Journal of Cellular Biochemistry 82:123–133 (2001)

# Inactivation of Multiple Targets by Nitric Oxide in CD95-Triggered Apoptosis

**Francesca Bernassola, Maria Valeria Catani, Marco Corazzari, Antonello Rossi, and Gerry Melino***

Biochemistry Laboratory, IDI-IRCCS, c/o Department of Experimental Medicine, University of Rome Tor Vergata, 00133 Rome, Italy

**Abstract** Nitric oxide (NO) plays an important anti-apoptotic role by inactivating both upstream and downstream apoptotic molecules. We now report that exogenously supplied NO protected Jurkat T cells from anti-CD95-stimulated apoptosis. We have recently shown that nitrosation of the activator protein-1 (AP-1) transcriptional factor is crucial for NO-mediated inhibition of cell death triggered by etoposide or ceramide. Since the inhibition of apoptosis by NO has been reported to involve AP-1, we evaluated its involvement in in CD95-mediated cell death. Cross-linking of CD95 enhanced AP-1 DNA binding activity and AP-1-dependent CD95L transactivation, which were both significantly reduced by different NO-donors compounds. However, AP-1 induction does not seem to significantly contribute to anti-CD95-triggered apoptosis, as cell death could not be prevented by using the recombinant Fas-Fc fusion protein which inhibits the CD95/CD95L interaction. We observed that caspase 3-like activity was negatively modulated by several NO-donors in vitro and that titratable thiol groups of purified caspases 3, 7, and 9 decreased in the presence of NO-releasing compounds. In conclusion, we demonstrated that NO-mediated inhibition of other targets, possibly caspases, but not AP-1, is a crucial event responsible for protection against anti-CD95-stimulated apoptosis. Even though NO affects multiple molecular mechanisms, the relevant target for exerting the cellular effects, may vary among different models. J. Cell. Biochem. 82: 123–133, 2001. 

**Key words:** apoptosis; cell death; nitric oxide; AP-1; S-nitrosylation; CD95

CD95 (APO-1/Fas), a member of the nerve growth factor/tumor necrosis factor (TNF) death receptor superfamily [Itoh et al., 1991], is activated by cross-linking either with its natural ligand CD95L [Suda et al., 1993], or with agonistic anti-CD95 monoclonal antibodies [Trauth et al., 1989]. Activation of CD95 triggers the assembly of the death-inducing signaling complex [Kischkel et al., 1995], in which pro-caspase 8 is recruited and proteolitically activated [Yang et al., 1998]. Subsequently, caspase 8 is released into the cytosol, where it can activate a cascade of downstream executioner caspases, such as caspase 3 [Salvesen and Dixit, 1997], leading to cell death. Caspase 8 activates the downstream caspases also indirectly through the cleavage of Bid [Li et al., 1998] and the cytochrome c/Apaf-1 signaling pathway [Li et al., 1997; Zou et al., 1997; Luo et al., 1998].

CD95 cross-linking also results in the generation of ceramide which initiates the c-Jun N-terminal kinase (JNK) signaling pathway, leading to the activation of the transcription factor activator protein-1 (AP-1) [Kyriakis et al., 1994]. AP-1 has been implicated as both positive and negative modulator of stress-induced apoptosis. Nevertheless, its role in cell death remains to be determined, given the controversial results present in the literature. It seems

Abbreviations used: TNF, tumor necrosis factor; CD95L, CD95 ligand; AP-1, activator protein-1; NO, nitric oxide; TCA, trichloroacetic acid; PBS, phosphate buffered saline; PI, propidium iodide; EMSA, electrophoretic mobility shift assay; SNAP, S-nitroso-N-acetylpenicillamine; SPER-NO, spermine-NO; SIN-1, 3-morpholinosydnonimine; GSNO, S-nitrosoglutathione; DTT, dithiothreitol

Grant sponsor: Min. Sanita', AIRC EU; Grant number: QLG1-1999-00739; Grant sponsor: MURST (40%); Grant sponsor: Progetto Finalizzato CNR-Biotecnologie and Telethon to G.M.; Grant numbers: E872, E417/bi; Grant sponsor: FIRC to F.B.

*Correspondence to: Gerry Melino, IDI-IRCCS, Biochemistry Lab, c/o Department of Experimental Medicine, Room D26/F153, University of Rome Tor Vergata, Via Tor Vergata 135, 00133 Rome, Italy.
E-mail: gerry.melino@uniroma2.it

Received 3 July 2000; Accepted 18 January 2001

likely that the expression of AP-1-sensitive genes is involved in the apoptotic pathway depending on the stimulus, the microenvironment, and the cell type.

The free radical nitric oxide (NO) and related nitroso compounds are important messengers playing central roles in pathophysiology. NO-mediated signaling pathways are classified as either cGMP-dependent or -independent; cGMP formation results from NO binding to the haem prosthetic group of soluble guanylyl cyclase, thus leading to the enzyme activation. The cGMP-independent mechanisms include inhibition of iron–sulfur centers, DNA damage, and protein modification due to nitrosation of thiol groups, thiol oxidation to sulfenic and sulfinic acid and tyrosine nitration [Stamler et al., 1992a, 1992b, 1994; Stamler and Hausladen, 1998]. Proteins which undergo a change in function upon nitrosation include membrane ion channel [Lipton et al., 1993; Xu et al., 1998], signaling proteins [Lander et al., 1995; So et al., 1998], cytosolic enzymes, such as tissue transglutaminase and caspases [Dimmeler et al., 1997; Melino et al., 1997], extracellular molecules [Catani et al., 1998], and transcriptional factors such as NF-κB [Matthews et al., 1996], OxyR [Hausladen et al., 1996], c-Myb [Brendeford et al., 1998], and AP-1 [Tabuchi et al., 1994; Nikitovic et al., 1998].

NO acts through several potential toxic mechanisms eliciting necrotic as well as apoptotic cell death. Protective actions from apoptotic pathways have also been implicated for NO [Dimmeler et al., 1997; Kim et al., 1997; Melino et al., 1997]. We have previously demonstrated that exogenous NO inhibits CD95 signaling in Jurkat T cells via nitrosation of caspase 3-like proteases [Melino et al., 1997]. NO-mediated inactivation of caspases has been also reported in TNF α-stimulated endothelial cell lines and rat hepatocytes [Dimmeler et al., 1997; Kim et al., 1997; Dimmeler and Zeiher, 1999; Li and Billiar, 1999; Liu and Stamler, 1999; Nicotera et al., 1999]. It has been recently found that caspases are endogenously nitrosated, and that CD95 cross-linking activates caspase 3 either by inducing the cleavage of the protease precursor, or by stimulating the denitrosation of its catalytic site cysteine [Mannick et al., 1999].

NO can also block the apoptotic pathway acting upstream of caspase activation. Indeed, the inhibition of mitochondrial permeability transition pore opening, and subsequent cytochrome c release, has been recently identified as a novel mechanism through which NO exerts its anti-apoptotic effects [Brookes et al., 2000]. We have recently reported that NO may prevent AP-1-dependent CD95L transactivation elicited by several cytotoxic stressors in Jurkat T cells [Melino et al., 2000]. Indeed, the promoter of the CD95L gene contains putative binding sites for different transcriptional factors including the consensus sequence for AP-1 [Takahashi et al., 1994]. In some cases, the inhibition of apoptosis is only transitory and possibly its mode changes to necrosis. NO can switch apoptosis to necrosis by impairing mitochondrial ATP generation, through the inhibition of the mitochondrial respiratory chain. It has been suggested that NO-mediated lowering of intracellular ATP may in fact affect caspase activation by preventing cytochrome c release from mitochondria or, alternatively, the formation of the apoptosome complex from pro-caspase 9, Apaf-1, and ATP [Leist et al., 1999].

Here, we explored the possibility that NO may prevent CD95-triggered apoptosis by influencing DNA binding ability of AP-1 and subsequent regulation of CD95L expression or by modulating caspase activity. To this end, we first examined the effects of NO-donor compounds on cell death induced by CD95 cross-linking. We then investigated whether caspases, AP-1 binding activity and transactivation of the AP-1 responsive CD95L promoter could be affected by NO.

## MATERIALS AND METHODS

### Materials

Ribonuclease A and propidium iodide (PI) were obtained from Sigma Chemical (St. Louis, MO). Ham's F-12 and minimal essential medium were from Gibco (Berlin, Germany) and fetal calf serum (FCS) from HyClone (Oud-Beijerland, Holland). All the NO-donors were purchased from Alexis Biochemicals (Läufelfingen, Switzerland). The anti–CD95 agonistic antibody (IgM), clone CH-11, was from Upstate Biotechnology (New York). The genomic clone of human CD95L containing the putative promoter region was kindly provided by Dr. S. Nagata, Department of Genetics, Osaka University Medical School, Japan. A 1.2 kb fragment, containing binding sites for several transcriptional factors including AP-1 consensus

sequence (TTAGTCAG), was subcloned by PCR into the eukaryotic expression vector HsLuc [Kasibhatla et al., 1998]. The mouse monoclonal anti-PARP antibody (clone C-2-10) was kindly provided by Dr. G. Poirier, Health and Environment Unit, CHUL Research Center, Quebec, Canada. The recombinant Fas-Fc fusion protein, used as a competitive inhibitor of CD95/CD95L interactions [Brunner et al., 1995], was kindly provided by Dr. Douglas R. Green, La Jolla Institute of Allergy and Immunology, San Diego, CA.

### Cell Cultures

Jurkat T cells were grown in a 1:1 mixture of minimal essential medium and Ham's F-12 medium supplemented with 10% heat-inactivated FCS, 1.2 g/l Na-bicarbonate, 1% non essential amino acids and 15 mM HEPES, at 37°C with 5% $CO_2$ in a humidified atmosphere. The amount of free NO released from the NO-donor compounds in the culture medium was determined by using the Iso-NO meter (WPI, World Precision Instruments Inc., Sarasota, FL).

### Determination of Cell Death

To estimate DNA fragmentation, cells subjected to different treatments were collected at 800*g* for 10 min and fixed with 1:1 PBS and methanol-acetone (4:1 v/v) solution at −20°C. The cell cycle was evaluated by flow cytometry using a propidium iodide (PI) staining (40 μg/ml) [Piacentini et al., 1993; Melino et al., 1994] in the presence of 13 kU/ml ribonuclease A (20 min incubation at 37°C) on a FACScalibur flow cytometer (Becton-Dickinson, CA). Cells were excited at 488 nm using a 15 mW Argon laser and the fluorescence was monitored at 578 nm at a rate of 150–300 events/sec. Ten thousand events were evaluated using the Lysis II Programme (ibid). Electronic gating forward side scatter (FSC)-a/vs/FSC-h was used, when appropriate, to eliminate cell aggregates. For ultrastructural examination, cell suspensions were fixed in 2% glutaraldehyde, postfixed in 1% $OsO_4$, and dehydrated in a graded series of ethanol, embedded in Epon resin and then semithin and ultrathin sections were cut on an ultramicrotome (Reichert Ultracut E, Leica, Wien, Austria). Ultrathin sections were mounted onto uncoated grids and stained with uranyl acetate and lead citrate, before observing in a transmission electron microscope (CM100, Philips, Eindhoven, The Netherlands).

### PARP Western Blotting

Cells ($15 \times 10^6$ cells/ml) were incubated at 37°C with the apoptotic stimulus alone or with the NO-donors for the indicated time. After treatment, cells were pelleted and then washed twice with phosphate buffered saline (PBS). Cell lysis was achieved with lysis buffer (50 mM Tris pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol) and sonication on ice (30 s, 40% duty cycle, microtips at limit), followed by Bradford protein determination. Proteins were normalised to 100 μg/lane, separated on 8% SDS-polyacrylamide gels and blotted onto nitrocellulose sheets. Filters were washed twice with PBS containing 0.1% Tween-20 before blocking non-specific binding with PBS/5% bovine serum albumine, 0.5% gelatin. The mouse anti-PARP antibody (clone C-2-10, 1:1000 in PBS + 5% BSA, 0.5% gelatin) was added and incubated overnight at 4°C. Nitrocellulose filters were washed five times and detection was performed by horseradish peroxidase-conjugated goat anti-mouse monoclonal antibody (1:2500) for 1 h at room temperature, using the ECL method (Amersham). Quantification of immunolabeled proteins was performed by scanning laser densitometry.

### Measurement of Caspase 3-Like Activity

Caspase activity was measured using continous fluorometric assay, as previously described [Nicholson et al., 1995]. Briefly, activity of caspase 3 was detected by measuring the proteolytic cleavage of the fluorogenic substrate 7-amino-4-coumarin (AMC)-DEVD and AMC as standard. Aliquots of caspase 3 (2 μg/100 μl) were combined with 500 μl of an enzymatic reaction mixture which consisted of a standard buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 10% sucrose, 5 mM dithiothreitol (DTT), and the protease inhibitors, including 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin and leupeptin each) containing 100 μM fluorescent substrate. The cleaved substrate was measured continuously at room temperature, using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. When the effect of NO was studied, caspase 3 was treated with increasing concentrations of NO-

generating donors for 10 min on ice, before starting the reaction.

### Titration of Thiol Groups

The highly reactive active site cysteine residue of caspases was titrated by alkylation with iodoacetamide. Reaction mixtures containing 0.1 M Tris-acetate pH 7.5, 20 mM $CaCl_2$, 0.5 mM [$^{14}$C] iodoacetamide (59 mCi/mmol) and 0.3 μg of caspases were incubated 5 min at 23°C. Reactions were terminated by addition of 100 mM DTT and 20 mM EDTA. Aliquots were spotted on 3 mm filter paper. Filters were washed in 20% trichloroacetic acid (TCA), 10% TCA, 5% TCA, and absolute ethanol dried and counted. When the effect of NO was studied, increasing concentrations of the NO-donors were added to the reaction mixture and incubated for 5 min, before the initiation of the reaction with [$^{14}$C] iodoacetamide.

### Electrophoretic Mobility Shift Assay (EMSA)

Nuclear mini-extracts obtained from $10^7$ cells were prepared according to Schreiber et al. [1989] with the modifications reported in Lee et al. [1996]. Mobility shift experiments were performed as described [Lee et al., 1996]. The AP-1 consensus oligonucleotide was CGCTTGATGAGTCAGCCGGAA, the AP-1 mutant oligonucleotide was CGCTTGATTAGTTAGCCGGAA. The complexes were resolved on nondenaturing 6% polyacrylamide gels in 0.5X TBE buffer for 1 h at 14 V/cm and autoradiographed overnight.

### Transient Transfections of Jurkat T Cells

Jurkat T cells were co-transfected with a reporter construct consisting of the luciferase gene under the control of the human CD95L promoter and the pCAT control vector (1 μg DNA/$10^6$ cells) by using Superfect Transfection Reagent (Qiagen; Hilden, Germany). Briefly, cells were washed once with PBS and plated in duplicate at $10^6$ cells/ml in complete medium. The formation of Superfect-DNA complexes (ratio Superfect: DNA 20:1) were allowed to proceed for 10 min at room temperature. Then, diluted complexes were applied to cells and incubation was prolonged for 48 h before treatment with the apoptotic stimulus and NO-donors. Transfected Jurkat T cells were incubated with 0.5 μg/ml anti CD95 antibody for 1 h, with or without different NO-donor compounds. After incubation, cells were harvested, washed once in PBS and lysed in 200 μl Reporter Lysis Buffer (Promega; Madison, WI). For gene expression assay, 20 μl of cell extracts were mixed with 100 μl of Luciferase Assay Reagent (Promega; Madison, WI) and light emission was measured with a highly sensitive LUMI-A (SEAS) luminometer [Roda et al., 1993]. Differences in transfection efficiency were normalised by chloramphenicol acetyl transferase activity, as described [Sambrook et al., 1989].

## RESULTS

### Anti CD95-Triggered Apoptosis is Prevented by NO-Donors in Jurkat T Cells

In order to investigate the effect of NO on CD95-induced cell death, Jurkat T cells were exposed to 1 μg/ml anti-CD95 agonistic antibody, for 1.5, 3, and 6 h, in the presence or the absence of increasing concentrations of the NO-donor SNAP (0.1–5 mM). Kinetic analysis showed that 1 mM SNAP released free NO concentrations in the nanomolar range, showing a maximum peak of release of 0.53 μM at 0.6 h, as evaluated by a modified Clarke electrode at 37°C in normal culture medium. Apoptosis was then assessed by flow cytometry analysis using PI staining. As shown in Figure 1A, CD95 ligation significantly increased the percentage of hypodiploid events within 3 h of treatment; nevertheless, co-incubation with SNAP resulted in a dose-dependently marked inhibition of CD95-mediated apoptosis. This result confirms our previous report which demonstrated the ability of NO to prevent receptor-induced apoptosis [Melino et al., 1997]. In order to characterise morphological features of apoptosis at ultrastructural level, we performed electron microscopy on anti-CD95-stimulated Jurkat T cells. Unlike the control (Fig. 1B), in anti-CD95-treated cells typical morphological features of apoptosis, such as highly condensed and cap-shaped marginated chromatin, could easily be seen (Fig. 1C). Co-incubation of Jurkat T cells with anti-CD95 and 1 mM SNAP resulted in a significant decrease in the percentage of apototic cells, even though cells with characteristic apoptotic changes were still evident (Fig. 1D). Apoptotic cell death was also confirmed by the absence of a significant release of lactate dehydrogenase (data not shown). Altogether, these data indicate the absence of necrosis in the experimental conditions used.

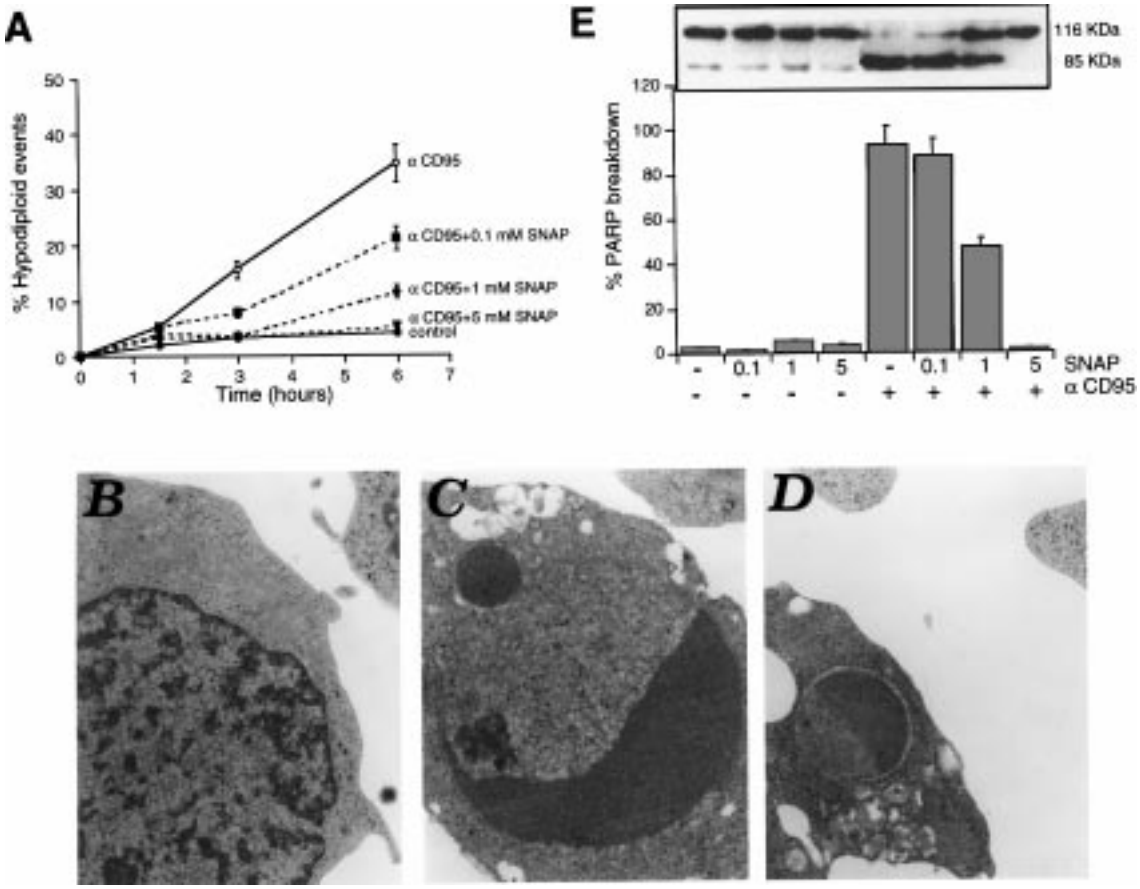


**Fig. 1.** Effect of NO-donors on CD95-triggered apoptosis in Jurkat T cells. **A:** Inhibition of apoptosis by increasing concentrations of SNAP (0.1–5 mM) after the addition of 1 μg/ml anti-CD95 antibody. Apoptotic events were evaluated by flow cytometry. Results are means±SE of duplicate determinations carried out on two different experiments. **B:** Electron microscopy of Jurkat cells without any treatment; a particular of the nucleus is reported (13,000×). **C:** Nucleus of cells undergoing apoptosis after 3 h treatment with 1 μg/ml anti-CD95 antibody (13,000×). **D:** Ultrastructural changes in the nucleus of Jurkat cells co-incubated with anti–CD95 antibody and 1 mM SNAP for 3 h (18,000×). **E:** Inhibition of anti–CD95-induced proteolytic cleavage of PARP by SNAP. Cells were left untreated, incubated with 0.1, 1, or 5 mM SNAP, 1 μg/ml anti CD95 antibody or co-incubated with anti CD95–antibody and the NO-donor for 90 min. Cell extracts were analysed by Western blotting (panel inside) and immunolabeled bands were quantified by gel scanning densitometry. PARP breakdown product was expressed as the ratio of the cleaved fragment density over the total immunoreactivity.

Since caspase 3-like enzymes are early activated in CD95-triggered cell death [Schlegel et al., 1996], we also tested the ability of the NO-donors to interfere with this protease activity. Jurkat T cells were exposed to 1 μg/ml anti-CD95 antibody or co-incubated with anti-CD95 and increasing concentrations of SNAP or S-nitrosoglutathione (GSNO) for 90 min, and then Western blotting analysis of PARP cleavage was performed. CD95 cross-linking led to proteolytic cleavage of the 116 kDa enzyme into the 85 kDa fragment, 90 min after incubation. Addition of SNAP (Fig. 1E) or GSNO (data not shown) dose-dependently blocked caspase-mediated PARP digestion, as revealed by densitometric analysis, in which PARP breakdown product was expressed as a percentage of total immunoreactivity. Treatment with SNAP alone had no effect on proteolytic digestion of PARP.

We then investigated the effect of NO and NO-related species on caspase activity in vitro, after incubation of purified recombinant caspase 3 with increasing concentrations of different NO-releasing compounds. As shown in Figure 2A, all the NO-donors employed were able to inhibit the enzymatic activity in a dose-dependent manner, although with distinct kinetics. SNAP and GSNO were the most effective inhibitors, leading to 100% inactivation at higher concentrations, whereas, spermine-NO (SPER-NO) and 3-morpholinosydnonimine (SIN-1) caused 71 and 56% inhibition,

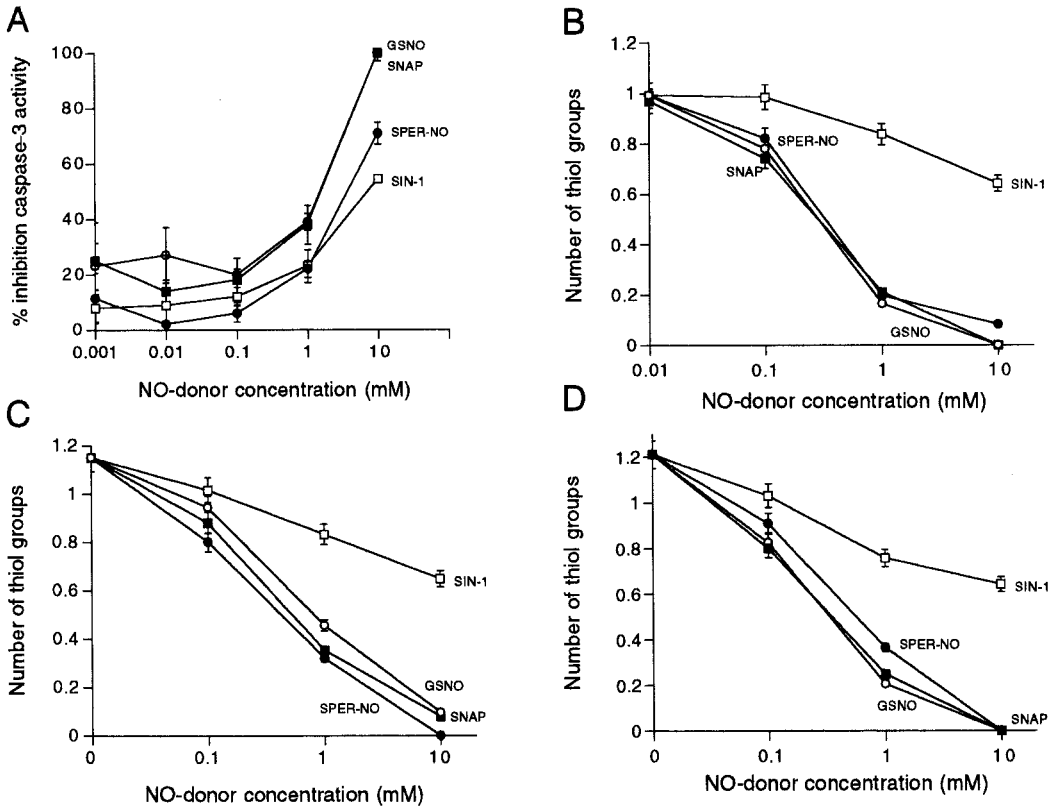


**Fig. 2.** Effect of NO-releasing compounds on caspase activity. **A:** Effect of NO on caspase 3 activity in vitro. Purified recombinant caspase 3 (2 μg/100 μl) was incubated with increasing concentrations of NO-generating compounds [SPER-NO (●), GSNO (○), SNAP (■), SIN-1 (□)] for 10 min on ice, before starting the assay, and the activity was monitored by continously measuring the proteolytic cleavage of the fluorogenic AMC-DEVD substrate. Data are means±SE of triplicate determinations of three separate experiments. **B-D:** Thiol groups titration of caspase 3 (panel B), 7 (panel C), and 9 (panel D), evaluated by alkylation with [$^{14}$C]-iodoacetamide. Purified enzymes were incubated with increasing concentrations of SPER-NO (●), GSNO (○), SNAP (■), SIN-1 (□) and titration was performed after 5 min. Data are means±SE of triplicate determinations carried out on duplicate different experiments.

respectively. The spermine has been shown to trigger intracellular caspase activation [Stefanelli et al., 1998], suggesting that the release of the polyamine from the NO-donor compound might counteract the inhibitory action of NO. Accordingly, the exposure of recombinant caspase 3 to increasing doses of spermine resulted in enzyme activation (9, 15, 22, 33, and 48% for 0.001, 0.01, 0.1, 1, and 10 mM spermine, respectively). The ability of SIN-1 to weakly inhibit caspase 3 may be due to the release of both NO and superoxide anion, which can rapidly react to produce peroxynitrite.

Caspase activity is strongly dependent on the presence of reducing agents. All the experiments were carried out using 5 mM DTT. To assess whether the inhibitory effect of NO on the protease enzymatic activity was influenced by the reducing agent DTT, we incubated purified recombinant caspase 3 with 1 mM NO-releasing compounds, in the presence of 0.5 mM DTT. As shown in Table I, 5 mM DTT reversed a significant portion of the effect of the NO-donors, thus indicating that inactivation by NO may proceed through nitrosation of catalytic cysteine residues, or through other reversible chemical modifications, such as disulfide or sulfenic acid formation.

To address whether caspase activity may be modulated by chemical modification of a reactive cysteine residue, we performed titration of thiol groups of purified caspases 3, 7, and 9 in

**TABLE I. Effect of DTT on NO-Mediated Inhibition of Caspase Activity**

| DTT (mM) | SNAP | GSNO | SPER-NO | SIN-1 |
|---|---|---|---|---|
| 5 | 30% | 19% | 34% | 3% |
| 0.5 | 37% | 57% | 57% | 71% |

the presence of NO-releasing compounds. Figure 2 (B–D) shows that the NO-donors were able to decrease the number of titratable thiol groups from 1 to 0; in accordance with the activity data, SIN-1 was unable to completely abolish the titratable cysteine residue. These data indicate that the mechanism by which NO or NO-related species inactivate caspases involves the chemical modification of their active cysteine residue.

### NO Inhibits the CD95-Stimulated Increase of AP-1 Activity

In agreement with our recent findings [Melino et al., 2000], we studied whether cross-linking of CD95 on Jurkat T cells did result in activation of AP-1 DNA binding activity. As determined by EMSA, nuclear extracts derived from Jurkat T cells stimulated with 1 μg/ml of anti-CD95 antibody, showed an enhanced ability to shift a TRE-containing oligonucleotide (Fig. 3A, lane 4), as compared to untreated cells (Fig. 3A, lane 3). Treatment with SNAP led to a dose-dependent decrease of AP-1 activity (Fig. 3A, lanes 5–7). The specificity of AP-1 binding was confirmed by competition studies, using 50-fold molar excess of AP-1 mutant oligonucleotide (Fig. 3A, lane 1), or unlabeled AP-1 consensus oligonucleotide (Fig. 3A, lane 2). We have previously reported that SNAP decreased the number of titratable cysteines per monomer of c-Jun from 3 to 2 [Melino et al., 2000]. Furthermore, analysis by nanoelectrospray tandem mass spectrometry of the NO-donor-treated c-Jun, revealed that Cys 269, on the DNA binding site, reacted with NO [Melino et al., 2000]. Hence, the loss of AP-1 DNA binding activity might be caused by the nitroso compound-induced modification of a cysteine residue of c-Jun.

The increase of AP-1 binding activity in CD95-ligated Jurkat T cells results in an enhanced AP-1-dependent gene transactivation, as demonstrated by transfection experiments. Jurkat T cells were transiently transfected with a reporter construct consisting of the luciferase gene under the control of the human CD95L promoter. Forty-eight hours after transfection, cells were stimulated for 1 h, either with 1 μg/ml anti-CD95 antibody alone or in combination with increasing concentrations (0.01–5 mM) of four NO-donors, SNAP, GSNO, SPER-NO, and SIN-1. As shown in Figure 3B, Jurkat T cells stimulated with anti-CD95 antibody displayed

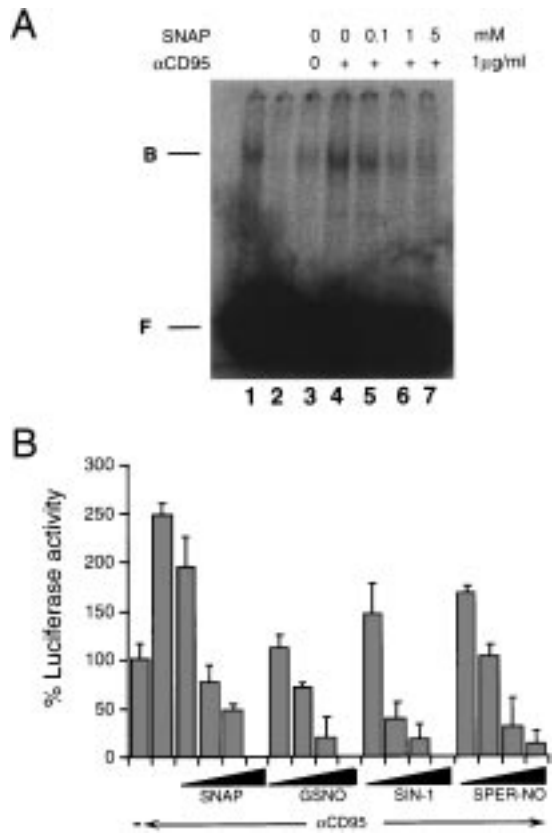


**Fig. 3.** Effect of NO-releasing compounds on AP-1 activity. **A:** Effect of NO on the increase of AP-1 binding activity induced by CD95 ligation. Cells were left untreated (**lane 3**), stimulated with 1 μg/ml anti-CD95 antibody (**lane 4**) or co-incubated with SNAP and anti-CD95 antibody (**lanes 5–7**) for 1 h. Molar excess of AP-1 mutant oligonucleotide (**lane 1**) and unlabeled AP-1 consensus oligonucleotide (**lane 2**) were used as specificity controls. The positions of DNA–protein complexes and free oligonucleotide probe are indicated by B and F respectively. **B:** Effect of NO-donors on CD95L promoter-regulated luciferase activity induced by cross-linking of CD95. Cells were transiently transfected with the luciferase construct containing the CD95L promoter and then left untreated or incubated with 1 μg/ml anti-CD95 antibody for 1 h. Where indicated, cells were co-incubated with increasing concentrations (0.01, 0.1, 1, 5 mM) of SNAP, GSNO, SIN-1 and SPER-NO. Results are means±SE of duplicate determinations carried out on two different experiments.

increasing levels of CD95L promoter-regulated reporter activity of 2.5-fold over untreated transfected cells. The CD95L promoter-driven luciferase transcription could be powerfully blocked by the different NO-releasing compounds, with complete inactivation occurring at the higher concentrations of NO-donor (Fig. 3B). We have previously reported the inhibitory effect of the NO-donors on the basal activity of AP-1-dependent luciferase activity

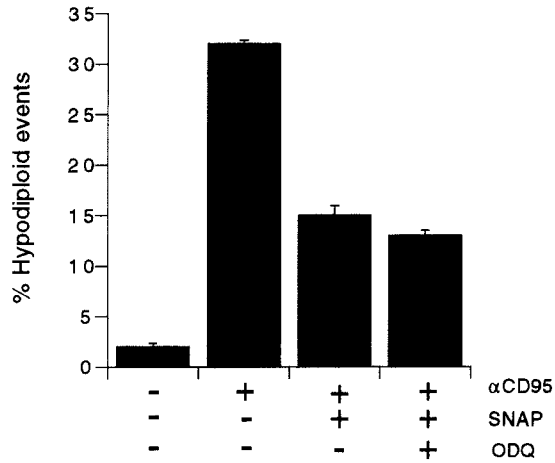


**Fig. 4.** Effect of guanylyl cyclase inhibition on CD95-triggered apoptosis. Jurkat T cells were left untreated or incubated for 6 h with anti-CD95 antibody (1 μg/ml), with or without SNAP (1 mM) and SNAP (1 mM) + ODQ (1 μM). Apoptotic events were evaluated by flow cytometry. Results are means±SE of duplicate determinations carried out on two different experiments.

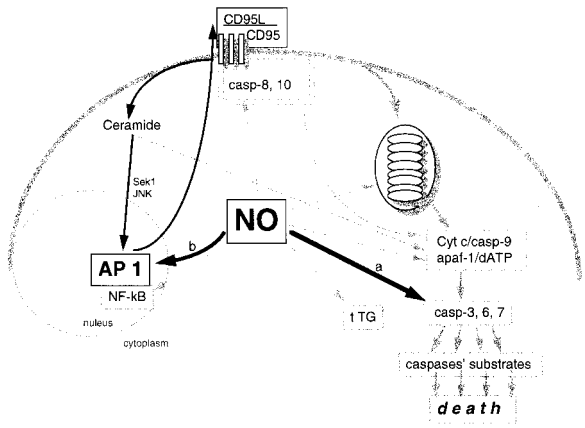


**Fig. 5.** Schematic model of the CD95/CD95L pathway and its regulation by NO. Ligation of CD95 activates two main pathways indicated as grey arrows: (i) downstream caspase activation via a direct or mitochondria-mediated activation and (ii) production of ceramide which might activate the downstream caspases. CD95 ligation may also induce AP-1-dependent CD95L transactivation. As indicated by the dashed arrows, potential molecular targets for NO may be signaling and executioner caspases, tissue transglutaminase, transcriptional factors (e.g., AP-1 and NF-κB) and the mitochondrial respiratory chain. Nitrosation of AP-1 and downstream caspases (grey arrows) may inhibit AP-1-dependent CD95L transactivation and cleavage of cellular substrates (e.g., I/CAD and PARP), thus preventing apoptosis. Not all pathways and connections are represented in this simplified scheme. a: Nitrosation of downstream caspases, (b) Nitrosation of AP-1.

[Melino et al., 2000]. Taken together, these findings indicate that NO may interfere with this circuit by inactivating primary components of the AP-1 complex.

In other cell types, NO-stimulated cGMP synthesis has been shown to prevent either caspase activation [Kim et al., 1997] or upstream signaling events [De Nadai et al., 2000]. We have therefore investigated whether a cGMP-dependent mechanism may contribute to the anti-apoptotic actions of NO in our experimental model. As shown in Figure 4, co–incubation of CD95-stimulated cells with SNAP and the soluble guanylyl cyclase inhibitor H-[1, 2, 4]oxadiazolo[4, 3-a]quinoxalin-1-one (ODQ) was not able to reverse the protective effect exerted by the NO-donor; ODQ alone had no effect (not shown). This result demonstrates that the anti-apoptotic actions of NO in CD95-induced apoptosis of Jurkat T cells are not mediated by cGMP generation.

As it is schematically described in Figure 5, it seems likely that the signaling pathway leading to CD95L expression and subsequent apoptosis, may be activated also through AP-1. Prevention of cell death by nitroso compounds may be therefore achieved through at least two independent molecular mechanisms, (i) inactivation of downstream caspases (Fig. 5, arrow a), and (ii) inhibition of AP-1-dependent CD95L transactivation (Fig. 5, arrow b). Even though it is difficult to comment on the reaction occurring in vivo, inactivation by NO may proceed through direct nitrosation of active cysteine residues, or through other reversible chemical modifications, such as disulfide or sulfenic acid formation [Stamler and Hausladen, 1998].

The involvement of AP-1 in CD95-triggered apoptosis was an unexpected result because this pathway proceeds without transcription. However, this result is relevant because we have recently demonstrated that AP-1 is a crucial target for NO in the inhibition of apoptosis [Melino et al., 2000]. To address whether AP-1-induced CD95L expression may amplify the CD95 death signal or may alternatively represent a delayed bystander event which follows apoptosis, we used the recombinant human Fas-Fc fusion protein as a competitive inhibitor of CD95/CD95L interactions [Brunner et al., 1995]. The Fas-Fc protein blocks the interaction between CD95 and CD95L by binding to the ligand, while it has no effect on anti-CD95 agonistic antibody activation of the receptor. We tested the effect of increasing concentrations (1–15 μg/ml) of Fas/Fc protein on CD95 ligation in Jurkat T cells.

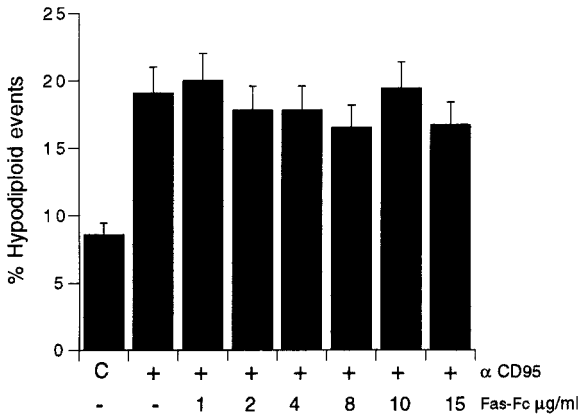


**Fig. 6.** Effect of the fusion protein Fas-Fc on anti CD95-stimulated apoptosis. Cell were left untreated or stimulated with 1 μg/ml anti-CD95 agonistic antibody in the absence or in the presence of increasing doses (1–15 μg/ml) of the Fas-Fc protein for 6 h. Apoptotic events were assessed by flow cytometry after PI staining. Results are means±SEM of duplicate determinations carried out on two different experiments.

As shown in Figure 6, the Fas/Fc protein was not effective in blocking anti-CD95 agonistic antibody-stimulated apoptosis. These results indicate that AP-1 activation, and subsequent CD95L induction and apoptosis, are not crucial events in CD95 signaling pathway and exclude the existence of an autocrine loop amplifying the death signal, at least in the experimental model tested. Furthermore, while AP-1 is essential for NO to inhibit apoptosis in certain models [Melino et al., 2000], this is not true for all systems. This indicates that, while NO has many targets in the apoptotic machinery, the relevant/crucial events differ among the cellular models and contexts used.

## DISCUSSION

In this paper, we show that exogenous sources of NO-related species may influence the apoptotic pathway elicited by CD95 cross-linking. Indeed, we found that the NO-donor SNAP strongly inhibited apoptotic cell death in a concentration-dependent manner. Moreover, NO-releasing compounds were able to reduce anti-CD95 antibody-induced caspase 3-like activation.

AP-1 activation participates in the induction of apoptosis triggered by cytokines [Verheij et al., 1996; Wilson et al., 1996], and various environmental and pharmacological stresses [Sawai et al., 1995; Chen et al., 1996; Verheij et al., 1996; Herr et al., 1997; Kasibhatla et al., 1998]. The role of JNK pathway in CD95-triggered apoptosis is controversial because it is not hindered by disruption of the JNK cascade [Lenczowsky et al., 1997]. AP-1 function was enhanced in our experimental model after CD95 ligation. This was an unexpected result, since there is strong evidence in the literature that CD95-triggered apoptosis does not require transcription. The same NO-donor concentrations which protected from cell death, also blocked AP-1 DNA binding activity and CD95L promoter-driven transcription. However, in this model, the absence of interference with the CD95/CD95L interaction by employing a recombinant Fas-Fc protein, indicated that anti-CD95-induced apoptosis does not seem to depend on AP-1-regulated CD95L expression. Therefore, the autocrine circuit may be a secondary event in cell death triggered by CD95 ligation, while it may be essential in other cellular models, such as chemotherapic drug-induced apoptosis, or pathologies, in which the leading death pathway is less efficient, and therefore requires to be potentiated.

The presented results indicate that despite the fact that nitroso compounds inactivate AP-1, this is not a relevant target in the inhibition of CD95-triggered apoptosis. This is in marked contrast with our recent data, indicating that AP-1 was the relevant target of NO in both etoposide- and ceramide-induced apoptosis [Melino et al., 2000]. Taken together, NO has many different targets in the cell death machinery (see Fig. 5), but the relevant target to exert a biological action clearly depends on the cellular model and context. NO overproduction has been implicated in the pathogenesis of many disorders, including inflammatory and autoimmune diseases and cancer. The NO-mediated inhibition of CD95 signaling might play a major role in modulating immune responses, as well as in the pathogenesis of autoimmune diseases. Resistance to CD95-mediated cell death might also contribute to the development of some forms of cancer, such as lymphoid neoplasms which may express CD95 without undergoing apoptosis.

## ACKNOWLEDGMENT

We are grateful to Dr. Gianna Zambruno for the helpful assistance in electron microscopy.

## REFERENCES


Brendeford EM, Andersson KB, Gabrielsen OS. 1998. Nitric oxide (NO) disrupts specific DNA binding of the transcription factor c-Myb in vitro. FEBS Lett 425:52–56.

Brookes PS, Padilla-Salinas E, Darley-Usmar K, Eiserich JP, Freeman BA, Darley-Anderson PG. 2000. Concentration dependent effects of nitric oxide on mitochondrial permeability transition and cytochrome C release. J Biol Chem 275:20474–20479.

Brunner T, Mogil RJ, LaFace D, Yoo NJ, Mahboubi A, Echeverri F, Martin SJ, Forcem WR, Lynch DH, Ware CF, Green DR. 1995. Cell-autonomous Fas (CD95) interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature 373:441–444.

Catani MV, Bernassola F, Rossi A, Melino G. 1998. Inhibition of clotting factor XIII activity by nitric oxide. Biochem Biophys Res Commun 249:275–278.

Chen YR, Wang X, Templeton D, Davis R, Tan TH. 1996. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and $\gamma$ radiation. J Biol Chem 271:31929–31936.

De Nadai C, Sestili P, Cantoni O, Lievremont J-P, Sciorati C, Barsacchi R, Moncada S, Meldolesi J, Clementi E. 2000. Nitric oxide inhibits tumor necrosis factor-$\alpha$-induced apoptosis by reducing the generation of ceramide. Proc Natl Acad Sci USA 97:5480–5485.

Dimmeler S, Haendeler J, Nehls M, Zeiher AM. 1997. Suppression of apoptosis by nitric oxide by inhibition of ICE-like and CPP32-like proteases. J Exp Med 185:601–608.

Dimmeler S, Zeiher AM. 1999. Nitric oxide-an endothelial cell survival factor. Cell Death Differ 6:964–968.

Hausladen A, Privalle CT, Keng T, DeAngelo J, Stamler JS. 1996. Nitrosative stress: activation of the transcription factor Oxy R. Cell 86:719–729.

Herr I, Wilhelm D, Böhler T, Angel P, Debatin KM. 1997. Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. EMBO J 16: 6200–6208.

Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66:233–243.

Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A, Green DR. 1998. DNA damaging agents induce expression of Fas-Ligand and subsequent apoptosis in T lymphocytes via the activation of NF-$\kappa$B and AP-1. Mol Cell 1:543–551.

Kim YM, Talanian RV, Billiar TR. 1997. Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. J Biol Chem 272:31138–31148.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME. 1995. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins from a death-inducing signalling complex (DISC) with the receptor. EMBO J 14:5579–5588.

Kyriakis J, Banergee P, Nikolakaki E, Dai T, Rubie E, Ahmad M, Avruch J, Woodgett J. 1994. The stress-activated protein kinase subfamily of c-Jun kinases. Nature 369:156–160.

Lander HM, Ogiste JS, Pearce SF, Levi R, Novogrodsky A. 1995. Nitric oxide-stimulated guanine nucleotide exchange on $p21^{Ras}$. J Biol Chem 270:7017–7020.

Lee JH, Jang SI, Markova NG, Steinert PM. 1996. The proximal promoter of the transglutaminase 3 gene. J Biol Chem 271:4561–4568.

Leist M, Single B, Naumann H, Fava E, Simon B, Kuhnle S, Nicotera P. 1999. Nitric oxide inhibits execution of apoptosis at two distinct ATP-dependent steps upstream and downstream of mitochondrial cytochrome c release. Biochem Biophys Res Commun 258:215–221.

Lenczowsky JM, Dominguez L, Eder AM, King LB, Zacharchuk CM, Ashwell JD. 1997. Lack of a role for Jun kinase and AP-1 in Fas-induced apoptosis. Mol Cell Biol 17:170–181.

Li P, Nijhavan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479–489.

Li H, Zhu H, Xu CJ, Yuan J. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94:491–501.

Li J, Billiar TR. 1999. The anti-apoptotic actions of nitric oxide in hepatocytes. Cell Death Differ 6:952–955.

Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, Loscalzo J, Singel DJ, Stamler JS. 1993. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature 364:626–632.

Liu L, Stamler JS. 1999. NO: an inhibitor of cell death. Cell Death Differ 6:937–942.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. 1998. Bid, a Bcl-2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94:481–490.

Mannick JB, Hausladen A, Liu L, Hess DT, Zeng M, Miao QX, Kane LS, Gow AJ, Stamler JS. 1999. Fas-induced caspase denitrosylation. Science 284:651–654.

Matthews JR, Botting CH, Panico M, Morris HR, Hay RT. 1996. Inhibition of NF-$\kappa$B DNA binding by nitric oxide. Nucleic Acids Res 24:2236–2242.

Melino G, Annicchiarico-Petruzzelli M, Piredda L, Candi E, Gentile V, Davies PJ, Piacentini M. 1994. Tissue transglutaminase and apoptosis: sense and antisense transfection studies with human neuroblastoma cells. Mol Cell Biol 14:6584–6596.

Melino G, Bernassola F, Knight RA, Corasaniti MT, Nisticò G, Finazzi-Agrò A. 1997. S-nitrosylation regulates apoptosis. Nature 388:432–433.

Melino G, Bernassola F, Catani MV, Rossi A, Corazzari M, Sabatini S, Vilbois F, Green DR. 2000. Nitric oxide inhibits apoptosis via AP-1-dependent CD95L transactivation. Cancer Res 60:2377–2383.

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Emulson ME, Yamin TT, Yu VL, Miller DK. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37–43.

Nicotera P, Bernassola F, Melino G. 1999. Nitric oxide (NO), a signaling molecule with a killer soul. Cell Death Differ 6:931–933.

Nikitovic D, Holmgren A, Spyrou G. 1998. Inhibition of AP-1 DNA binding by nitric oxide involving conserved cysteine residues in Jun and Fos. Biochem Biophys Res Commun 242:109–112.

Piacentini M, Fesus L, Melino G. 1993. Multiple cell cycle access to the apoptotic death programme in human neuroblastoma cells. FEBS Lett 320:150–154.

Roda A, Girotti S, Motolese G, Ghini S, Ferri E, Ursini F, Maiorino M. 1993. In: Szalay AA, Kricka LJ, Stanley P, editors. Proceedings of the VIIth International Symposium on Bioluminescence and Chemiluminescence. Chichester: John Wiley and Sons.

Salvesen GS, Dixit VM. 1997. Caspases: intracellular signaling by proteolysis. Cell 91:443–446.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning. A laboratory manual. 2nd edition, Cold Spring Harbor Laboratory Press.

Sawai H, Okazaki T, Yamamoto H, Okano H, Takeda Y, Tashima M, Sawada H, Okuma M, Ishikura H, Umehara H. 1995. Requirement of AP-1 for ceramide-induced apoptosis in human leukemia HL-60 cells. J Biol Chem 270:27326–27331.

Schlegel J, Peters I, Orrenius S, Miller DK, Thornberry NA, Yamin TT, Nicholson DW. 1996. CPP32/Apopain is a key interleukin 1β converting enzyme-like protease involved in Fas-mediated apoptosis. J Biol Chem 271:1841–1844.

Schreiber E, Muller MM, Schaffner W. 1989. Rapid detection of octamer-binding proteins with "mini-extracts", prepared from a small number of cells. Nucleic Acids Res 17:6419.

So HS, Park RK, Kim MS, Lee SR, Jung BH, Chung SY, Jun CD, Chung HT. 1998. Nitric oxide inhibits c-Jun N-terminal kinase 2 (JNK2) via S-nitrosylation. Biochem Biophys Res Commun 247:809–813.

Stamler JS, Singel DJ, Loscalzo J. 1992a. Biochemistry of nitric oxide and its redox-activated forms. Science 258:1898–1902.

Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ, Loscalzo J. 1992b. S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. Proc Natl Acad Sci USA 89:444–448.

Stamler JS. 1994. Redox signaling: nitrosylation and related target interactions of nitric oxide. Cell 78:931–936.

Stamler JS, Hausladen A. 1998. Oxidative modifications in nitrosative stress. Nat Struct Biol 5:247–249.

Stefanelli C, Bonavita F, Stanic' I, Mignani M, Facchini A, Pignatti C, Flamigni F, Caldarera CM. 1998. Spermine causes caspase activation in leukaemia cells. FEBS Lett 437:233–236.

Suda T, Takahashi T, Golstein P, Nagata S. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the TNF family. Cell 75:1169–1178.

Tabuchi A, Sano K, Oh E, Tsuchiya T, Tsuda M. 1994. Modulation of AP-1 activity by nitric oxide (NO) in vitro: NO-mediated modulation of AP-1. FEBS Lett 351:123–127.

Takahashi T, Tanaka M, Inazawa J, Abe T, Suda T, Nagata S. 1994. Human Fas Ligand: gene structure, chromosomal localization and species specificity. Int J Immunol 6:1567–1574.

Trauth BC, Klas C, Peters AMJ, Matzku S, Moller P, Falk W, Debatin KM, Krammer PH. 1989. Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 245:301–305.

Verheij M Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN. 1996. Requirement for ceramide-initiated SAPK-JNK signalling in stress-induced apoptosis. Nature 380:75–79.

Wilson DJ, Fortner KA, Lynch DH, Mattingly RR, Macara IG, Posada JA, Budd RC. 1996. JNK, but not MAPK, activation is associated with Fas-mediated apoptosis in human T cells. Eur J Immunol 26:989–994.

Xu L, Eu JP, Meissner G, Stamler JS. 1998. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. Science 279:234–237.

Yang X, Chang HY, Baltimore D. 1998. Autoproteolytic activation of pro-caspases by oligomerization. Mol Cell 1:319–325.

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90:405–413.