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Review

Nitric oxide can inhibit apoptosis or switch it into necrosis

G. Melino*, M. V. Catani, M. Corazzari, P. Guerrieri and F. Bernassola

IDI-IRCCS, Biochemistry Lab, c/o Department of Experimental Medicine, D26/F153, University of Rome Tor Vergata, Via Tor Vergata 135, I-00133 Rome (Italy), Fax + 39 6 20427290, e-mail: gerry.melino@uniroma2.it

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Abstract. Nitric oxide (NO) and its related molecules are important messengers that play central roles in pathophysiology. Redox modulation of thiol groups on protein cysteine residues by S-nitrosylation can modulate protein function. NO has emerged as a potent regulator of apoptosis in many cell types, either preventing cell death or driving an apoptotic response into a necrotic one. NO protects neuroblastoma cells from retinoid- and cisplatin-induced apoptosis, without significantly increasing necrotic cell damage. Nitrosylation of thiol groups of several critical factors may be important for cell survival. Indeed, S-nitrosylation of the active-site cysteine residue of apoptotic molecules, such as caspases and tissue transglutaminase, results in the inhibition of their catalytic activities and has important implications for the regulation of apoptosis by NO. On the other hand, NO is able to shift the anti-CD95- and ceramide-triggered apoptotic response of Jurkat T cells into necrotic cell death. In these apoptotic models, NO is therefore unable to solely inhibit cell death, indicating that it may act below the point of no return elicited by CD95-ligation and ceramide stimulation.

Key words. Nitric oxide; S-nitrosylation; apoptosis.

Introduction

Nitric oxide (NO) is a hydrophobic gaseous molecule and highly diffusible free radical, generated from the oxidation of L-arginine to L-citrulline by a family of constitutive or cytokine-inducible nictonamide adenine dinucleotide phosphate (NADPH)-dependent isoenzymes, the NO synthases (NOS) (fig. 1). Neuronal NOS (nNOS) and endothelial NOS (eNOS), usually constitutively expressed, are transiently activated by elevation of intracellular Ca^{++} concentration. Unlike nNOS and eNOS, the cytokine-inducible NOS (iNOS) is transcriptionally regulated, producing NO at basal Ca^{++} levels for up to several days [1]. Besides NOS activation, a chemically heterogeneous group of spontaneously decomposing NO-donors preserve NO in their molecular structure and exhibit biological activity after NO liberation. NO-releasing compounds are valuable biochemical tools to investigate the signal transduction pathway of NO.

NO is an extremely versatile messenger in biological systems, and has been implicated in a number of different physiopathological roles, such as smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation, cell differentiation and tissue morphogenesis [2], and cytotoxicity [3]. Cell death elicited by endogenously derived NO plays central role in pathologic phenomena such as septic shock, nonspecific 'host' defense against tumours and intracellular pathogens, acute and chronic neurodegenerative diseases, pancreatic β-cell destruction in diabete mellitus and transplant rejection [4–8].

NO is highly reactive, and its half-life is short in biological systems. The chemistry of NO involves interrelated redox forms ($NO^{\bullet}$, NO^{+}, NO^{-}), which show different chemical reactivity toward various target groups, thus

* Corresponding author.

explaining the pleiotropic effects of NO in biological systems (see fig. 1) [9].

$NO^{\bullet}$ reacts with molecular oxygen, superoxide anion and transition metal cations, leading to the formation of reactive nitrogen intermediates that directly or indirectly support additional nitrosative reactions (fig. 1A). In particular, $NO^{\bullet}$ binds to hemoproteins and iron-sulfur centers. The nitrosonium cation (NO^+) character found in nitroso compounds in biological systems is involved in nitrosation reactions with nucleophilic groups such as thiols, amines, carboxyls, hydroxyls and aromatic rings (fig. 1A). The nitroxyl anion (NO^-) rapidly undergoes dimerization and dehydration, thus generating dinitrogen oxide, in addition to reacting with thiols leading to sulfhydryl oxidation and with metals (fig. 1A).

NO-mediated signaling pathways are classified as either cyclic guanylic acid (cGMP)-dependent or -independent (fig. 1B); cGMP formation results from NO binding to the heme group of soluble guanylyl cyclase, thus leading to enzyme activation. The cGMP-independent mechanisms include inhibition of iron-sulfur centers, DNA damage, poly(adenosine 5′-diphosphate-ribosylation) activation with cellular energy deprivation and protein modification due to S-nitrosylation of thiol groups, thiol oxidation to sulfenic and sulfinic acid and tyrosine nitration [10–13]. cGMP-independent NO-induced responses account for the antimicrobial, cytostatic and cytotoxic ability of NO (fig. 1B).

S-nitrosylation of cysteine residues is a common protein modification achieved by NO in the presence of oxygen and usually associated with loss of protein function.

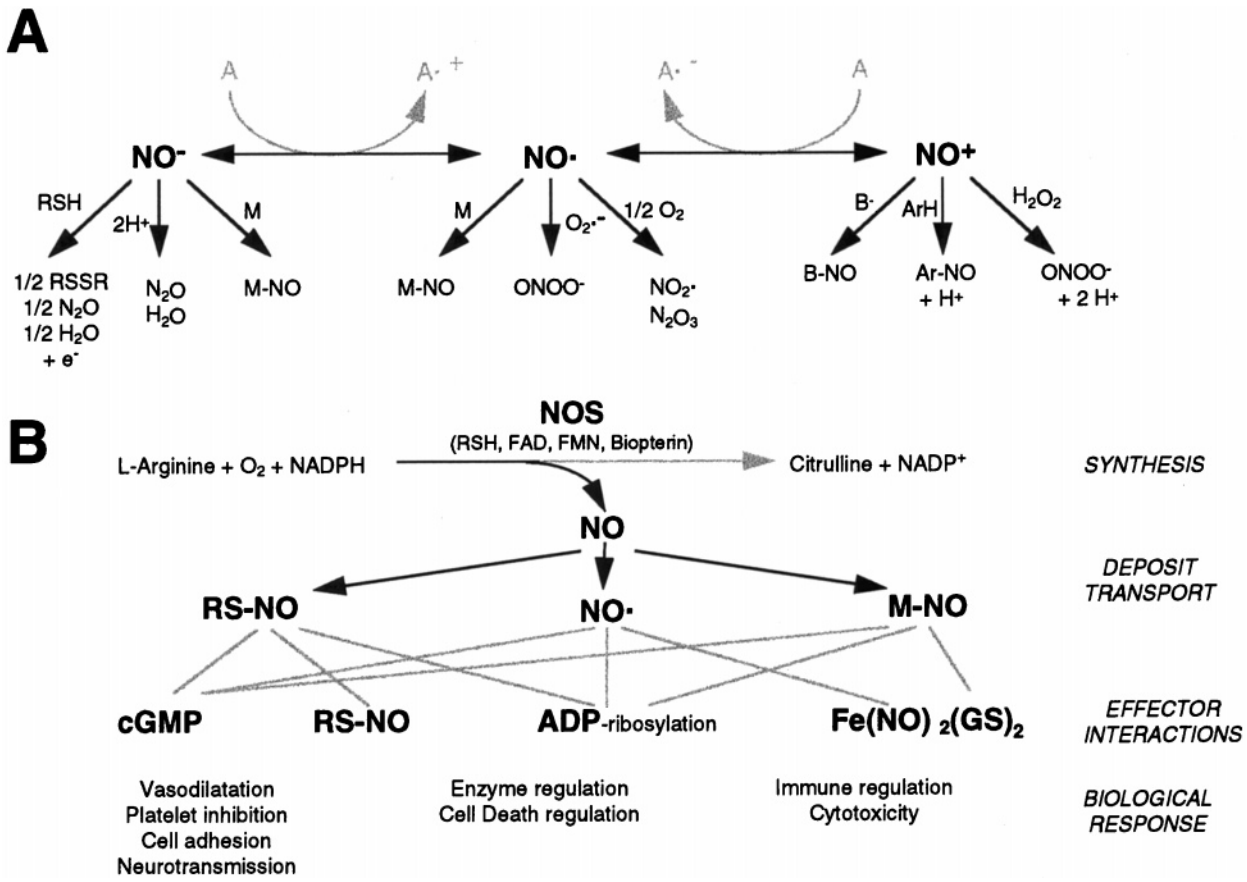

Figure 1. NO exists as three interconverting species, nitroxyl anion (NO^-), nitric oxide radical ($NO^{\bullet}$) and nitrosonium cation (NO^+), with 1^+, 2^+ and 3^+ nitrogen oxidation number, respectively. The chemical properties vary significantly with a bond length of 1.26 (NO^-), 1.15 ($NO^{\bullet}$) and 0.95 (NO^+), expressed in Å. Similarly, the N–O stretch is 1290 (NO^-), 1840 ($NO^{\bullet}$) and 2300 (NO^+) cm^{-1}. These strong differences in chemical properties result in very different reactions and biological effects. Panel A shows a simplified and schematic diagram of the redox-interrelated forms of NO, produced with metals (M), electron acceptors (A), thiols (RSH), aromatic substitutions (Ar), addition to bases (B^-), as well as reactions with oxygen, superoxide anion, hydrogen peroxide and NO^- itself. Panel B shows the synthesis (by three isoforms of NOS, nNOS, eNOS and iNOS), deposit and effector interactions of NO to produce a biological response. The major pathways of biological significance are (i) reactions with metals forming nitrosyl complexes, (ii) charge transfer to electron acceptors and (iii) coupling to thiol/disulfide redox reactions. Intracellular storage is mainly related to glutathione-NO (RS-NO) or diglutathionyl-dinitroso-iron [$Fe(NO)_2(GS)_2$], known as dinitrosyl iron complex (DNIC). The schemes are modified from Stamler et al. [9].

S-nitrosylation may regulate cellular signal transduction by influencing signaling proteins such as $p21^{ras}$ [14] and c-Jun N-terminal kinase 2 [15]. It affects the cellular gene transcription machinery by inhibiting the DNA binding activity of nuclear factor κB (NF-κB) [16] and activator protein-1 (AP-1) [17] or by activation of OxyR [18]. NO nitrosylates thiol groups of cytosolic enzymes such as tissue transglutaminase (tTG) [19], caspases [19, 20], glyceraldehyde-3-phosphate dehydrogenase [21] and hemoglobin [22, 23], and it can modulate the biological activity of extracellular molecules such as coagulation factor XIII [24]. As we will discuss later, S-nitrosylation can finely regulate cell death.

The role of NO in cell death

NO appears to be a bifunctional modulator of cell death capable of either stimulating or inhibiting cytotoxicity.

Mechanisms of NO inhibition of cell death

NO as a scavenger of radical species

NO has been proposed to be protective against cellular damage resulting from cerebral and myocardial ischemia/reperfusion. NO may exert its protective actions through the reaction with alkoxyl and peroxyl radicals, thus inhibiting lipid peroxidation [25]; it may suppress the superoxide/hydrogen peroxide-mediated cytotoxic effect by acting as a scavenger of reactive oxygen species (ROS) [3, 26, 27].

Induction of protective proteins

Exogenous NO or preinduction of iNOS has been shown to stimulate the expression of the inducible heat shock protein 70 (Hsp70) and subsequently prevent hepatocytes from tumour necrosis factor α (TNFα) and actinomycin D-induced apoptosis [28]. Even though it has been recently suggested that Hsp70 acts downstream of caspase activation [29], the mechanisms through which it suppresses apoptosis remain largely unknown. NO can also inhibit cell death by inducing the expression of other protective proteins such as cyclooxygenase-2 [3], heme oxygenase-1 [30, 31] and metallothionein [32]. Recent studies have demonstrated that protection from ultraviolet-A (UVA)-induced apoptosis is tightly correlated with NO-elicited upregulation of Bcl-2 expression [33].

cGMP-dependent mechanisms

In hepatocytes, NO prevents increases in caspase activity by a cGMP-dependent mechanism that does not involve S-nitrosylation [34, 35]. Furthermore, eNOS activation, which exerts a protective effect on the susceptibility of activated T lymphocytes to CD95-triggered apoptosis, acts via cGMP-dependent pathways [36]. The effect of cGMP on apoptosis seems to be mediated through cGMP-dependent kinase [34].

S-nitrosylation

S-nitrosylation of several critical factors may be important for cell survival. Protection from excitotoxic *N*-methyl-D-aspartate (NMDA)-mediated neuronal cell death has been demonstrated to be associated with downregulation of excessive NMDA receptor (NMDAR) activity following S-nitrosylation of the receptor [37, 38]. Protective actions from apoptotic pathways have been implicated for NO. Figure 2 shows several protein targets of the apoptotic machinery, which may be potentially inactivated by NO through direct S-nitrosylation of catalytic cysteine residues. NO may interfere with the downstream apoptotic machinery acting either at the level of the executioner caspases and tTG, or upstream of these events to inhibit initiator caspases such as caspase 8 and transcriptional factors such as AP-1 and NF-κB.

We have demonstrated that exogenous NO inhibits CD95 signaling in Jurkat T cells via S-nitrosylation of caspase 3-like proteases [19]. Other studies have shown NO-mediated inactivation of caspases in apoptotic models such as CD95-ligated human leukocytes [39] and TNFα-, proatherosclerotic factor-, and ROS-stimulated rat hepatocytes and endothelial cell lines [34, 35, 40]. The effects of NO can directly inhibit caspase activity by S-nitrosylation of the active-site cysteine residue [20, 41]. Kim et al. [42] have reported that both Bcl-2 cleavage and cytochrome c release are inhibited in TNFα- and actinomycin D-treated MCF-7 adenocarcinoma cells exposed to NO-donors. This indicates that NO is able to suppress a mechanism for the initiation or amplification of apoptotic signaling, leading to a failure to cleave death substrates, such as DNA fragmentation factor/inhibitor of caspase-activated DNase (DFF/ICAD), with subsequent inhibition of apoptosis [43]. Therefore, NO may suppress both upstream and downstream caspase activation. A recent study has shown that caspase zymogens are endogenously S-nitrosylated in unstimulated human lymphocyte cell lines, and that CD95 ligation activates caspase-3 also by stimulating the denitrosylation of its catalytic site cysteine [44, 45]. In previous studies we have shown that both purified and cellular tTG, whose activation leads to the irreversible assembly of a cross-linked protein scaffold in cells undergoing apoptosis, are inactivated by NO [19]. The cysteine thiol active center is a prerequisite for tTG catalytic activity. Therefore, NO may regulate tTG en-

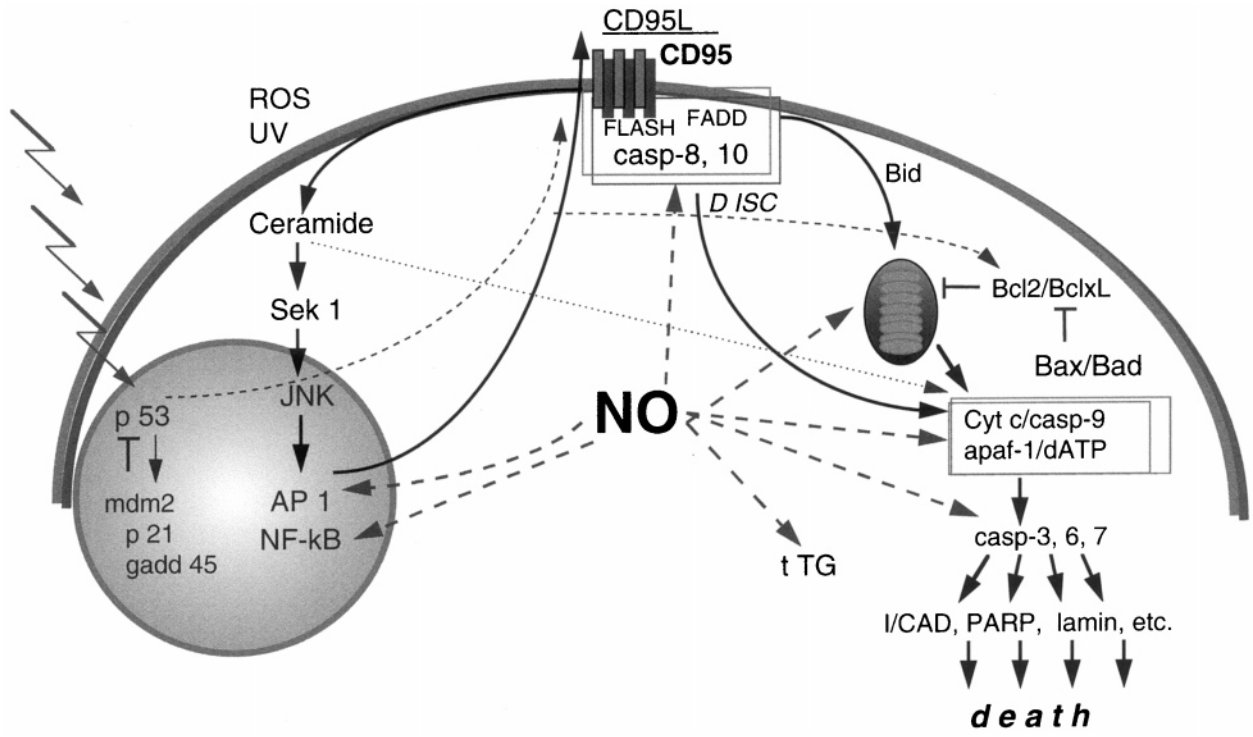

Figure 2. Schematic model of NO interference with several regulator/effector target proteins of the apoptotic program, which may be potentially inactivated by S-nitrosylation of their active cysteine residues. Cell exposure to different apoptotic stimuli (CD95L, TNFα, ceramides, ROS, DNA-damaging agents) may lead to the activation of two main pathways. For example, CD95 ligation results in downstream caspase activation via a direct or mitochondria-mediated activation, and production of ceramide which might activate the downstream caspases. NO may inhibit apoptotic molecules, including initiator and executioner caspases, tTG and transcriptional factors such as AP-1 and NF-κB, or directly inactivate the mitochondrial respiratory chain, thus preventing apoptosis.

zymatic activity via S-nitrosylation of its crucial sulfhydryl group.

NO protects from retinoic acid- and cisplatin-induced apoptosis in human neuroblastoma cells

We have demonstrated that apoptosis induced by retinoids or chemotherapeutic agents such as cisplatin in human neuroblastoma cells is associated with an increase of tTG activity [46, 47]. To investigate the antiapoptotic effect of NO in drug-induced cell death of neuroblastoma cells, we coincubated the SK-N-BE(2) cell line with the NO-donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 1 μM retinoic acid (RA) or 5 μM cisplatin, for 72 and 48 h respectively. As shown in figure 3, exposure of SK-N-BE(2) cells to the NO-donor dose-dependently reduced both RA- (panel A) and cisplatin-induced (panel B) apoptotic cell death. Therefore, the inhibition of tTG enzymatic activity observed in vitro [19] correlates with the ability of exogenously supplied NO to prevent the induction of apoptosis.

Although NO was able to protect neuroblastoma cells from drug-induced apoptosis, we could not detect a significant increase in RA- and cisplatin-induced lactate dehydrogenase (LDH) release by SNAP treatment (fig. 3C and D), indicating that in these two models, the NO-donors were able to prevent apoptosis without significantly enhancing necrosis.

Induction of cell death by NO

NO derived from spontaneously decomposing NO-donor compounds or endogenously produced by NOS enzymes causes cell death. Indeed, NO is implicated in both apoptotic and necrotic cell death depending on the chemistry that NO undergoes in a given cellular biological redox state. NO-mediated toxicity is also highly dependent on its concentration and on the exposure time to the stimulus. For example, severe energy depletion induced by acute exposure of neuronal cells to high concentrations of NO results in necrotic cell damage; in contrast, the long-lasting generation of relatively low doses of NO leads to neuronal injury with apoptotic features [48]. Cell susceptibility toward NO depends on the differential cellular ability to scavenge or to detoxify NO (e.g. intracellular glutathione, antioxidant enzymes), and/or on DNA repair mechanisms.

Mechanisms of NO-mediated necrotic cell damage

There are several potential mechanisms through which NO induces necrosis. Indeed, multiple target interactions, with thiol groups, heme, iron-sulfur and tyrosine residues containing proteins and DNA, have been implicated in NO-mediated cytotoxicity. Some of these effects may be direct, whereas others may arise from the reaction of NO with superoxide anion and, at high concentrations, with oxygen to form peroxynitrite ($ONOO^-$) and nitrosative agents, respectively. Cell damage by high concentrations or short-term exposure to NO has been associated with the inhibition of a number of cellular processes, such as DNA synthesis, mitochondrial respiration and metabolic reactions. For example, NO can react with ribonucleotide reductase, targeting both thiol groups and the tyrosyl free radical required for catalysis, thus inhibiting deoxyribonucleotide synthesis [49]. NO can disrupt mitochondrial function by reversibly inactivating cytochrome-c oxidase, thus stimulating superoxide anion production by the respiratory chain [50]. The resulting superoxide anion may be responsible, through the formation of $ONOO^-$, of irreversible inhibition of complexes I and III of the mitochondrial electron transport chain [51]. It has also been implicated in the inactivation of key metabolic enzymes such as aconitase, by forming iron-nitrosyl complexes with its Fe-S centers. Even though inhibition of aconitase does not necessarily induce adenosine 5′-triphosphate (ATP) depletion depending on the cell model, [52], it may lead to a reduction in glucose and amino acid metabolism and subsequent energy loss [53].

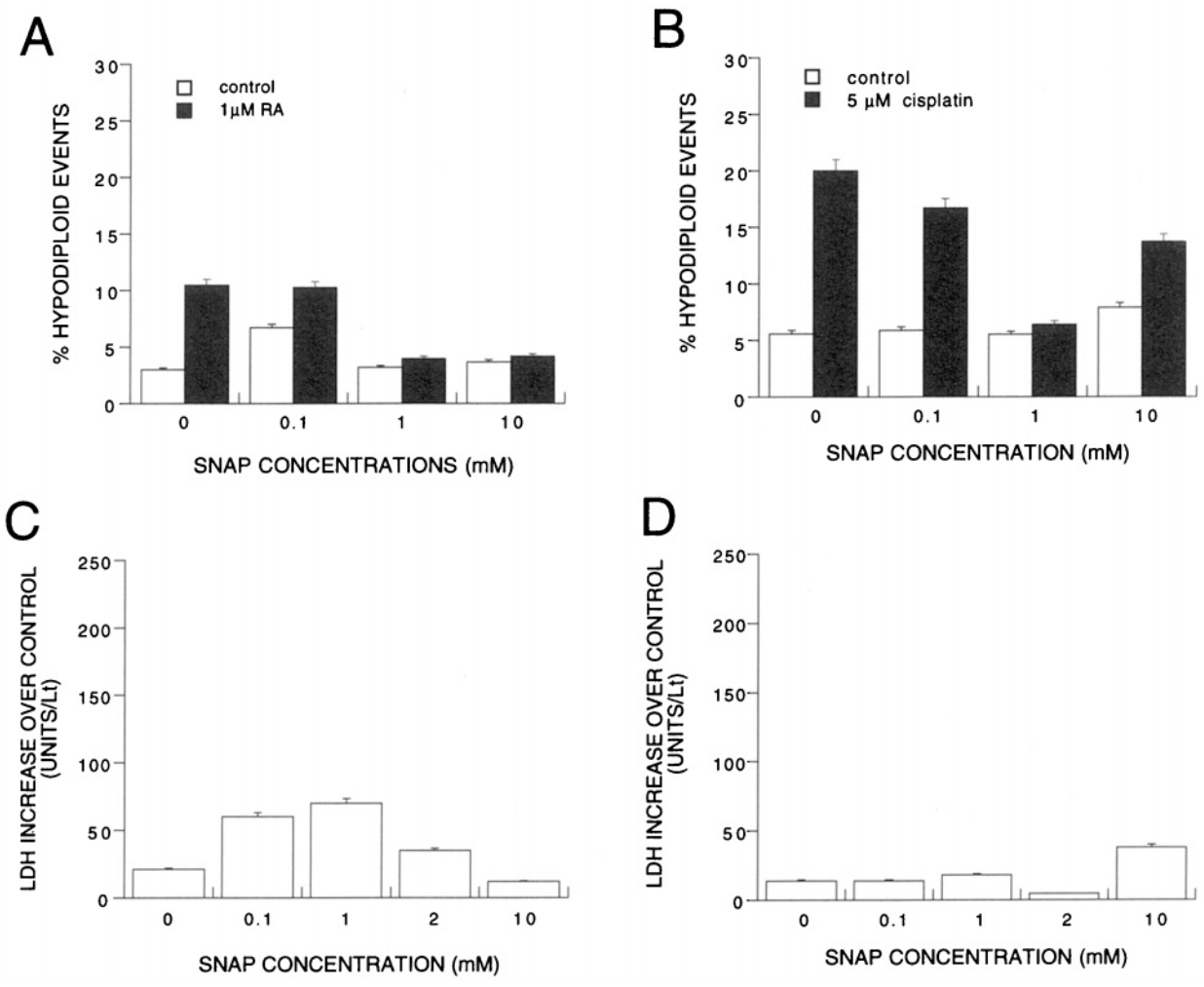

Figure 3. Effect of increasing concentrations SNAP on RA- and cisplatin-induced cell death in the SK-N-BE(2) human neuroblastoma cell line. Cells were left untreated, or incubated with 1 μM RA (*A*) and 5 μM cisplatin (*B*) for 72 and 48 h, respectively, in the absence or in the presence of SNAP. DNA fragmentation was measured by flow cytometry using propidium iodide staining and is expressed as percentage of hypodiploid events. At the same time points, necrotic cell death was determined by measuring LDH release (*C* and *D*, for RA and cisplatin, respectively). Results are means ± SE of duplicate determinations carried out on two different experiments.

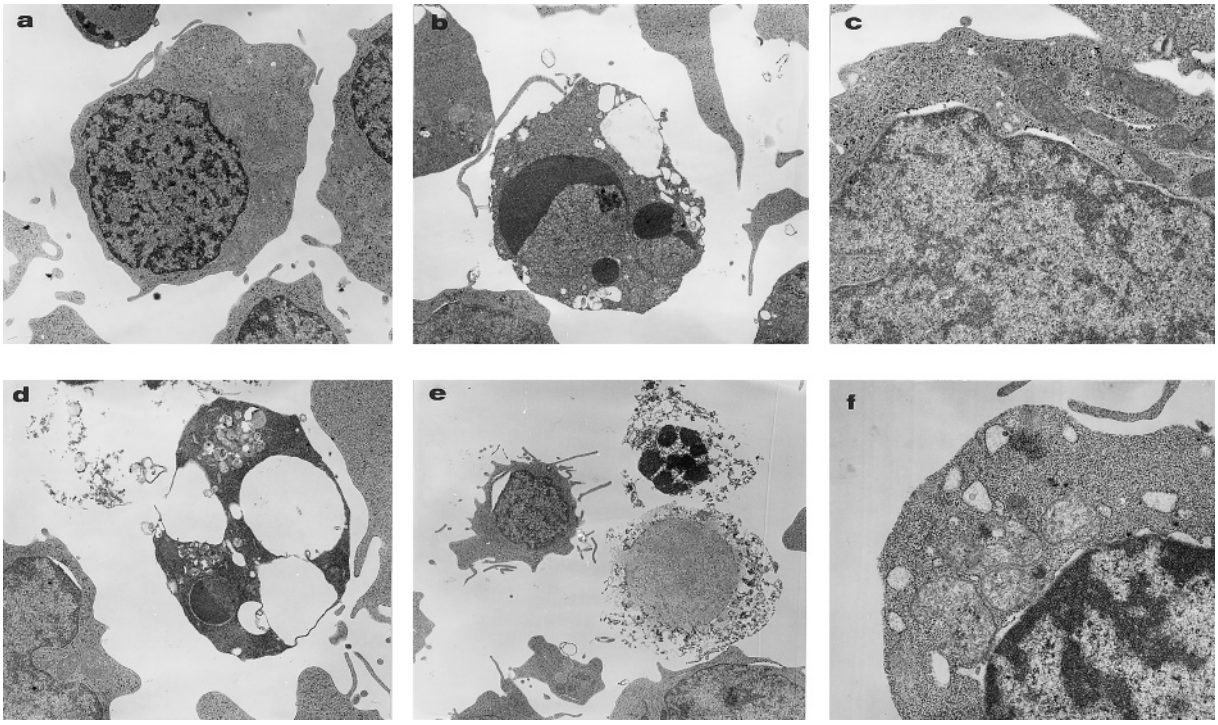

Figure 4. Transmission electron micrographs of Jurkat T cells. Compared with normal cells (*a*), the anti-CD95-stimulated cells (6 h) display typical apoptotic morphological alterations including aggregation of chromatin and fragmentation of the nucleus (*b*); these cells also show normal mitochondria (*c*). Cells coincubated with anti-CD95 and the NO-donor SNAP display characteristic necrotic ultrastructural changes including large vacuoles (1 mM SNAP, *d*), cell swelling and lysis (5 mM SNAP, *e*) and swollen mitochondria (*f*).

NO can lead to cytotoxicity via $ONOO^-$-mediated pathways. Indeed, the formation of the highly reactive oxidant $ONOO^-$ contributes to cellular injury. $ONOO^-$ causes lipid peroxidation [54], chemical cleavage of DNA [55] and nitration of both free and protein-bound tyrosine with subsequent alterations of protein phosphorylation or perturbation of protein tertiary structure [56]. $ONOO^-$ seems to contribute to cell death through the activation of the nuclear enzyme poly(adenosine 5′-diphosphoribose) synthetase, which in turn results in ATP depletion [57].

Mechanisms of induction of apoptosis by NO

NO-mediated cell death occurs through apoptosis in a variety of cell types including macrophages [3], neurons [37, 48], pancreatic β-cells [6, 58], thymocytes [59], chondrocytes [60] and hepatocytes [61]. The signaling pathways that lead to apoptosis remain still poorly understood. NO-induced apoptosis is often accompanied by accumulation of the tumour suppressor gene p53, changes in the expression of pro- and antiapoptotic Bcl-2 family members, caspase 3-like protease activation and cytochrome c translocation [3]. In excitotoxic death of cultured neurons, NO-triggered apoptosis requires a Ca^{++} signal triggered by activation of the NMDAR channels [62]. Depletion of ATP in PC12 cells, caused via the NO-mediated inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, is one of the mechanisms responsible for NO neurotoxicity [63]. However, NO-mediated glyceraldehyde-3-phosphate dehydrogenase modification cannot necessarily be linked to energy depletion, at least in monocytic macrophage cell lines [64].

NO shifts the apoptotic response elicited by different stimuli into necrotic cell death

The combined exposure of Jurkat T cells to an agonist anti-CD95 antibody and increasing concentrations of SNAP prevented apoptosis triggered by CD95 ligation in a concentration-dependent manner and led to a decrease in cell viability assessed by trypan blue exclusion and LDH release, within 3 h after stimulation [19]. These results show that NO shifts the anti-CD95-triggered apoptotic response of Jurkat T cells into necrotic cell death. Indeed, the NO-donor was unable to solely inhibit cell death, indicating that NO may act below the point of no return elicited by CD95 ligation.

In order to characterize morphological features of cell death at an ultrastructural level, we performed electron microscopy on Jurkat T cells. Unlike the control (fig. 4A), typical morphological hallmarks of apoptosis, such as highly condensed and cap-shaped marginated chromatin and fragmentation of nuclei into several DNA-containing vesicles, were evident in anti-CD95-treated cells (fig. 4B); however, the mitochondria appeared normal in CD95-exposed cells (fig. 4C). Most of the cells co-incubated with anti-CD95 and the NO-donor showed characteristic necrotic ultrastructural changes, including large vacuoles, cell swelling and lysis (fig. 4D and E) and swollen mithocondria (fig. 4F). Interestingly, some apoptotic hallmarks such as chromatin condensation and nuclear compaction were still evident in cells co-incubated with anti-CD95 and the NO-donor, even though other apoptotic features such as nuclear fragmentation could not be detected [19]. The absence of nuclear fragmentation is probably a consequence of direct caspase inhibition, as this event is caspase-dependent [43, 65].

To determine whether the reversion of apoptosis into necrosis by NO may be generalized, we treated Jurkat T cells with 30 μM C_6-ceramide with or without different concentrations of SNAP, and necrotic cell death was measured 24 h later. As shown in figure 5, a dose-dependent increase in the percentage of necrotic cell death, assessed by trypan blue uptake (panel A) and

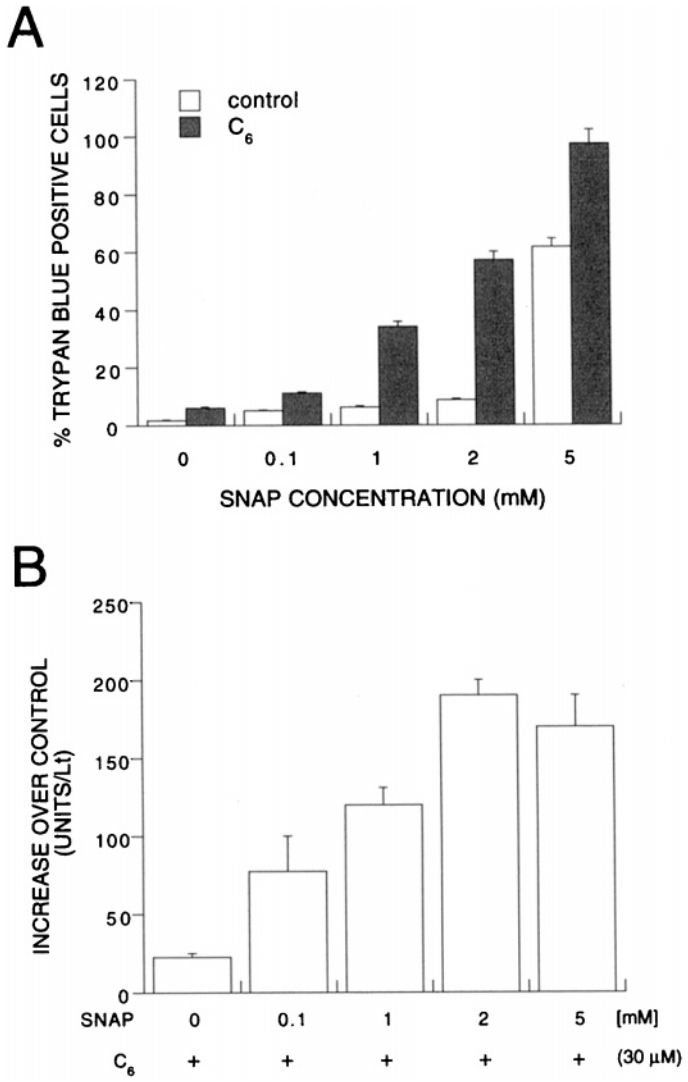

Figure 5. Effect of the NO-donor SNAP on C6-induced necrotic cell death in Jurkat T cells. Increase of C6-triggered cell death by SNAP in Jurkat T cells. Cells were incubated with increasing doses of SNAP with or without C6 (30 μM) for 24 h and necrotic cell death was assessed by trypan blue uptake (*A*) and LDH release (*B*). Data are given as the mean ± SE of duplicate determinations of two separate experiments.

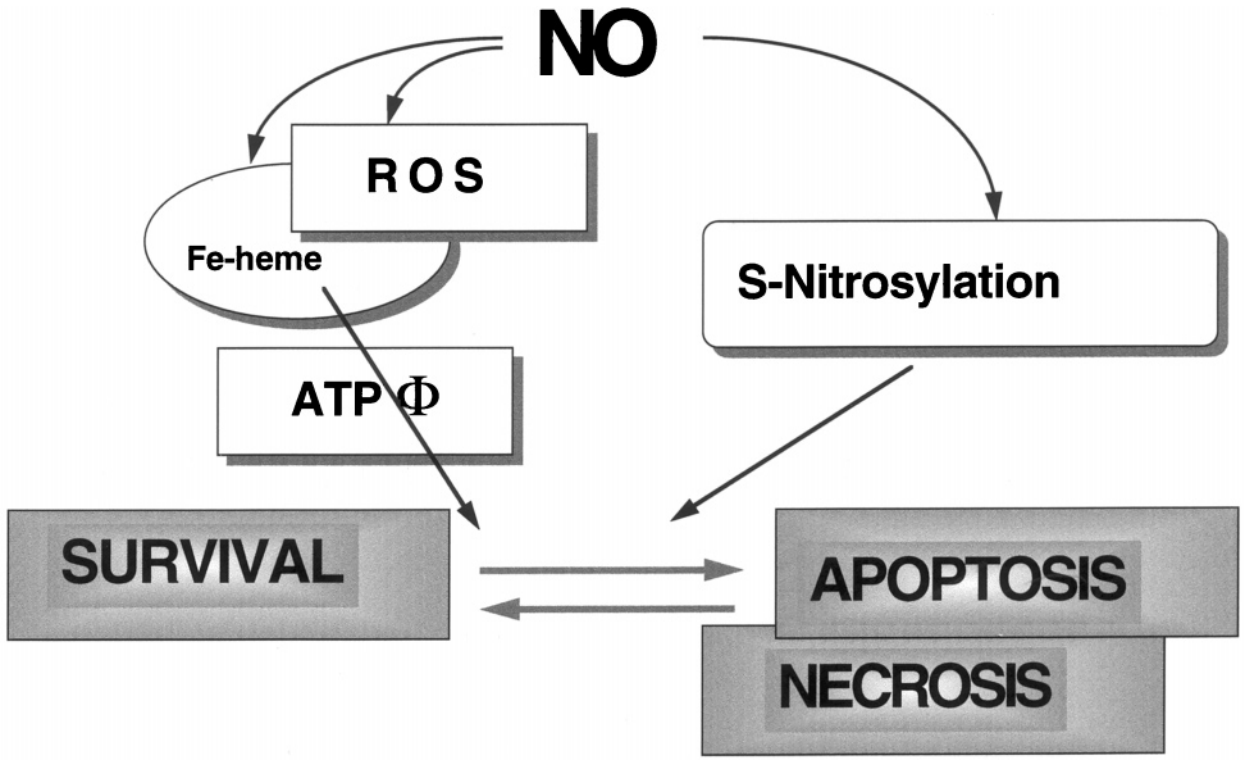

Figure 6. Schematic representation of the mechanisms which might be possibly involved in controlling the balance between the NO-mediated protective and destructive actions.

LDH release (panel B), could be observed in cells co-incubated with the apoptotic stimulus and the NO-releasing compound. Similarly to the CD95-triggered model [19], also in the C_6-triggered model the NO-donors were able to prevent apoptosis by converting it into necrosis. However, this is significantly less evident compared with the CD95 apoptotic model (data not shown). A major difference occurs between the two models: the exposure time to the NO-donor is different. Indeed, CD95 ligation kills cells in 3–6 h, whereas C_6 kills in 12–24 h, possibly offering significant differences to short-lived molecules derived from the NO-donors.

The apparent conflict between the results shown (shift from apoptosis into necrosis) and drug-induced apoptosis of neuroblastoma cells (inhibition of apoptosis leading to cell survival), might be explained by considering that the experimental model significantly differed in the type of cells employed, as well as in the exposure time to the NO-donor (see also later).

Conclusions

NO has emerged as a potent inhibitor of apoptosis in many cell types [1, 35, 40, 45]. The ability of NO to rapidly diffuse intracellularly and from cell to cell would represent an efficient mechanism to prevent the activation of apoptotic effector molecules such as caspases and tTG, resulting from cell injury or exposure to other activators (CD95L, TNFα, ceramides, retinoids, chemotherapeutic drugs).

The ability of NO to both induce and prevent cell death might be influenced by the biological milieu, its redox state, concentration, exposure time and the combination with oxygen, superoxide and other molecules. As shown in figure 6, ATP depletion might be relevant for NO to affect cell death. Indeed, mitochondrial function and intracellular energy levels are critical factors in determining the type (apoptosis or necrosis) of neuronal cell death during glutamate excitotoxicity, with ATP depletion leading to necrosis [66, 67]. S-nitrosylation and subsequent inactivation of critical regulator/effector elements of the apoptotic program may represent an additional mechanism influencing the balance between apoptosis and necrosis (fig. 6).

Even though the mechanisms which determine the role of NO in cell death and survival have yet to be fully elucidated, we can speculate that NO may differently influence cell fate, depending on its interaction with the apoptotic pathway. Indeed, NO might interfere at different levels with a cell which has received an apoptotic signal (fig. 7A and fig. 2). The inactivation of the

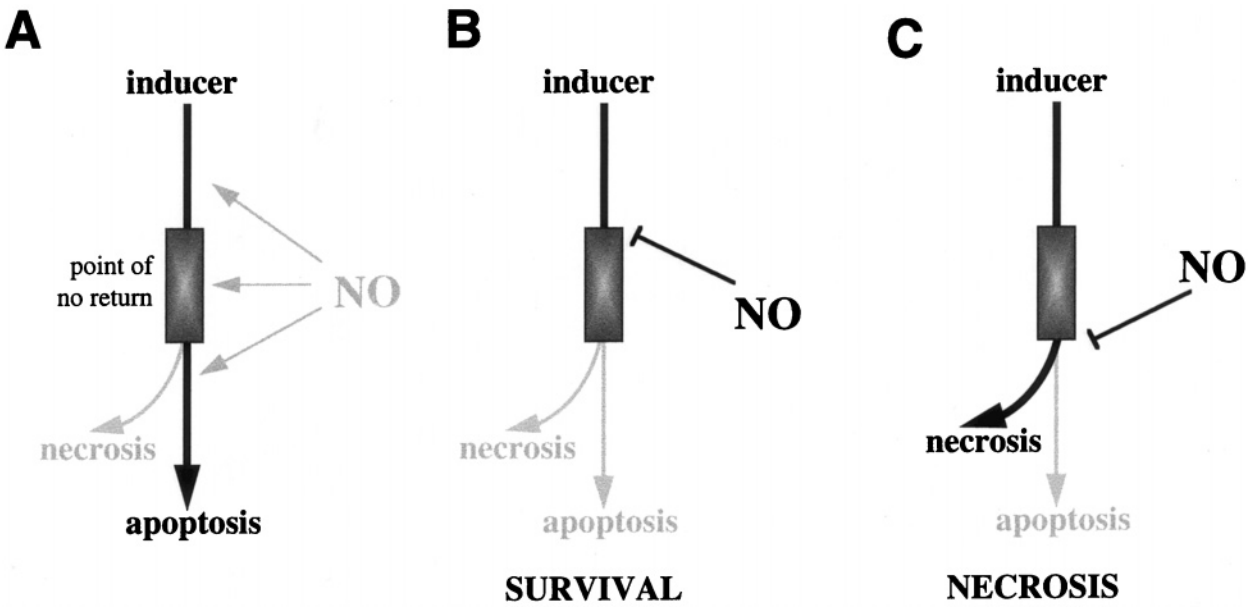

Figure 7. Schematic model of NO interference with the apoptotic pathway elicited by several inducers (e.g. CD95L, TNFα, ceramides, retinoids, chemotherapeutic drugs). NO might act at different levels of the apoptotic cascade (*A*), see also figure 2. NO might inhibit apoptosis before a cell has been committed to die, resulting in prevention of apotosis and resistance to death (*B*), or it might act downstream of the point of no return, thus switching from apoptotic to necrotic cell death (*C*).

execution machinery in a cell before the point of no return might result in prevention of apotosis and resistance to death (fig. 7B). Alternatively, the inhibition of apoptosis downstream of the point of no return, might lead to a change of the type of cell death from apotosis toward necrosis (fig. 7C).

NO can therefore finely regulate cell death under physiological circumstances as well as in pathologies, such as cancer and susceptibility to cancer therapy. For example, the NO-mediated inhibition of apoptosis may protect the liver from inflammatory injury [35] and the vessel wall from atherogenesis [40]. On the other hand, cytotoxic actions of NO could lead to pathological states or cell destruction [3]. To elucidate the underlying mechanisms controlling the balance between the NO-mediated protective and destructive actions might be important to understand the switch from physiology to pathology.

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