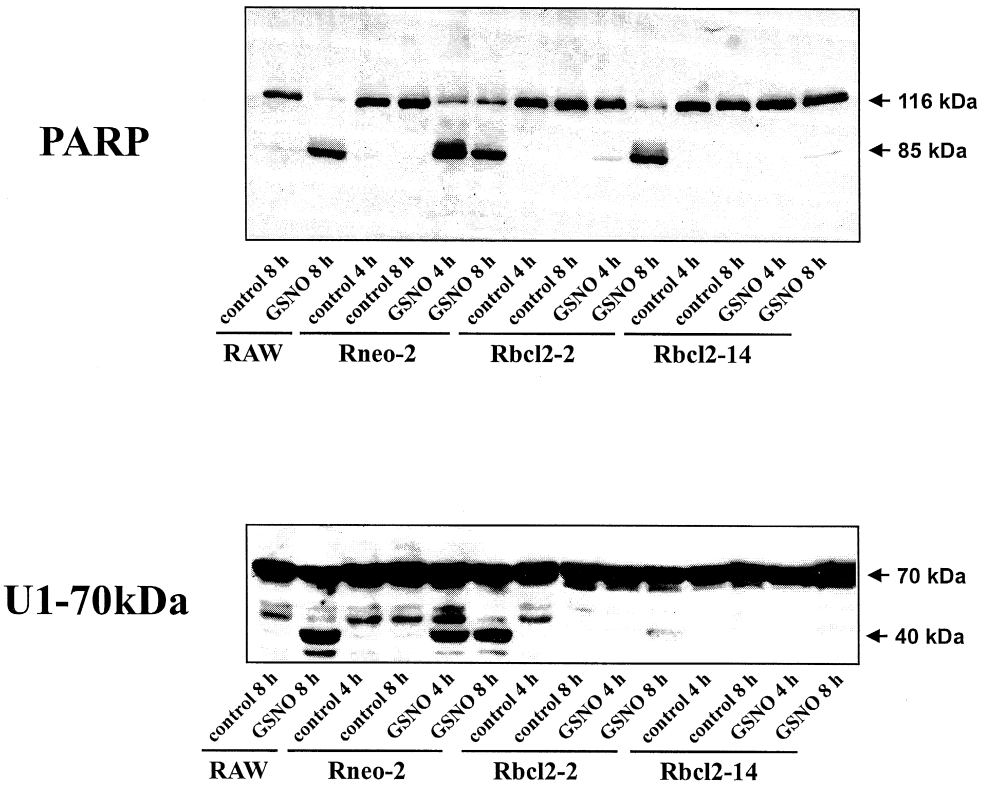

Fig. 8. Bcl-2 delays poly(ADP-ribose) polymerase and U1-70kDa cleavage in response to NO-donor exposure. RAW 264.7 macrophages, a neomycin-vector control clone (Rneo-2), and two Bcl-2 transfected clones (Rbcl2-2 and Rbcl2-14) were incubated for 4 or 8 h with vehicle (control) or 1 mM GSNO as indicated. Proteins were normalized to 100 μg/lane followed by examination of poly(ADP-ribose) polymerase (116 kDa holoenzyme and 85 kDa fragment) and U1-70kDa (70 kDa homolog and 40 kDa fragment) by Western blot analysis. Details are as in Figs. 5 and 6.

tion was strongly suppressed in both, the Rbcl2-2 and Rbcl2-14 clones (Fig. 8). Therefore, Bcl-2-transfected macrophages further allowed to compare conditions of poly(ADP-ribose) polymerase and U1-70kDa cleavage, as well as DNA fragmentation.

4. Discussion

With the use of protease inhibitors, we addressed the role of serine and caspase-like proteases during NO-induced apoptosis in RAW 264.7 macrophages and U937 cells. We conclude that specific peptide-based inhibitors of caspase-3 (Ac-DEVD-CHO) and caspase-1 (Ac-YVAD-CHO) do not block NO-toxicity. This assumption is based on the observation that these inhibitors neither block endonuclease-mediated DNA fragmentation, nor inhibit the activation of essential executive apoptotic steps that result in caspase activation and concomitant poly(ADP-ribose) polymerase, or U1-70kDa digestion, although Ac-DEVD-CHO blocked caspase-3 activity in intact cells. In contrast, *Z*-Asp-CH_2-DCB, a caspase inhibitor, attenuated apoptotic DNA fragmentation, morphological apoptotic alterations, and U1-70kDa protein cleavage in response to NO. One plausible explanation would be that *Z*-Asp-CH_2-DCB, apart from inhibition of caspase-3, also affected a caspase-3 and caspase-1 distinctive cysteine protease that evoked NO-mediated apoptosis. Furthermore, the U1-70kDa protein is a likely substrate for the *Z*-Asp-CH_2-DCB-sensitive protease.

In our previous studies, we characterized early NO-initiated apoptotic alterations which implicate accumulation of the tumor suppressor p53 as an early and convenient apoptotic marker. However, by using antisense p53-transfected macrophages, we established p53-independent signal transmission during NO-toxicity as well. In line are the present results indicating apoptosis in the p53-negative cell line U937 when exposed to NO-generating substances (Meßmer and Brüne, 1996b). In a simple model, p53-dependent and p53-independent signaling converge upon a final common pathway leading to death. Recently, activation of a protease cascade as a common feature of many forms of cell death became apparent. Our observations tie NO-mediated apoptotic signaling and increased caspase activity, which ultimately resulted in proteolytic cleavage of poly(ADP-ribose) polymerase and the U1-70kDa protein (Figs. 6 and 7). This substantiates a link between NO-signaling and activation of specific apoptotic-associated cellular responses, thus making an undirected toxic interference of NO in cellular metabolism dispensible. Both, poly(ADP-ribose) polymerase and U1-70kDa are well characterized substrates for caspase-3 (Casciola-Rosen et al., 1996). In turn, caspase-3 was established as the key protease in many apoptotic processes, such as Fas/CD95 and TNF-induced apoptosis (Schlegel et al., 1996; Armstrong et al., 1996). However, using a potent and highly selective caspase-3 inhibitor (Ac-DEVD-CHO) in RAW 264.7 macrophages and U937 cells, our studies make an essential role of this protease in NO-induced apoptosis improbable. These results, as well as several other reports, imply apoptotic cell death without caspase-3 involvement. Darmon and Bleackley (1996) described Fas-mediated killing independently of caspase-3 activation in EL4 and YAC-1 mouse lymphoma cells, whereas Fas-induced U937 apoptosis was blocked by a caspase-3 isoenzyme-specific inhibitor indicating variable protease activation by different apoptotic stimuli (Gamen et al., 1996). Our data further argue against the involvement of the prototypic cysteine protease caspase-1 in NO-mediated apoptotic cell death. Lack of inhibition by pharmacological protease inhibitors based on inefficient target cell uptake (Thornberry et al., 1992) was ruled out by raising inhibitor concentrations to 200 μM, which far exceeded necessary in vitro inhibitory concentrations (Darmon and Bleackley, 1996), and by confirming inhibition of caspase-3 proteolytic activity in intact cells.

Previous work has demonstrated induction of apoptosis by serine proteases exemplified by granzyme B (Quan et al., 1996), or a purified 24 kDa serine-like protease with TPCK-sensitive elastase activity (Wright et al., 1995, 1996). In turn, effective serine protease inhibitors such as TLCK and TPCK have been examined for their suppressive action in different apoptotic models. We established activation of TPCK and TLCK-sensitive proteases during NO-induced apoptosis in human U937 cells only, whereas these inhibitors failed to protect mouse RAW 264.7 macrophages. This implies a different set of active proteases during NO-mediated apoptosis, depending on the cellular system studied. For conclusive interpretation, one should keep in mind that TPCK and TLCK, although widely used as selective chymotrypsin-like and trypsin-like serine protease antagonists, are potent inhibitors of some cysteine proteases, including cathepsin B, cathepsin L (Kaufmann et al., 1993), and caspases (Patel et al., 1996) as well.

The aspartate-based protease inhibitor *Z*-Asp-CH_2-DCB was recently described as a caspase inhibitor (Mashima et al., 1995b). *Z*-Asp-CH_2-DCB blocked apoptotic cell death of U937 cells induced by etoposide, camptothecin, adriamycin, and Ara-C (Mashima et al., 1995a,b). We extended potent inhibition by *Z*-Asp-CH_2-DCB towards NO-induced apoptosis in U937 cells and RAW 264.7 macrophages ($K_i = 30-50$ μM). In contrast to TPCK/TLCK inhibition, which is cell-selective, *Z*-Asp-CH_2-DCB interfered in both systems, thus pointing to a protease more generally involved in NO-mediated apoptosis. Conclusively, protease activation in RAW 264.7 macrophages is downstream of the p53-response, because accumulation of the tumor suppressor protein is not intercepted by protease inhibition (Fig. 5).

Our results establish poly(ADP-ribose) polymerase and U1-70kDa digestion during NO-mediated apoptosis. How-

ever, a general cause–effect relationship between apoptosis and ICE-like target destruction must be questioned. An efficient block in DNA degradation is paralleled by preserved integrity of U1-70kDa only, whereas poly(ADP-ribose) polymerase breakdown is only partially inhibited or proceeded unaltered. Conclusively, poly(ADP-ribose) polymerase-destruction seems dispensible for the NO-propagated death pathway, because proteolytic protein cleavage was not associated with an apoptotic phenotype. This is in analogy to the observation of functional apoptotic responses in cells from poly(ADP-ribose) polymerase$^{-/-}$ mice (Leist et al., 1997; Wang et al., 1997). However, in contrast to these observations, genetic disruption of PARP provides profound protection against glutamate-NO-mediated ischemic insults and decreased infarct volume after reversible middle cerebral artery occlusion (Eliasson et al., 1997).

Our results are consistent with multiple protease activation during NO-mediated apoptosis and positions a *Z*-Asp-CH_2-DCB sensitive protease that cleaves U1-70kDa in the executive degradation pathway (Greidinger et al., 1996). Assuming that caspase-3 proteolytically degrades poly(ADP-ribose) polymerase, one possible conclusion is that *Z*-Asp-CH_2-DCB targets a different isoenzyme. Caspase-7 would be a likely candidate, as the protease cleaves both, poly(ADP-ribose) polymerase and U1-70kDa (Orth et al., 1996b). However, other explanations such as different catalytic efficiency of the caspase on poly(ADP-ribose) polymerase vs. U1-70kDa, or a different subcellular location of poly(ADP-ribose) polymerase and U1-70kDa with separate accessibility to *Z*-Asp-CH_2-DCB cannot be ruled out.

Further, we present evidence that poly(ADP-ribose) polymerase-digestion not necessarily coincides with other apoptotic parameters by using Bcl-2 overexpressing cells. Several reports established Bcl-2 interference upstream of ICE/CED-cysteine proteases (Chinnaiyan et al., 1996; Shimizu et al., 1996) and downstream of the p53 response (Meßmer et al., 1996a). Overexpression of Bcl-2 to various extent (low expression of Bcl-2 in clone 2; higher expression of Bcl-2 in clone 14) reflected again differences between poly(ADP-ribose) polymerase and U1-70kDa cleavage. High expression of Bcl-2 totally suppressed NO-mediated DNA fragmentation paralleled by intact poly(ADP-ribose) polymerase and U1-70kDa. Low Bcl-2 expression allowed poly(ADP-ribose) polymerase digestion, whereas U1-70kDa breakdown and DNA fragmentation were still suppressed to a great extent. Most notably, the sensitivity of protease activation is differently affected by Bcl-2. This suggests that Bcl-2 interferes at distinguished sites in the protease cascade rather than blocking protease activation in a more general way. Assuming that anti-Fas may trigger a different set of (caspase) proteases depending on the cellular system, our suggestion would also be an explanation for earlier studies on the influence of Bcl-2 in protecting tumor cells from anti-Fas-mediated cytotoxicity, which are contradictory (Jäättelä et al., 1995; Chiu et al., 1995).

Pharmacological intervention and gene transfer of Bcl-2 allowed a comparison of the role of poly(ADP-ribose) polymerase and U1-70kDa protein cleavage. The observation that poly(ADP-ribose) polymerase cleavage not necessarily parallels apoptosis may imply that protease activation leading to poly(ADP-ribose) polymerase breakdown does not causatively lead to NO-induced cell death. Further work that identifies the proteases involved in NO-mediated apoptosis will be essential to understand cytotoxic NO actions and may help to explain cellular sensitivity variations towards the potential toxic molecule.

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