Nitric oxide donors inhibit formation of the Apaf-1/caspase-9 apoptosome and activation of caspases

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Caspases are critical for the initiation and execution of apoptosis. Nitric oxide (NO) or derived species can prevent programmed cell death in several cell types, reportedly through S-nitrosation and inactivation of active caspases. Although we find that S-nitrosation of caspases can occur *in vitro*, our study questions whether this post-translational modification is solely responsible for NO-mediated inhibition of apoptosis. Indeed, using Jurkat cells as a model system, we demonstrate that NO donors block Fas- and etoposide-induced caspase activation and apoptosis (downstream of mitochondrial membrane depolarization) and cytochrome *c* release. However, caspase activity was not restored by the strong reducing agent dithiothreitol, as predicted for S-nitrosation reactions, thereby excluding active-site-thiol modification of caspases as the only anti-apoptotic mechanism of NO donors in cells. Rather, we observed that processing

of procaspases-9, -3 and -8 was decreased due to ineffective formation of the Apaf-1/caspase-9 apoptosome. Gel-filtration and *in vitro* binding assays indicated that NO donors inhibit correct assembly of Apaf-1 into an active approx. 700 kDa apoptosome complex, and markedly attenuate caspase-recruitment domain (CARD)–CARD interactions between Apaf-1 and procaspase-9. Therefore we suggest that NO or a metabolite acts directly at the level of the apoptosome and inhibits the sequential activation of caspases-9, -3 and -8, which are required for both stress- and receptor-induced death in cells that use the mitochondrial subroute of cell demise.

Key words: caspase processing, cytochrome *c*, Fas, nitric oxide, nitrosation.

INTRODUCTION

Apoptosis is an important form of programmed cell death required for maintaining appropriate cell numbers in both developing and mature tissues. Consequently, an inappropriate rate of apoptosis versus that of cell growth has been implicated in both malignancy and neurodegenerative diseases [1,2]. Various stimuli can induce apoptosis through activation of receptor- or stress-induced signal-transduction pathways [3,4] and, in most cases, cysteine proteases, known as caspases, play a critical role in both propagating and executing the death signal.

Fas (also known as Apo-1 or CD95), tumour necrosis factor (TNF) receptor-1 and DR5 represent well-characterized death receptors that are activated by their cognate ligands: Fas ligand, TNF- α and TRAIL ('tumour-necrosis-factor-related apoptosis-inducing ligand') respectively. Ligand binding stimulates receptor trimerization and sequential recruitment of the adapter molecule FADD (Fas-associated death domain) and the initiator caspase-8 to the death-inducing signalling complex (DISC) [5,6]. In contrast, loss of trophic stimuli, chemicals and radiation stimulate release of cytochrome *c* from mitochondria, often by involving pro-apoptotic Bcl-2 proteins, such as Bax or Bak [7,8]. Cytosolic cytochrome *c*, in co-operation with dATP/ATP, induces formation of the apoptosome by stimulating oligomerization of the adapter protein Apaf-1 (apoptotic protease-activating factor 1) and, consequently, recruitment of the initiator caspase-9 [9]. Thus, in receptor- and stress-induced apoptosis, initiator caspases-8 and -9 are specifically recruited to unique caspase-activating complexes through their interactions with FADD and Apaf-1 respectively.

One model for initiator caspase activation proposes that clustering of initiator caspases within the DISC and apoptosome results in proximity-induced, *trans*-catalytic activation [10]. Once activated, caspases-8 and -9 process downstream effector caspases, such as caspases-3, -6 and -7. Interestingly, in some cells, the amount of caspase-8 activated at the DISC appears to be minimal following death-receptor stimulation. Thus, in order to activate sufficient effector caspases to induce apoptosis, the apoptotic signal must be amplified through formation of the apoptosome [5,6]. Specifically, caspase-8 cleaves and activates Bid, a pro-apoptotic Bcl-2 family member, which facilitates pore formation in the outer mitochondrial membrane and promotes release of cytochrome *c* [9,11]. A number of endogenous inhibitors of apoptosis, including decoy receptors, FLIPs ('FADD-like interleukin-1 β converting enzyme inhibitory proteins'), anti-apoptotic Bcl-2 family members and IAP ('inhibitor-of-apoptosis') proteins, appear to act by inhibiting formation of functional caspase-activating complexes or by directly inhibiting active caspases [12,13].

Recently, however, the small signalling molecule nitric oxide (NO) has been shown to inhibit apoptosis in some experimental systems [14,15]. Generation of NO can be achieved pharmacologically through the addition of NO-releasing drugs, or endogenously through activation of the inducible NO synthase (NOS). Since NO preferentially reacts with transition metals, oxygen and reactive protein thiol groups [16], it was initially proposed that NO might block apoptosis through S-nitrosation of the active-site cysteine residue in caspases. Indeed, S-nitrosation and inactivation of processed caspases occurs *in vitro* for several caspase family members [17,18]. However, detailed studies

Abbreviations used: (Ac-)DEVD-AMC, (acetyl-)Asp-Glu-Val-Asp-aminomethylcoumarin; Apaf-1, apoptosis protease-activating factor 1; CARD, caspase-recruitment domain; DiOC₆, 3,3'-dihexyloxycarbocyanide iodide; DISC, death-inducing signalling complex; DTT, dithiothreitol; ECL, enhanced chemiluminescence; FADD, Fas-associated death domain; GST, glutathione S-transferase; NOS, NO synthase; SNAP, S-nitroso-N-acetylpenicillamine; TCA, trichloroacetic acid; TNF, tumour necrosis factor.

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to substantiate this mechanism of action *in vivo* are scarce and, indeed, inhibition of apoptosis by NO may require formation of cGMP or up-regulation of protective proteins [14,19]. In the present study, we examined Fas-induced apoptosis in Jurkat cells, a model system known to be negatively regulated by NO. We demonstrate that NO donors block processing of procaspases, in addition to inhibiting active enzymes. Specifically, NO or NO-derived species interfere with the apoptotic pathway downstream of mitochondria by interfering with caspase-9/Apaf-1 interactions and preventing correct assembly of the apoptosome.

EXPERIMENTAL

Cell culture

The human T-cell line Jurkat was maintained in RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany). Apoptosis was induced with 100 ng/ml Fas antibody 7C11 (Coulter Immunotech, Krefeld, Germany), or 100 µM etoposide. *S*-Nitroso-*N*-acetyl-DL-penicillamine (SNAP; Alexis, Gruenberg, Germany) was dissolved in ethanol, and appropriate solvent controls were performed.

Quantification of DNA fragmentation

DNA fragmentation was measured with the diphenylamine assay, as reported elsewhere [20]. Briefly, following treatment, cells (2×10^6) were harvested, resuspended in 250 µl of TE buffer [10 mM Tris/HCl (pH 8.0)/1 mM EDTA] and incubated with an additional 250 µl of lysis buffer [5 mM Tris/HCl (pH 8.0)/20 mM EDTA/0.5% (v/v) Triton X-100] for 30 min at 4 °C. Thereafter, 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 overnight by adding 500 µl of 10% (w/v) trichloroacetic acid (TCA) at 4 °C. DNA fragments were pelleted by centrifugation (4000 *g* for 10 min) and the supernatant was removed. After addition of 150 µl of 5% (w/v) TCA, samples were boiled for 15 min. The samples were then assayed for DNA concentration using the diphenylamine reagent [21]. The percentage of fragmented DNA was calculated as the ratio of the DNA content in the supernatant to that of the pellet.

DNA agarose-gel electrophoresis

Jurkat cells (6×10^6) were lysed and centrifuged to separate DNA fragments from intact chromatin. DNA fragments in the supernatants were precipitated overnight with 1 ml of 100% ethanol and 50 µl of 5 M NaCl at -20 °C. After centrifugation (14000 *g* for 15 min), pellets were incubated in 500 µl of TE buffer containing 100 µg/ml RNase A at 37 °C for 30 min. The samples were then extracted with phenol/chloroform/3-methylbutanol (25:24:1, by vol.) and precipitated again overnight at -20 °C. DNA fragments were separated electrophoretically on a 1% (w/v) agarose gel and visualized by UV fluorescence after staining with ethidium bromide (1 µg/ml).

Determination of caspase-3-like activity

Caspase-3-like activity was determined by the cleavage of the fluorescent substrate, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC; Biomol, Hamburg, Germany). Cells (1×10^6) were lysed in buffer containing 50 mM Tris/HCl, pH 7.5, 500 mM sucrose, 0.1% (w/v) CHAPS, 1 mM EDTA, 1 mM PMSF and 1% Triton X-100 for 10 min at 4 °C. Cell

supernatants (30 µg of protein) were incubated in caspase assay buffer [100 mM Hepes (pH 7.5)/500 mM sucrose/0.1% CHAPS] at 30 °C with 25 µM Ac-DEVD-AMC, in the absence or presence of 10 mM dithiothreitol (DTT). The increase in fluorescence (or AMC release) was monitored at 460 nm. Caspase activity was expressed as the percentage of maximal activity achieved by Fas (26 nmol of AMC/mg of protein per min) or etoposide (14 nmol of AMC/mg of protein per min).

Western blot analysis

Cell lysates were prepared (25 µg for caspases-3 and -8; 75 µg for caspase-9) as described above, subjected to SDS/PAGE (15% gels) and transferred on to nitrocellulose. Membranes were then blocked and incubated overnight at 4 °C with primary antibodies directed against caspase-3 (Transduction Laboratories, Heidelberg, Germany), caspase-8 (kindly provided by Professor P. Krammer (DKFZ, Heidelberg, Germany), caspase-9 (New England Biolabs, Schwalbach, Germany) or an antibody directed against a His₆-tag (Qiagen, Hilden, Germany). Horseradish peroxidase ('HRP')-conjugated polyclonal antibodies were used for enhanced chemiluminescence (ECL) detection (Amersham, Freiburg, Germany).

Preparation of cell-free extracts

Cells (1×10^8) were pelleted, washed with PBS and resuspended in 50 µl of buffer containing 50 mM Hepes, pH 7.5, 50 mM KCl, 5 mM EDTA, 2 mM MgCl₂, 250 mM sucrose, 1 mM DTT and protease inhibitors. Following addition of 50 µl of the same buffer supplemented with 8 mg/ml digitonin, cells were lysed for 3 min on ice and centrifuged at 10000 *g* for 15 min. Supernatants were used immediately or frozen at -80 °C. Cytosolic fractions contained approx. 20–30 µg of protein/µl.

Coupled *in vitro* transcription/translation of caspases

[³⁵S]Methionine-labelled caspases were translated *in vitro* using the TNT kit (Promega, Mannheim, Germany) according to the manufacturer's instructions. Plasmids for caspase-3, -8 and -9 were kindly provided, respectively, by Professor Guy S. Salvesen (The Burnham Institute, La Jolla, CA, U.S.A.), Professor P. Krammer and Professor Y. Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.). For cleavage assays, 0.5 µl of ³⁵S-labelled caspases-3 and -8 and 2 µl of caspase-9 were used.

In vitro cleavage of ³⁵S-labelled caspases

Cell-free extracts (10 µg/µl) were incubated with *in vitro*-translated caspases in caspase assay buffer supplemented with 10 mM DTT for 3 h at 37 °C. Caspase processing was analysed by SDS/PAGE, followed by detection on X-ray film.

Mitochondrial membrane potential (ΔΨ)

Following incubation, cells were loaded with 40 nM of the fluorochrome 3,3'-dihexyloxycarbocyanide iodide (DiOC₆; Molecular Probes, Leiden, Netherlands) for 10 min, and the ΔΨ was measured on a Coulter Epics XL flow cytometer. At least 1×10^5 cells were counted for analysis. Results are given as a decrease in membrane potential compared with control cells.

Cytochrome *c* release

Mitochondrial cytochrome *c* release was determined by Western blot analysis of cytosolic cytochrome *c*, as described elsewhere

[22]. Briefly, cells (4×10^6) were harvested by centrifugation and resuspended in 250 μ l of PBS at room temperature. Next, 250 μ l of a digitonin/sucrose solution (40 μ g digitonin in 500 mM sucrose/ 4×10^6 cells) was added for 30 s, and the samples were subsequently centrifuged at 10000 g for 1 min. Cytosolic supernatants (30 μ g) were subjected to SDS/PAGE (15% gels) and Western blot analysis using the anti-(cytochrome *c*) antibody 7H8.2C12 (PharMingen, Hamburg, Germany). In order to confirm their purity, individual fractions were probed with antibodies against the cytosolic protein tubulin (Roche, Mannheim, Germany), and the mitochondrial protein cytochrome oxidase II (COX II; Molecular Probes).

Cytochrome *c*/dATP activation of the apoptosome

Unstimulated cell-free extracts (10 μ l; 15 μ g of protein/ μ l) were pre-incubated with caspase assay buffer [100 mM Hepes (pH 7.5)/500 mM sucrose/0.1% (w/v) CHAPS/1 mM DTT] containing SNAP (dissolved in buffer) or oxidized SNAP for 45 min at 4 °C. Apoptosome formation and caspase activation was then induced by adding cytochrome *c* (75 μ g/ml) and dATP (1 mM) for 3 h at 37 °C. Samples were subsequently subjected to SDS/PAGE (15% gels) and immunoblotted for caspases-9, -3 and -8.

Immunoprecipitation of the apoptosome

Jurkat cell extracts were incubated with or without dATP and cytochrome *c* in the presence or absence of SNAP, as described above. Incubations were terminated after 5 min with the addition of the pan-caspase inhibitor Z-ADCB [¹benzoyloxycarbonyl-Asp-CH₂OC(O)-2,6-dichlorobenzene¹]. Extracts (0.5 mg) were then incubated for 12 h at 4 °C with 1 μ g of anti-(caspase-9) antibody (kindly provided by Y. Lazebnik) and 25 μ l of 50% Protein A-Sepharose. Pellets were washed with SNTE buffer [5% sucrose/1% Nonidet P40/0.5 M NaCl/50 mM Tris/HCl (pH 7.4)/5 mM EDTA], as described previously [23], and proteins were subjected to SDS/PAGE (10% gels) and Western blotting using an Apaf-1 antibody (kindly provided by Y. Lazebnik). Input controls were performed by Western blot analysis of Apaf-1 and caspase-9.

In vitro caspase-9/Apaf-1 association assay

A plasmid (kindly provided by Professor S. J. Martin, Department of Genetics, Trinity College, Dublin, Ireland) encoding a glutathione S-transferase (GST)-tagged Apaf-1¹⁻⁹⁷ fusion protein [GST-Apaf-1^{CARD} (where CARD represents caspase-recruitment domain)] was transformed in *Escherichia coli* strain DH5 α . Bacteria were induced with 100 μ M isopropyl β -D-thiogalactoside ('IPTG') for 4 h at room temperature to express the recombinant protein, which was subsequently purified with GSH-Sepharose (Pharmacia, Freiburg, Germany) by following the standard procedure. For interaction studies, GSH-Sepharose-bound GST (control) or GST-Apaf-1^{CARD} was incubated with unstimulated cell-free extracts (50 μ g of protein) in 200 μ l of buffer [50 mM Hepes (pH 7.5)/50 mM KCl/5 mM EDTA/2 mM MgCl₂] overnight at 4 °C. To study the effect of SNAP on protein-protein interactions, cell extracts were pre-incubated with SNAP (0.75–3 mM) or oxidized SNAP (3 mM) for 45 min at 37 °C before the addition of GST-Apaf-1^{CARD} bound to GSH-Sepharose. Binding of caspase-9 to GST-Apaf-1^{CARD} was analysed by Western blotting for caspase-9. Equal loading of GST-Apaf-1^{CARD} was confirmed by Ponceau-S staining of the blotting membrane, ruling out that GST can be nitrosated, a

modification that may interfere with GST binding to GSH-Sepharose.

Using purified GST-Apaf-1-412 (plasmid provided by S. J. Martin; prepared as described for GST-Apaf-1^{CARD}) and purified His₆-tagged recombinant caspase 9 (wild-type) or a Cys²⁸⁷ → Ala mutant (plasmids kindly provided by E. Shiozaki, Princeton University, Princeton, NJ, U.S.A.; also see [24]) we tested the ability of SNAP to interfere with complex formation using a cell-free system. Binding of caspase-9 to GST-Apaf-1-412 was analysed by Western blotting for the His₆-tag of caspase-9.

Oligomerization of Apaf-1

Jurkat cell extracts (approx. 10 mg/ml) were activated with dATP and cytochrome *c* in the presence or absence of SNAP, and were then fractionated by size-exclusion chromatography using an FPLC protein-purification system with a Superose 6 HR 10/30 column (Amersham Biosciences, Little Chalfont, Bucks., U.K.), as described previously [25]. Briefly, apoptosome complexes (of approx. 700 kDa and 1.4 MDa) were eluted using column buffer [20 mM Hepes (pH 7.0)/0.1% CHAPS/5 mM DTT/5% sucrose/50 mM NaCl]. The fractions were subsequently subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with an Apaf-1-specific antibody (R & D Systems, Wiesbaden, Germany). The data presented in Figure 8(C) were generated within the same experiment, i.e. the gel-filtration columns were run on the same day, the fractions were separated by gel electrophoresis the following day, and the transferred membranes were immunoblotted/visualized by ECL using the same piece of film within the same cassette to guarantee that film exposure times were identical.

Statistics

Experiments were repeated at least twice. Results are given as mean values $\pm$ S.D.; otherwise representative data are shown.

RESULTS

NO donors attenuate Fas-induced DNA fragmentation

Previously, it has been documented that NO attenuates cell death in several cellular systems. In the present study, we characterized the inhibitory effect of NO donors on Fas-induced apoptosis in

Figure 1 Attenuation of Fas-induced apoptosis mediated by SNAP

Jurkat cells were stimulated with 100 ng/ml Fas, 500 μ M SNAP, or a combination of Fas and SNAP, for the times indicated. Controls received vehicle for 8 h. (A) DNA fragmentation was quantified using the diphenylamine assay (mean values $\pm$ S.D., $n = 3$) as described in the Experimental section. (B) DNA laddering was analysed by agarose-gel electrophoresis, as described in the Experimental section.

Figure 2 Inhibition of caspase-3-like activity mediated by SNAP

(A) cells were exposed to 100 ng/ml Fas or to Fas in combination with increasing concentrations of SNAP for 4 h. Controls received SNAP or vehicle. Cell lysates were prepared as described in the Experimental section, and 30 μg of the total cell extract was analysed for caspase-3-like activity using the fluorescent substrate DEVD-AMC. (B) Caspase-3-like activity was determined in the presence or absence of 10 mM DTT added to the assay buffer. Cell extracts were prepared as described for (A). Data represent the means ± S.D. for three individual experiments.

Jurkat cells with the intention of identifying targets of NO-redox species that interfere with the execution of cell death. In Jurkat cells, Fas antibody (100 ng/ml) induced DNA fragmentation in a time-dependent manner, as detected by the diphenylamine assay (Figure 1A). Approx. 30% of cells exhibited DNA

fragmentation by 5 h, and this value increased to 50% following an 8 h incubation period.

Remarkably, when Fas-treated cells were co-treated with the NO donor SNAP (500 μM), DNA fragmentation was reduced to 10% at 8 h. Similar results were observed when DNA fragmentation was visualized by agarose-gel electrophoresis (Figure 1B). Importantly, NO donors did not simply shift cell death from apoptosis to necrosis, as neither lactate dehydrogenase release nor propidium-iodide-positive cells were detected (results not shown). In order to prove that a NO-redox species released from SNAP was responsible for inhibiting apoptosis in itself, we utilized the chemically distinct NO donor, sodium nitroprusside (up to 500 μM), and oxidized SNAP (500 μM). Whereas sodium nitroprusside mimicked SNAP, oxidized SNAP was without effect (results not shown). Therefore we concluded that NO donors specifically inhibited Fas-induced cell death.

NO donors inhibit processing of caspase-9, -3 and -8

Since activation of caspases is essential for the execution of apoptotic cell death, and given that NO-redox species S-nitrosate caspases *in vitro*, we analysed whether active caspases were targets of NO donors in Fas-treated Jurkat cells. Effector caspase-3-like activity ('DEVDase') was maximal (26 nmol/mg per min) following a 4 h treatment with Fas (Figure 2A), and this activity was dose-dependently inhibited by SNAP (EC₅₀ approx. 100 μM).

Similarly, sodium nitroprusside (100 μM) inhibited DEVDase activity by approx. 50% (results not shown). Control treatments with SNAP or sodium nitroprusside did not increase DEVD-

Figure 3 Suppression of the processing of caspase-3, -9 and -8 by SNAP

Jurkat cells were treated with 100 ng/ml Fas in the absence or presence of 500 μM SNAP for the times indicated. Caspase processing was detected by Western blot analysis. (A) Caspase-3 processing is shown by the cleavage of the 32 kDa pro-form to the 20 kDa and 17 kDa fragments. (B) Cleavage analysis of the 56 kDa procaspase-8 to a 43 kDa intermediate and to the active small 18 kDa fragment. The lower part of Figure 3(B) reveals a longer exposure of the Western blot to visualize the 18 kDa fragment more clearly. (C) Caspase-9 processing is detected by the cleavage of the 47 kDa procaspase to the 35 kDa fragment. Results are representative of three independent experiments.

Figure 4 *In vitro* cleavage of ^{35}S -labelled caspases by cytosol of Fas-treated cells

Cells were stimulated for 4 h with 100 ng/ml Fas, Fas in combination with 500 μM SNAP, SNAP alone or vehicle as control. Cell-free extracts were prepared as described in the Experimental section. Samples of the extracts (100 μg) were incubated with ^{35}S -labelled caspases for 3 h at 37 $^{\circ}\text{C}$ in the presence of 10 mM DTT. ^{35}S -labelled caspases were synthesized by coupled *in vitro* transcription/translation. Molecular masses of procaspases and the cleavage fragments are indicated, as follows: (A) procaspase-3, 32 kDa; fragments, 20, 17 and 12 kDa; (B) procaspase-8, 56 kDa; fragments, 43, 18 and 10 kDa; (C) procaspase-9, 47 kDa; fragment, 35 kDa. Data are representative of two or three independent experiments.

Figure 5 Mitochondrial membrane depolarization and cytochrome *c* release remain unaffected by SNAP

Jurkat cells were exposed to 100 ng/ml Fas or Fas in combination with 500 μM SNAP for the times indicated. (A) Stimulated cells were loaded with the fluorescent dye DiOC₆ for 10 min and analysed by flow cytometry for mitochondrial membrane depolarization. Data shown are the mean values $\pm$ S.D. for three independent experiments, showing the mitochondrial membrane potential $\Delta\Psi$ in terms of the percentage of control values. (B) Cell lysates of stimulated cells were prepared as described in the Experimental section, and analysed for cytochrome *c* (cyt. *c*) by immunoblotting. In another set of experiments blots were probed for cytochrome *c*, tubulin and cytochrome oxidase II (COX II) to confirm the purity of the cytosolic versus the mitochondrial fractions. Results are representative of three independent experiments.

AMC cleavage. Thus NO donors appeared to inhibit effector caspase activity, although the mechanism of action was unknown.

Since nitrosative and oxidative inhibition of caspases is reversible with DTT (10 mM) [18], we used this reducing agent to determine whether catalytic function of the caspase-3-like enzymes could be restored. Experimentally, we examined DEVDase activity in the absence or presence of 10 mM DTT. In cells stimulated with Fas and SNAP, but also in cells treated with Fas alone, DTT slightly enhanced DEVD-AMC cleavage (Figure

2B). However, the absence of any major DTT reversibility indicated that neither S-nitrosation nor thiol oxidation to mixed disulphides accounted for caspase-3 inhibition. Thus it appeared more likely that NO species inhibited the initial processing of caspase-3 to its active form. In order to confirm this possibility, we followed processing of procaspase-3 to its active subunits using Western blot analysis (Figure 3A). Both caspases-8 and -9 initially process procaspase-3 between its large and small subunit (IETD↓S; the arrow denotes the site of cleavage),

Figure 6 Attenuation of etoposide-induced apoptosis by SNAP

Cells were treated with 100 μ M etoposide in the presence or absence of 500 μ M SNAP for 3 to 6 h. **(A)** Cell lysate (30 μ g) was analysed for caspase-3-like activity by cleavage of fluorescent substrate DEVD-AMC in the presence of 10 mM DTT. Data represent mean values $\pm$ S.D. for three individual experiments. **(B)** Caspase-8 processing was determined by Western blot analysis. The positions of procaspase-8 (56 kDa) and its cleaved fragments (43 kDa and 18 kDa) are indicated. The lower part of the Figure shows a longer exposure of the Western blot to visualize the 18 kDa fragment. Data are representative of three independent experiments.

leading to formation of the p20 form of the enzyme, and this active p20 then autocatalytically removes its pro-domain (ESMD↓S) to form the fully mature p17 form of the enzyme. Indeed, treatment of Jurkat cells with Fas for 2–4 h led to increased processing of procaspase-3 to its fully mature 17 kDa form. However, in the presence of SNAP, processing of procaspase-3 was inhibited, as was conversion of the p20 subunit into the fully mature 17 kDa fragment (Figure 3A). These data suggested that SNAP inhibited either caspase-8 or caspase-9 to prevent initial processing of caspase-3, and that some inhibition of processed caspase-3 (p20) also occurred.

Stimulation of Jurkat cells with Fas also caused processing of procaspase-8, as indicated by the presence of its 43 and 41 kDa fragments. At later time points (3–4 h), these fragments were processed further to 18 kDa fragments. The apparent late processing of caspase-8 is typical for type II cells, where the major portion of caspase-8 is processed downstream of mitochondria and is not processed in the DISC. Thus, not surprisingly,

Figure 7 Inhibition of cytochrome *c*/dATP-induced apoptosis *in vitro* by SNAP

Cell-free extracts (150 μ g of protein in 10 μ l) of untreated Jurkat cells were supplemented with 75 μ g/ml cytochrome *c* and 1 mM dATP for 3 h at 37 °C. SNAP, at the concentrations indicated, was pre-incubated prior to cytochrome *c*/dATP addition for 45 min. Oxidized SNAP (ox.) was used as a control. Processing of caspases was followed by Western blot analysis and bands are labelled according to their molecular masses, as follows: **(A)** procaspase-9, 47 kDa; fragment, 35 kDa; **(B)** procaspase-3, 32 kDa; fragments, 20 and 17 kDa; **(C)** procaspase-8, 56 kDa; fragments, 43, 26 and 18 kDa, with longer exposure of the lower part of the Western blot to visualize the 18 kDa fragment. Results are representative of two or three independent experiments.

procaspase-8 was not cleaved in the presence of SNAP, as this treatment inhibited caspase-3 processing and activity (Figure 3A). As already noted, caspase-9 is activated following mitochondrial cytochrome *c* release and formation of the apoptosome. In our Fas-stimulated cells, procaspase-9 was processed to its 35 kDa cleavage product by 2 h, and was completely processed by 3 h (Figure 3C). Importantly, however, processing was significantly inhibited by SNAP, implying that this NO donor might be inhibiting the mitochondrial-mediated caspase amplification loop.

As an additional functional assay for active caspases, we examined cytosols from Fas- and Fas/SNAP-treated Jurkat cells for their ability to process *in vitro*-translated 35 S-labelled

Figure 8 Attenuation of apoptosome formation by SNAP

(A) Immunoprecipitations of the apoptosome from Jurkat cytosol were performed as described in the Experimental section. Apoptosome assembly in the absence (lane 2) or presence (lane 3) of SNAP was initiated for 5 min by the addition of 1 mM dATP and 75 μ g/ml cytochrome *c*. Controls were performed without dATP/cytochrome *c*. Caspase-9 was precipitated, and Western blots were analysed for the presence of Apaf-1. Input controls for Apaf-1 and caspase-9 were performed by Western blot analysis. (B) Cell lysates were exposed to increasing concentrations of SNAP, followed by overnight incubations with GST-tagged Apaf-1^{CARD} bound to GSH-Sepharose. SNAP or 3 mM oxidized SNAP (ox.) was supplied to cell lysates for 45 min before the addition of GST-Apaf-1^{CARD}-bound GSH-Sepharose. Beads were washed and subjected to SDS/PAGE (15% gels). Binding of procaspase-9 was detected by Western blot analysis. As a control, cell extracts were incubated with the GST protein bound to Sepharose (mock). Data are representative of two independent experiments. (C) Binding of wild-type (wt) or a Cys²⁸⁷ → Ala mutant (mut) purified His₆-tagged caspase-9 to GST-Apaf-1-412 (bound to GSH-Sepharose) in the presence or absence of 3 mM SNAP was performed in a cell-lysate-free system. Caspase-9 was detected by using antibodies directed towards the His₆-tag. (D) Jurkat cell extracts (approx. 10 mg/ml) were activated with cytochrome *c*/dATP in the presence or absence of 3 mM SNAP, and were then fractionated by size-exclusion chromatography using an FPLC protein-purification system with a Superose 6 HR 10/30 column. Apoptosome complexes (approx. 700 kDa and approx. 1.4 MDa) were eluted using column buffer, and the resulting fractions were immunoblotted for Apaf-1.

procaspases-3, -8 and -9 (Figure 4). These assays were performed in the presence of 10 mM DTT in order to reverse any possible S-nitrosation of endogenous caspases. Cytosol from Fas-stimulated cells cleaved ³⁵S-labelled caspase-3, detected by the characteristic large 17 kDa and small 12 kDa subunits (Figure 4A). In contrast, processing was not detected in Fas/SNAP-stimulated cells. Similarly, ³⁵S-labelled caspase-8 was processed to its 43 kDa, 18 kDa and 10 kDa fragments, and ³⁵S-labelled procaspase-9 was processed to its 35 kDa fragment in the presence of cytosol from Fas-stimulated cells, but not from Fas/SNAP-treated cells (Figures 4B and 4C).

NO donors do not affect mitochondrial alterations

Since SNAP appeared to inhibit the activation of caspase-9 and, consequently, caspases-3 and -8, we examined the effects of SNAP on mitochondrial membrane potential and the release of cytochrome *c*. Fas stimulation led to a time-dependent and significant decrease in the mitochondrial membrane potential, compared with untreated cells. However, SNAP did not inhibit Fas-induced membrane depolarization; nor did it affect the mitochondrial membrane potential in control cells (Figure 5A).

Next, we investigated mitochondrial release of cytochrome *c* by Western blot analysis. Similarly, Fas stimulation led to a significant release of cytochrome *c* from mitochondria within 2–4 h, and this accumulation of cytosolic cytochrome *c* was not inhibited by co-treatment with SNAP (Figure 5B). Immunoblotting for tubulin and cytochrome oxidase II confirmed the purity of the cytosolic and mitochondrial fractions. Thus SNAP appears to interact with the Fas-signalling pathway downstream of mitochondria, but upstream of caspase activation.

NO donors suppressed etoposide-induced apoptosis

Since SNAP attenuated Fas-induced apoptosis downstream of mitochondrial alterations, we anticipated that NO donors might also inhibit stress-induced apoptosis, mediated by toxic stimuli. Indeed, the chemotherapeutic and DNA-damaging agent, etoposide (100 μ M), induced a time-dependent activation of caspase-3 in Jurkat cells, as determined by an increase in DEVDase activity (approx. 14 nmol/mg per min by 6 h) and cleavage of caspase-8 from its 56 kDa pro-form to its active 43 kDa and 18 kDa fragments (Figures 6A and 6B).

Significantly, however, co-treatment of cells with SNAP (500 μ M) totally inhibited etoposide-induced DEVDase activity and processing of caspase-8 (Figures 6A and 6B). Consistent with these data, etoposide (100 μ M)-induced DNA fragmentation was decreased from 40% to 15% in the presence of 500 μ M SNAP at 8 h (results not shown). Thus, since SNAP attenuated both Fas- and etoposide-mediated apoptosis, NO-derived species appeared to be acting at the level of the apoptosome.

NO donors attenuate caspase activation in a cell-free system

To gain further insight into the inhibitory mechanism(s) of NO donors, we examined the activation of caspases in a cell-free system. Cell lysates from unstimulated Jurkat cells can be activated by the addition of cytochrome *c* and dATP. This results in formation of the apoptosome and sequential recruitment and activation of caspases-9, -3 and -7 [26]. Therefore we incubated lysates with cytochrome *c* (75 μ g/ml) and dATP (1 mM) for 3 h and monitored the cleavage of endogenous procaspases by Western blotting. Procaspases-9, -3 and -8 were processed to their active 35 kDa, 17 kDa and 26/18 kDa subunits respectively

(Figure 7). Importantly, however, SNAP dose-dependently suppressed caspase cleavage. At the highest concentration of SNAP (3 mM), the NO donor totally prevented the autocatalytic processing/activation of caspase-9 to its 35 kDa form within the apoptosome (Figure 7A) and, consequently, inhibited processing of caspases-3 and -8 (Figures 7B and 7C).

However, at somewhat lower concentrations, the effects of SNAP appeared to be mixed. Indeed, SNAP (1.5 mM) did not inhibit the initial processing of caspase-9 to its 35 kDa fragment (Figure 7A), but did appear to inhibit the activity of the processed enzyme, because less procaspase-3 was converted into its 20 kDa form (Figure 7B). The 20 kDa form of caspase-3 normally removes its pro-domain autocatalytically to form the fully mature 17 kDa enzyme; however, SNAP (1.5 mM) also appeared to inhibit this autocatalytic step, probably via nitrosation or oxidation of the enzyme (Figure 7B). Caspase-8 processing was totally inhibited at both concentrations of SNAP (Figure 7C) because, in each case, caspase-3 was either not processed at all (Figure 7B; 3 mM SNAP) or the small amount of processed enzyme that was present was inactivated (Figure 7B; 1.5 mM SNAP). Lower concentrations of SNAP (0.75 mM), as well as oxidized SNAP, were without effect. Higher concentrations of NO donors were required for the *in vitro* experiments, compared with cell-culture experiments, because cell lysates must be prepared under reducing conditions (1 mM DTT) in order to maintain full activity.

SNAP inhibits the interaction between procaspase-9 and Apaf-1

Since SNAP (3 mM) appeared to inhibit the processing and activity of caspase-9 (Figure 7A), we next examined the effects of this NO donor on formation of the apoptosome. Given that caspase-9 is only active when bound to Apaf-1 [27], we speculated that SNAP might interfere with caspase-9–Apaf-1 interactions. Consequently, we activated Jurkat lysates with cytochrome *c*/dATP in the presence and absence of SNAP, immunoprecipitated caspase-9 and examined the precipitates for the presence of Apaf-1 (Figure 8A). As expected, caspase-9 was processed and co-precipitated with Apaf-1 in the cytochrome *c*/dATP-activated lysates, but not in the unactivated control lysates (Figure 8A). More importantly, however, SNAP totally prevented the association of caspase-9 with Apaf-1 in the activated lysates and, consequently, inhibited the activation of caspase-9 (Figure 8A). In each case, equivalent levels of caspase-9 and Apaf-1 were available for precipitation (Figure 8A), and similar amounts of caspase-9 were precipitated (data not shown).

As Apaf-1 associates with procaspase-9 specifically through CARD–CARD interactions, we examined further the ability of NO donors to directly interfere with these interactions. GST–Apaf-1^{CARD} bound to GSH–Sepharose was incubated with control or SNAP-treated Jurkat lysates, followed by extensive washing. Binding of GST–Apaf-1^{CARD} to endogenous procaspase-9 was dose-dependently inhibited by SNAP, compared with the naïve and oxidized SNAP controls (Figure 8B). GST alone (mock) did not pull down any procaspase-9 (Figure 8B), and SNAP did not interfere with GST binding to GSH–Sepharose. This was analysed by detecting GST–Apaf-1^{CARD} on nitrocellulose membranes during Western blot analysis. To determine the Apaf-1–caspase-9 interaction in a cell-lysate-free system, we expressed GST–Apaf-1–412, which subsequently was bound to GSH–Sepharose. A wild-type His₆-tagged purified procaspase-9 or a Cys²⁸⁷ → Ala mutant was then added to GST–Apaf-1–412 in the presence or absence of 3 mM SNAP (Figure 8C). Using a His-antibody to detect caspase-9 showed binding of caspase-9 to Apaf-1, and revealed that in a cell-lysate-

free system SNAP did not interfere with caspase-9 binding to Apaf-1.

In order to better understand the impact of SNAP on the formation of apoptosome complexes in native lysates, we directly examined their formation *in vitro* using a Superose-6 gel-filtration assay [25]. Indeed, it was previously demonstrated that Apaf-1 can oligomerize into either an approx. 700 kDa apoptosome complex, which is biologically active and capable of processing effector caspases, or an inappropriately oligomerized and inactive approx. 1.4 MDa complex [25,26,28]. In the unactivated Jurkat lysates, Apaf-1 eluted predominantly in its unbound, monomeric form (Figure 8D, fractions 20–21), with some of it eluting at a higher molecular mass (Figure 8D, fraction 6). Following activation with cytochrome *c*/dATP, Apaf-1 oligomerized into two distinct approx. 1.4 MDa (Figure 8D, fractions 5–8) and approx. 700 kDa (Figure 8D, fractions 10–13) complexes. However, most importantly, when lysates were co-treated with cytochrome *c*/dATP and the NO donor SNAP, Apaf-1 oligomerized exclusively into the inactive approx. 1.4 MDa apoptosome complex (Figure 8D, fractions 6–7). Thus SNAP inhibited appropriate oligomerization of Apaf-1 into a functional caspase-activating complex.

DISCUSSION

Here we delineate the impact of NO donors on apoptosis in Jurkat cells. NO donors blocked Fas- as well as etoposide-induced apoptosis, downstream of the mitochondria. As a result, processing of caspases-9, -3 and -8, as well as DNA fragmentation, was inhibited. Interestingly, this suggests that, in Jurkat cells, the caspase-activated DNase ('CAD') must be primarily responsible for mediating DNA fragmentation during apoptosis, in contrast with the mitochondrial proteins apoptosis-inducing factor ('AIF') and the recently identified, endonuclease G [29]. Our results suggest that in addition to S-nitrosation of active caspases [30–32], SNAP inhibits apoptosis by inhibiting the Apaf-1/caspase-9 apoptosome. Indeed, SNAP prevented the association of caspase-9 with Apaf-1 and promoted formation of the inactive approx. 1.4 MDa apoptosome complex, rather than the active approx. 700 kDa complex.

Inhibition of apoptosis by NO donors or transfection of inducible NOS has been described for several systems, including hepatocytes [31,33], endothelial cells [34], and T-cells [32]. Mechanistically, it is proposed that S-nitrosation of caspases, especially caspase-3, blocks the execution of cell death. This assumption is based on experiments *in vitro*, which show that NO donors inactivate several members of the caspase family by S-nitrosation or oxidation of the active-site cysteine residue [17,18]. Importantly, *in vitro*, both modifications are fully reversed under strong reducing conditions, i.e. in the presence of 10 mM DTT. Therefore, assuming S-nitrosation of active caspases in cells, we expected these modifications to be reversed *in vitro* under reducing conditions.

We selected Jurkat cells as the model system because this T-cell line is protected from apoptosis by NO donors [30,35]. Jurkat cells require a mitochondrial amplification loop in order to execute cell death, because only small amounts of caspase-8 are activated at the DISC [5,6]. Hence cytochrome *c*-induced activation of caspase-9 within the apoptosome is the critical step required for the activation of downstream effector caspases [36]. Membrane damage, in response to high concentrations of SNAP [32], can result in a loss of ATP and, consequently, in a decrease in apoptosis [37]. However, in our studies, we carefully excluded a shift towards necrosis as an explanation for an apparent block in apoptosis. Initially, we confirmed inhibition of caspases in

Fas-stimulated cells as a result of SNAP addition. We were surprised, however, that DTT did not fully restore caspase activity, ruling out S-nitrosation or oxidation as the sole underlying inhibitory mechanism. Western blots indicated instead that SNAP inhibited the processing/activation of caspase-9, in addition to inhibiting the activity of some processed caspase-3. We conclude that NO donors attenuate the processing of caspases, in addition to inhibiting their activity.

Most studies have suggested that NO/NO-derived species inhibit apoptosis by interfering with caspase-3 activity. In hepatocytes, SNAP was reported to suppress the processing of caspase-8 in response to TNF- α /actinomycin D [33]. However, given that hepatocytes require the mitochondrial death amplification loop, it is unclear whether NO inhibited receptor-mediated activation of caspase-8 at the DISC or caspase-3-mediated processing of caspase-8, downstream of cytochrome *c* release and activation of the apoptosome. In the case of caspase-3 or caspase-9, modification of proenzymes by NOS- or SNAP-derived NO species has been demonstrated [38,39]. Importantly, we and others [39] still noticed cytochrome *c* release from mitochondria under conditions of SNAP treatment, which pointed to some interference in the apoptotic cascade downstream of mitochondria. Inducible NOS-derived NO was equipotent compared with an approximate concentration of 300 μ M SNAP [39], indicating that the impact of NO donors at concentrations used for cellular studies in our experiments are somehow reflected by the impact evoked by NOS-derived species.

Our results from the Apaf-1/caspase-9 co-precipitation, GST pull-down experiments and Apaf-1 oligomerization assays suggest that NO may affect the recruitment of caspase-9 to the apoptosome complex by interfering with CARD–CARD interactions. Considering that apoptosome assembly, in general, and CARD–CARD interactions, in particular, are proposed targets for anti-apoptotic heat-shock proteins, it is interesting that NO-derived species appear to function in a similar way [40,41]. One might hypothesize that NO induces a conformational change in either Apaf-1–CARD or procaspase-9. However, there are no thiol groups directly involved in CARD–CARD interactions [42], and S-nitrosation of the active-site cysteine in procaspase-9 seems an unlikely explanation, since an active-site mutant of procaspase-9 (Cys²⁸⁷ $\rightarrow$ Ala) still binds to the Apaf-1 apoptosome [26,43]. Considering that the active-site cysteine in caspase-9 is not required for apoptosome assembly, and taking into account that oligomerization of Apaf-1 occurs, even in the absence of caspase-9 [26], we conclude that even if S-nitrosation of the active-site cysteine were to occur, it would not be expected to interfere with apoptosome assembly. Observing SNAP-evoked inhibition of Apaf-1–caspase-9 interactions when working with a cell lysate, but not seeing inhibition when using a cell-lysate-free Apaf-1–caspase-9 interaction system, implies that NO donors might interfere with other proteins involved in apoptosome assembly. Possible candidates may include heat-shock proteins, or the haem protein cytochrome *c* itself. These options will be addressed in further experiments.

Apoptosome assembly and DISC formation possess similar features [44], and thus NO-redox species could potentially interfere with caspase activation at both sites. In a broader sense, inhibition of apoptosis as a result of NO generation is mechanistically not restricted to one signalling target. Actions of cGMP, in response to NO, may attenuate apoptotic pathways, at least in endothelial cells [19], and NO may induce expression of various protective proteins [14]. Most intriguing is the fact that NO can be protective or pro-apoptotic, depending upon the concentration of NO used and the cell type treated [45]. Recently, it has been proposed that cellular non-haem iron is able to

convert NO-sensitive cells into NO-resistant ones [46]. In that paper, Kim and co-workers claimed that preloading macrophages with iron facilitated S-nitrosation and inactivation of caspase-3, and thus attenuated apoptosis. However, they were unable to increase caspase-3 activity with DTT, implying that simple thiol modification of caspases was unlikely to be NO's mechanism of action. It will be challenging to explain the differences in the sensitivity towards NO between several cell types on the basis of the redox status of the cell and/or the ability of the cell to promote or reverse NO-derived post-translational modifications. However, the specific mechanism(s) of NO-mediated inhibition of apoptosis are important to understand, as they may be relevant to understand the role of NO in carcinogenesis and tumour progression.

In conclusion, our data describe a new action of NO donors in attenuating protein–protein interactions, thus interfering with apoptosome formation and cell-death execution. In Jurkat cells, NO donors block the processing of caspases, as well as the activity of some caspases. The finding that NO and/or NO-derived species affect formation of a functional apoptosome appears to be an important function of NO-redox species in affecting cell death.

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