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Inhibition of Caspase-3 by S-Nitrosation and Oxidation Caused by Nitric Oxide

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Apoptotic signaling cascades converge in the activation of caspases (interleukin-1β converting enzyme like proteases). Treatment of the human promyelocytic leukaemia cell line U937 with actinomycin D resulted in the activation of caspase-3 also known as CPP32. Protease activity was measured in cytosolic extracts by fluorometric analysis of the time-dependent cleavage of acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC), a caspase-3 substrate. Caspase activity was inhibited by thiol modifying agents such as N-ethylmaleimide or iodoacetamide and NO donors such as S-nitrosoglutathione (GSNO), BF_4NO, and spermine-NO. NO-mediated enzyme inhibition was fully reversible upon the addition of DTT (dithiothreitol). $NO^{\bullet}$ itself was not primarily responsible for downregulation of caspase-3, as we found no correlation between rates of $NO^{\bullet}$ release and the magnitude of enzyme inhibition. It is likely that S-nitrosation accounts for enzyme inhibition by various NO donors. SIN-1 and peroxynitrite were inhibitory as well. In this case, however, enzyme activity was not restored upon DTT addition, suggesting oxidation as an additional thiol modification mechanism. Our studies provide evidence that caspases are targeted by NO via S-nitrosation and oxidation of critical thiol groups.

Apoptotic cell death is characterized by morphological and biochemical changes (1,2). Initiation of programmed cell death is achieved by the action of drugs or toxins (3,4), while the executive phase of apoptosis includes the processing of proteases (for references, 5-8). In mammalian cells multiple cDNAs encode ICE-related proteases that are termed "caspases" based on two catalytic properties of these enzymes (9), a cysteine protease activity, and a specificity for cleavage after aspartic acid residues. Caspases are broadly divided into subdivisions, based on sequence homology. There are proteins resembling ICE (caspase-1) and enzymes more closely resembling CPP32/Yama/apopain (caspase-3) (for review, 6,10). All members of the ICE/Ced-3 family are found as inactive zymogens that are activated by proteolytic cleavage to the active dimeric or tetrameric species. Proteases may act in either a sequential or a parallel manner (11) and cleave multiple substrates (5,6,12).

NO is recognized for its participation in diverse biological processes in nearly all aspects of life (13-16). Signal transduction pathways for NO are classified as either cyclic GMP-dependent or -independent (17). The cyclic GMP-independent mechanisms are more closely associated with nitration, nitrosation, and oxidation (18,19). In this regard S-nitrosation of proteins (20), covalent protein modifications in the presence of NAD^+ (21), and peroxynitrite ($ONOO^-$)-derived reactions have received significant attention (22). S-nitrosothiols are common in biological systems, and they might serve as bioactive reservoirs of NO that target reactive sulfhydryl centers. Heterolytic pathways of S-nitrosothiol decomposition have been invoked in the allosteric regulation of receptors, the antimicrobial effects of S-nitrosothiols, the inhibition of sulfhydryl containing enzymes, and the modification of transcription factors (18,23).

With the notion that S-nitrosation may be a regulating mechanism of cellular function, we reasoned that the active sulfhydryl center of caspase enzymes may represent a potential target for NO-mediated protein modification. Our present information indicates that caspases are targeted by oxidative and nitrosative modifications.

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Abbreviations: GSNO, S-nitrosoglutathione; NO, nitric oxide; Act D, actinomycin D; DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; AMC, aminomethylcoumarin; SIN-1, 3-morpholinosydnonimine; BF_4NO, nitrosonium terafluoroborate; DTT, dithiothreitol.

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EXPERIMENTAL PROCEDURES

Materials. Spermine-NO was provided by Biotrend. SNP, GSH, HEPES, PMSF, pepstatin, and leupeptin were purchased from Sigma. Detergents came from Fluka. DEVD-AMC was bought from Biomol. The anti CPP32 antibody was from Transduction laboratories, while anti-rabbit IgG was from BioRad. RPMI 1640, cell culture supplements, and foetal calf serum were ordered from Biochrom. All other chemicals were of the highest grade of purity commercially available.

Cell culture. The human promyelocytic leukaemia cell line U937 was maintained in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated foetal calf serum (complete RPMI). All experiments were performed using complete RPMI. For caspase-3 activation, cells were incubated with actinomycin D (Act D) or S-nitrosoglutathione (GSNO) as indicated. To test caspase-3 inhibition by GSNO on a cellular level, cells were treated with Act D (1 μg/ml) for 6 h before GSNO (1 mM) was added for an additional 2 h period. Caspase-3 activity was determined in the cytosolic cell fraction.

Preparation of cell lysates. Cells (4×10^6) were incubated with GSNO or Act D for 8 h (otherwise as indicated), recovered from culture plates, and centrifuged (1200 $\times$ g, 4°C, 5 min). Cell pellets were resuspended in lysis buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mM EDTA) and left on ice for 30 min. Following sonification (10 sec, Heart System sonicator, 10% pulse) lysates were centrifuged (10000 $\times$ g, 10 min, 4°C) and stored at −20°C. Protein determinations were performed with the BioRad assay.

GSNO and peroxynitrite synthesis. GSNO (S-nitroso derivative of glutathione) was synthesized as described previously (24). Solutions were prepared fresh, prior to the experiment. Peroxynitrite was synthesized as indicated previously (25) and determined by UV-absorbance spectroscopy at 302 nm in 1N NaOH.

Fluorogenic caspase-3 assay. Cell lysate (50 μg protein) was incubated in 100 mM HEPES, pH 7.5, 10% sucrose, 0.1% Chaps, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin at 30°C with 20 μM of DEVD-AMC in a total volume of 0.5 ml. Substrate cleavage was followed fluorometrically with excitation at 360 nm and emission at 460 nm. Inhibitors were added 20 min prior to DEVD-AMC.

Caspase-3 western blot analysis. Cleavage of caspase-3 holoenzyme was detected in cell lysates by western blot analysis. Following protein separation in 15% SDS-gels, proteins were transblotted onto nitrocellulose sheets. Membranes were washed 3 times with phosphate-buffered saline (PBS)/0.1% Tween-20 and blocked in 5% non fat dry milk/PBS/0.1% Tween-20 for 2h. The monoclonal antibody directed against CPP32 (1/1000 dilution) was added and membranes were incubated overnight at 4°C. The blot was then washed 5 times with PBS/0.1% Tween-20, and incubated with a horseradish peroxidase conjugated anti-rabbit IgG antibody for 1 h, followed by ECL-detection.

Apoptotic index. Following incubations, U937 cells (4×10^6) were centrifuged at 1200 $\times$ g at 4°C, washed once with 5 ml ice-cold PBS, permeabilized, and fixed in 1 ml 80% ice-cold ethanol on ice for 30 min. Following centrigugation (3000 $\times$ g, 4°C, 5 min), cells were washed with 1 ml PBS, and stained overnight at 4°C with propidium iodide in PBS/0.1% Trition X-100. The apoptotic index was measured by an ELITE profile fluorescence activated cell sorter (Coulter) using a cell cycle analysis doublet discrimination protocol.

The oxyhemoglobin assay. Detection of NO-release from NO donors is based on the reaction of oxyhemoglobin with NO to form methemoglobin and nitrate as described elsewhere (26). For NO release studies it was important to have a linear correlation between methemoglobin formation and NO donor decomposition.

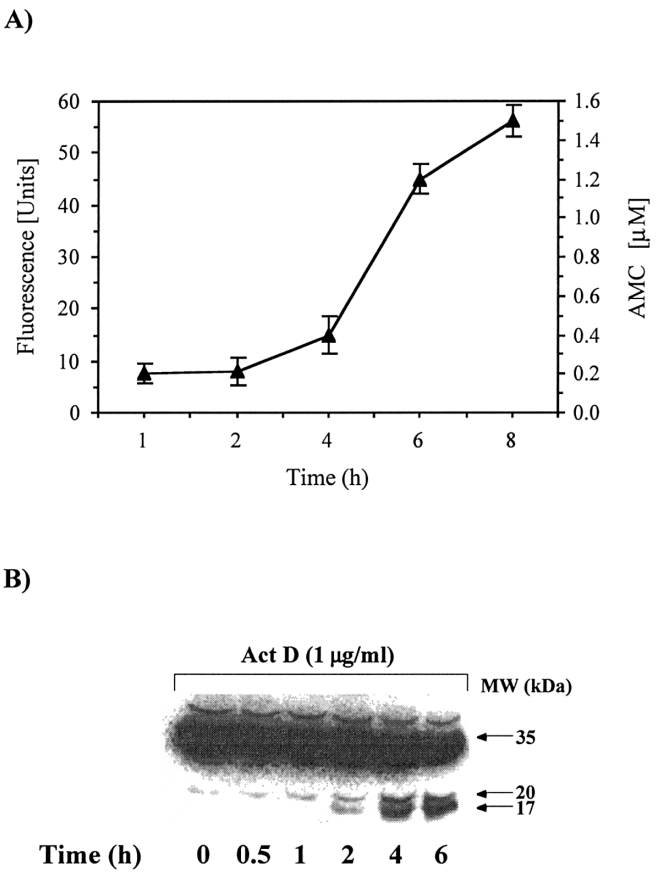

FIG. 1. Activation of caspase-3 by actinomycin D. A: U937 cells were treated with Act D (1 μg/ml) for different times as indicated. DEVD-AMC cleavage was measured in the cell lysates as described in Experimental Procedures. Values are means ± S.D. of four experiments. B: Proteolytic digestion of caspase-3 was followed by western blot analysis in cell lysates of Act D stimulated cells. Positions of the holoenzyme (35 kDa) and cleaved fragments (20 and 17 kDa) are indicated by arrows. The western blot is representative of three similar experiments.

Statistical analyses. Each experiment was performed at least three times and statistical analysis was performed using the two tailed Student's t-test. In some cases, a representative experiment is shown.

RESULTS

Actinomycin D (Act D) is a commonly used apoptotic initiating agent. Incubation of U937 cells with Act D resulted in activation of caspases. Protease activation was measured by following the cleavage of the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC) in the cytosolic fraction of activated cells. Accumulation of the fluorescent aminomethylcoumarin portion of DEVD-AMC was quantitated and correlated to enzyme activity. Act D activated caspase-3 in a time-dependent manner. Protease activity was significantly elevated 3 h after stimulation and reached a plateau after 6-8 h (Figure 1A). This was

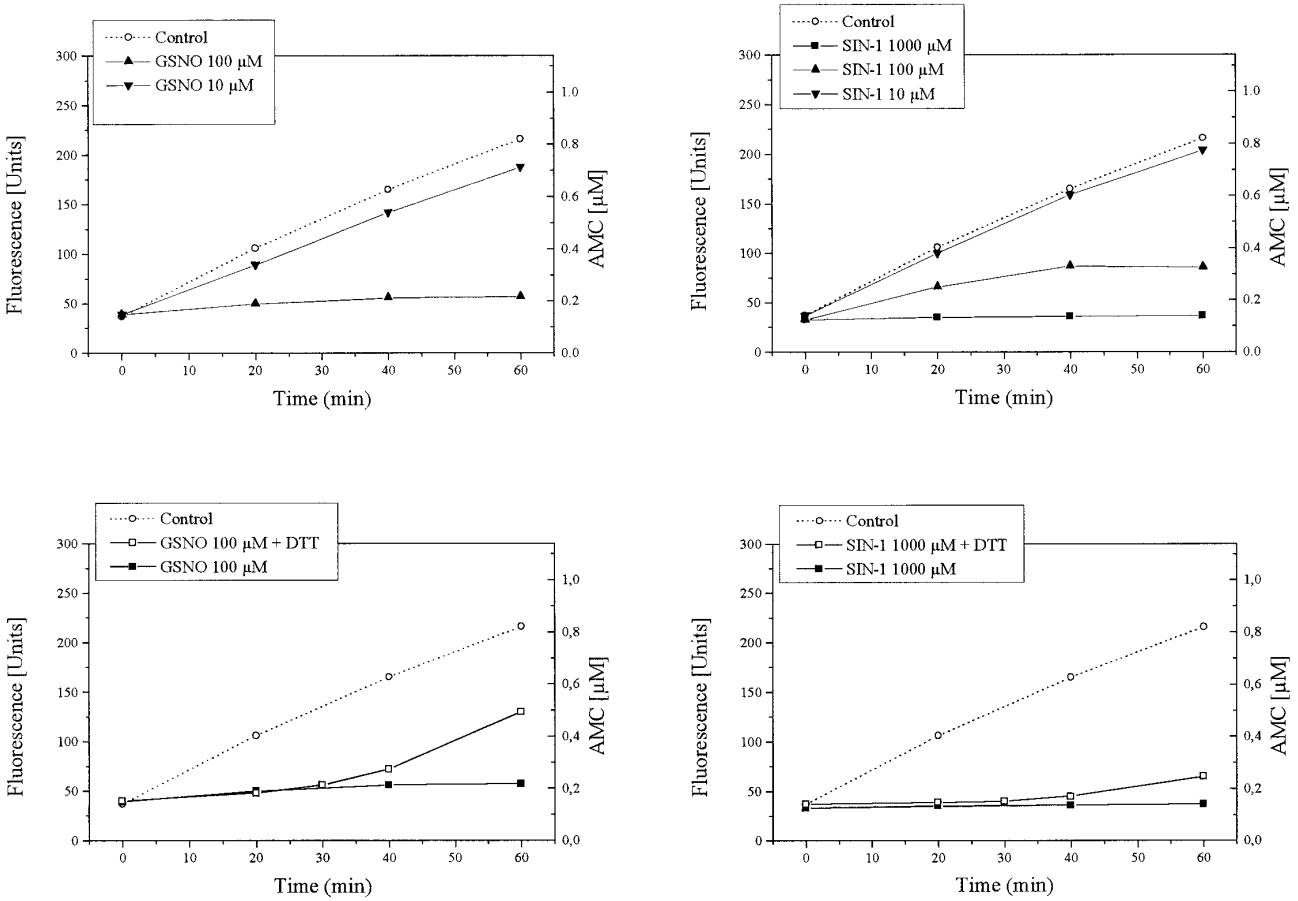

FIG. 2. Inhibition of caspase-3 by GSNO and SIN-1 and its reversion by DTT. U937 cells were treated with Act D (1 μg/ml) for 6 h in order to activate caspase-3. Enzymatic activity was measured as described under Experimental Procedures by DEVD-AMC cleavage in the presence of the GSNO or SIN-1. DTT (10 mM) was added at 30 min and DEVD-AMC cleavage was followed for an additional 30 min. Values are representative of four experiments.

closely correlated by cleavage of caspase-3 holoenzyme (35 kD) to the active form (17 kD) and an additional fragment (20 kD), as determined by western blot analysis (Figure 1B).

Proteolytic processing of caspase-3 and its enzymatic activity was in good agreement. Further analysis established potent inhibition of Act D-activated caspase-3 by the selective CPP32 inhibitor DEVD-CHO. Incubation of intact U937 cells with 10 μM DEVD-CHO fully blocked activation of caspase-3, when assayed in the cytosolic fraction of Act D-treated cells (data not shown). Moreover, DEVD-CHO produced half maximal inhibition of enzyme acticvity at 100 pM when assayed in lysates from cells treated with Act D. YVAD, a caspase-1 inhibitor produced no inhibition.

It is known that a reduced thiol group in the active site of caspase-3 is essential for its catalytic activity. Therefore, we employed typical thiol modulating agents such as N-ethylmaleimide or iodoacetamide. Inhibitors were added to a cytosolic fraction obtained from Act D treated cells. Both N-ethylmaleimide (1 μM) and iodoacetamide (1 μM) blocked DEVD-AMC cleavage completely. Activation of caspase-3 was achieved by incubating U937 cells for 6 h with 1 μg/ml Act D; enzymatic activity was then measured in the cytosolic fraction after addition of GSNO, spermine-NO, BF_4NO, peroxynitrite, or SIN-1. GSNO dose-dependently inhibited DEVD-AMC cleavage (Figure 2). A concentration of 10 μM GSNO blocked caspase-3 activity by 10-20%, while 100 μM GSNO produced more than 90% inhibition.

In similar experiments, SIN-1 efficiently interfered with caspase-3 activity (Figure 2), although inhibition was less pronounced as compared to GSNO. A concentration of 10 μM SIN-1 did not block caspase-3 activity. An intermediate inhibition was achieved with 100 μM SIN-1, while 1 mM was required for complete enzyme inhibition.

After establishing NO-mediated caspase-3 inhibition experiments we examined the reversible nature of those modifications. Protease inhibition by 100 μM GSNO is fully reversible upon addition of 10 mM DTT,

TABLE 1

NO-Mediated Caspase-3 Inhibition in Its Correlation to $NO^{\bullet}$ Release

	Concentration [μM]	% Inhibition (at 60 min)	NO release [$\mu M \times min^{-1}$]
GSNO	10	16 ± 4	n.d.
	100	100	0.019 ± 0.002
Spermine-NO	100	25 ± 3	n.d.
	1000	100	0.425 ± 0.08
BF_4NO	100	20 ± 4	n.d.
	1000	100	n.d.
$ONOO^-$	10	70 ± 2	n.d.
	100	100	n.d.
SIN-1	100	65 ± 3	n.d.
	1000	100	0.186 ± 0.07

Note. Caspase-3 enzyme activity was assayed in the cytosolic fraction of Act D treated cells in the presence of different NO donors. Enzymatic activity was compared to an uninhibited control (100% activity). NO release was measured by the hemoglobin assay as outlined in Experimental Procedures. n.d., not determined.

as the rate of enzyme activity after the addition of DTT became indistinguishable as compared to controls (Figure 2). Conversely, it was noticed that caspase-3 inhibition with SIN-1 was not reversed by DTT (Figure 2).

Spermine-NO, BF_4NO, and $ONOO^-$ caused inhibition of capase-3 as well (Table 1). Spermine-NO fully block enzyme activity at a concentration of 1 mM, whereas 100 μM produced roughly 50% inhibition. BF_4NO produced complete inhibition at 1 mM, although effective inhibitory concentrations probably were much lower, considering the rapid hydrolysis of this compound in water. Inhibition by spermine-NO and BF_4NO were reversed following DTT addition (data not shown). Peroxynitrite (100 μM) fully blocked enzyme activity, and a 10 μM concentration of $ONOO^-$ produced half maximal inhibition of the enzyme. In this case enzymatic activity was not restored upon DTT addition.

In further studies we sought to correlate the amount of $NO^{\bullet}$ generated by the different NO donors and caspase-3 inhibition. Release of $NO^{\bullet}$ from the NO donors was quantitated by oxidation of oxyhemoglobin to methemoglobin. Decomposition of GSNO, spermine-NO, and SIN-1 revealed a discrepancy between methemoglobin formation and their ability to block caspase activity (Table 1). GSNO, the most potent enzyme inhibitor released the lowest amounts of $NO^{\bullet}$, whereas spermine-NO, which generated much more $NO^{\bullet}$ produced a roughly 10-fold weaker inhibition. Although SIN-1 and spermine-NO produced comparable enzyme inhibition at 100 μM, the generation of $NO^{\bullet}$ from SIN-1 was half of that produced by spermine-NO.

Incubation of U937 cells with Act D for 6 to 8 h caused cleavage of the caspase-3 holoenzyme and subsequent protease activation. After initial Act D challenge for 6 h, 1 mM GSNO was applied for another 2 h period. Exogenously supplied GSNO inhibited the activity of caspase-3 by approximately 50%, which is not as high as the suppressive potency of GSNO observed in cytosolic extracts. However, exposure of U937 cells for 8 h to increasing concentrations of GSNO in the absence of Act D resulted in caspase-3 processing to the active 17 kD fragment as seen by western blot analysis (Figure 3).

Evidently, GSNO promoted caspase-3 activation rather than inhibition in intact cells.

DISCUSSION

NO affects different targets, thus resulting in the formation of surrogates that retain NO-like bioactivity. This is exemplified in the case of S-nitrosothiols (18). The ability of S-nitrosothiols to undergo decomposition assures reversibility of thiol modification and provides the basis for biological actions. Another important biological reaction of $NO^{\bullet}$ is its coupling with O_2^-, which results in the extremely rapid production of peroxynitrite (27). Peroxynitrite mediates the oxidation sulfhydryls, sometimes with an arsenite nonreducible product, suggesting oxidation of thiols beyond sulfenic acid (28). Therefore, enzymes that contain critical thiols at their active site, such as caspases, can be modified by the covalent attachment of the NO group or by oxidative reactions.

In our studies, we have examined catalytically required cysteine residue of caspase-3 as a potential NO target. Inhibition of cysteine protease activity was initiated with a diverse class of NO donors/NO-derived species and was confirmed with classical thiol blocking agents such as N-ethylmaleimide or iodoacetamide. The critical SH-group in caspase-3 was affected by NO but mechanistic differences emerged when we performed reversibility studies by DTT addition. Enzyme inactivation by BF_4NO indicates a NO^+-mediated S-nitrosation mechanism (NO^+ transfer), as the agent decomposes exclusively to NO^+ and $[BF_4]^-$. Excessive DTT addition reversed enzyme inhibition, thereby regenerating a catalytically active center in caspase-3.

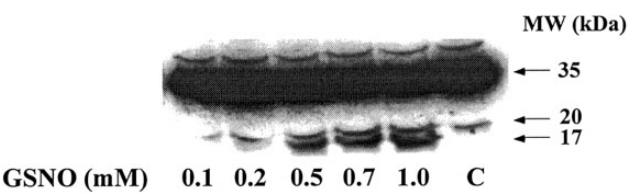

FIG. 3. Activation of caspase-3 by GSNO. U937 cells were treated with increasing concentrations of GSNO for 8 h. Proteolytic digestion of caspase-3 was followed by western blot analysis in cell lysates of GSNO-stimulated cells. Positions of the holoenzyme (35 kDa) and cleaved fragments (20 and 17 kDa) are indicated by arrows. The western blot is representative of three similar experiments.

Enzyme activity blocked by GSNO and spermine-NO most likely resembles a similar inhibition profile because DTT in both cases restored an active enzyme. The effectiveness of GSNO is best explained by a transnitrosation mechanism transferring NO^+ from GSNO to the protein thiol group. The fact that NO^+ rather than $NO^\bullet$ is involved in thiol modification is supported by studies showing that the efficacy of various NO donors at inducing caspase-3 modification seemed independent of rates of $NO^\bullet$ release (Table 1).

SIN-1- and $ONOO^-$- mediated caspase-3 inhibition revealed a different mechanism, since excess DTT did not reverse enzyme activity. Thiol oxidation may result in disulfide formation or higher oxidation states such as sulfenic, sulfinic, or sulfonic acids. Associated oxidation states of 0, +2, and +4 are not any longer reversed by reducing agents such as DTT. Therefore, our data substantiate NO-mediated thiol targeting by two different mechanisms which involved DTT-reverible and -irreversible modifications.

This study shows that treatment of U 937 cells with either Act D or GSNO causes cleavage of the caspase-3 proform with a concomitant formation of the active 17 kDa subunit (Figure 1 and 3). This clearly indicates caspase activation rather than inhibition upon NO exposure. However, if sequential treatment of these cells is performed with Act D and GSNO, the 17 kDa active subunit of caspase-3 generated by Act D will then be partially inhibited by GSNO. However, the high NO donor concentrations required for enzyme inhibition preclude predictions on the importance of caspase inhibition by endogenous NO formation. Notably, our results indicate that NO negatively affects the activity of the 17 kDa subunit of caspase-3 but not the holoenzyme. Even if NO affects the cysteine of the proform, this has no influence on the cleavage and activity of the 17 kDa subunit. The thiol group of the proform and the active 17 kDa subunit might have different pKa's, therefore influencing the affinity of these thiol groups for NO^+. Further, a variable sensitivity of cellular systems towards NO has been previously suggested. While NO induces apoptosis in some cells, it blocks apoptosis in others (29,30,31). It would be important to know the effect of NO on caspase modification in those cells in which NO inhibits apoptosis like lymphocytes, hepatocytes, or endothelial cells (32). Caspase modification by NO under cellular conditions remains to be established and may be important during conditions of oxidative stress thus leading to modification of the critical thiol group or in cells that contain a low antioxidative potential and are not able to reverse S-nitrosation of proteins.

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