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Forum Review

The Intimate Relation Between Nitric Oxide and Superoxide in Apoptosis and Cell Survival

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ABSTRACT

Intra- and intercellular communication in or between cells allows adaptation to changes in the environment. Formation of reactive oxygen (ROS) and nitrogen (RNS) species in response to external insults gained considerable attention in provoking cell demise along an apoptotic subroute of cell death, thus attributing radical formation to pathologies. In close association, stabilization of the tumor suppressor p53 and activation of caspases convey proapoptotic signaling. Complexity was added with the notion that ROS and RNS signals overlap and/or produce synergistic as well as antagonistic effects. With respect to nitric oxide (NO) signaling, it became clear that the molecule is endowed with pro- or antiapoptotic signaling capabilities, depending to some extend on the concentration and cellular context, *i.e.,* ROS generation. Here, some established concepts are summarized that allow an explanation of p53 accumulation under the impact of NO and an understanding of NO-evoked cell protection at the level of caspase inhibition, cyclic GMP formation, or expression of antiapoptotic proteins. In addition, the overlapping sphere of ROS and RNS signaling is recapitulated to appreciate cell physiology/pathology with the notion that marginal changes in the flux rates of either NO or superoxide may shift vital signals used for communication and cell survival into areas of pathology in close association with apoptosis/necrosis. *Antioxid. Redox Signal.* 7, 497–507.

INTRODUCTION

THE LANDMARK DISCOVERY that nitric oxide (NO) is synthesized by mammalian cells initiated a tremendous number of studies demonstrating that this free radical plays crucial roles in the homeostatic regulation of the cardiovascular, neuronal, and immune systems. Interestingly, NO taught us to revise traditional thinking that radicals are harmful molecules and highlighted new concepts of cellular communication that previously had been excluded from our pictures of biology/medicine. We now appreciate that biological actions often can be attributed to "reactive nitrogen species" (RNS) rather than NO itself. The term RNS refers to oxidation states and adducts of the products of nitric oxide synthases (NOSs), ranging from nitric oxide (•NO) to nitrate (NO_3^-). In analogy, reactive oxygen species (ROS) encompass intermediate products when oxygen is reduced to water. It is clear that biologically significant NO-redox and -additive reactions include those with ROS and transition metals that, in turn, dictate NO chemistry (28, 33, 87). In particular, NO^+ is a redox species with the ability to undergo addition or substitution reactions with nucleophiles, among others sulfur, resulting in -S-NO (*S*-nitrosothiol) formation. Under cellular conditions, NO^+ carrier species such as N_2O_3 or a N_2O_3-like species, as well as a Fenton-type reaction, may account for protein nitrosation (17, 23, 96). Furthermore, *S*-nitrosothiol formation is reversible, noticed under cellular conditions and considered the phenotypic redox-based NO-signaling mechanism (88). Of note, these signaling pathways are cyclic guanosine monophosphate (cGMP)-independent. However, the "classic" signal transduction event of NO can be attributed to activation of soluble guanylyl cyclase, concomitant cGMP production with cellular responses arising through cGMP-dependent signaling pathways, with the most prominent one that facilitates phosphorylation (43). Generally, to understand cytostatic, cytotoxic, or protective NO effects, the aforementioned cGMP-independent pathways, such

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as reactions with oxygen, superoxide (O_2^-), thiols, and transition metals, appear to predominate. Figure 1 summarizes major biological effects attributed to RNS and shows most relevant chemical effects that may occur in response to RNS production.

Endogenous NO is synthesized from L-arginine by a family of NOS isoenzymes, and it is becoming evident that NOS activity is associated with human diseases and disorders (47, 55). NO formation under inflammatory conditions influences gene expression, affects the relationship between mammalian hosts and microbial pathogens, modulates immune responses, and may contribute to cell demise by affecting apoptosis (12, 71).

Apoptosis, or programmed cell death, is a major form of cell death characterized by a series of stereotypic morphological and biochemical features (38). It is important in normal cell development and in many different diseases (26, 63). Apoptosis occurs in two phases known as an initial commitment phase followed by an execution phase. The latter involves condensation and fragmentation of nuclear chromatin as well as alterations at the cell membrane that guarantee recognition of apoptotic cell debris, followed by its removal via phagocytosis. In brief, apoptotic routes of death can be divided into two components, involving either mitochondria or death receptors. In the death receptor pathway, receptors such as tumor necrosis factor-α receptor1 or Fas/CD95 are activated by their cognate ligands, which allow the recruitment of downstream signaling partners that ultimately provoke caspase activation. Death receptor activation attracts and activates procaspase-8 through interactions between the death effector domains of these two proteins. Active caspase-8 by cleaving, *e.g.,* Bid generates signals to connect the death receptor with the intrinsic death pathway. In the mitochondrial pathway, cytochrome *c* is released from the intermembrane space to associate in an ATP- or dATP-dependent manner with apoptosis protease-activating factor 1 (Apaf-1) to form a multimeric complex, known as the apoptosome, that recruits and activates procaspase-9. This is followed by activation of executioner caspases such as caspase-3 or -7. Thus, in death receptor-mediated apoptosis, caspase-8 is the most apical caspase, whereas in the mitochondrial pathway this position is taken by caspase-9. As one might imagine, apoptosis is highly regulated, among others by the Bcl-2 family of proteins (42) that control the release of mitochondrial components, *e.g.,* cytochrome *c*. Figure 2 summarizes major routes of apoptosis as a result of extrinsic as well as intrinsic death-stimulating pathways.

At the end, the delicate balance between pro- and antiapoptotic signals will affect the outcome to direct cell survival and cell death. One important proapoptotic factor is the tumor suppressor p53 (46, 102). The tumor suppressor p53 may serve as a sensor of cellular stress and, once activated, can initiate apoptosis in a transcription-dependent and/or -independent manner (102). This makes p53 a master regulator of the apoptotic program, capable of coordinating the death process at multiple levels by affecting expression of pro- versus antiapoptotic modulators.

With regard to RNS signaling and apoptosis, the situation appeared complex. On one side RNS have the ability to provoke apoptosis, whereas on the other side RNS can antagonize cell death pathways. In part, this can be attributed to the concentration, *i.e.,* the flux rate of NO formation, but to a large extend may depend on the cellular milieu, *i.e.,* the intracellular redox state and redox capacity of a cell. The following sections will touch upon pro- and antiapoptotic actions attributed to RNS.

PROAPOPTOTIC ACTIONS OF RNS (NO)

In 1993, the first reports on NO-evoked apoptosis appeared (1, 84). Since then, apoptosis by RNS has been shown and

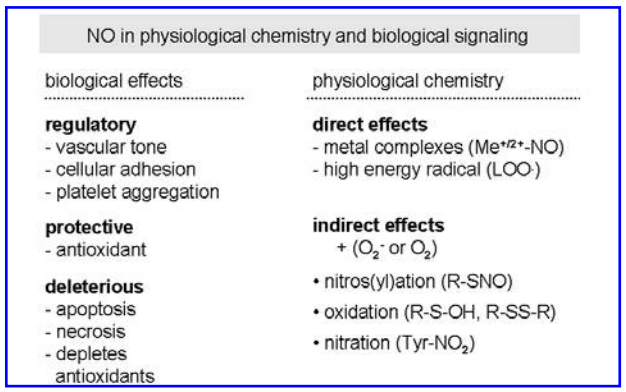

FIG. 1. Biological effects and physiological chemistry of RNS. Potentially relevant regulatory, protective, and deleterious biological effects, as well as physiological chemical reactions of NO, are summarized. See text for further details.

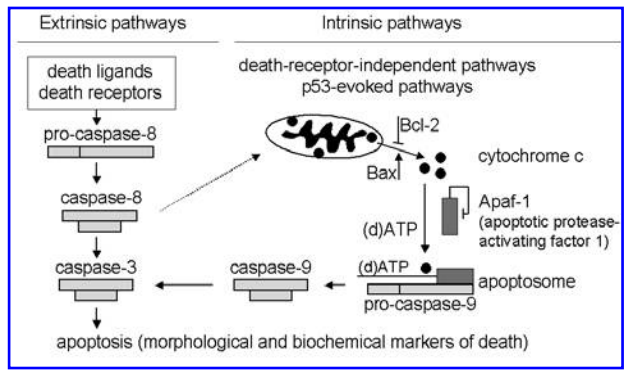

FIG. 2. Intrinsic and extrinsic apoptotic pathways. Extrinsic pathways are induced by death receptor stimulation by death ligands, *e.g.,* CD95/Fas ligand. Caspase-8 processing may directly process procaspase-3 to become active or may amplify the signal via the mitochondrial route of cell death execution. The intrinsic death receptor-independent pathway is triggered by the majority of apoptotic stimuli, such as cytotoxic drugs, ceramide, irradiation, and/or RNI, and leads to the loss of the mitochondrial membrane potential and release of cytochrome *c* into the cytosol. Cytochrome *c* together with (d)ATP, Apaf-1, and procaspase-9 then forms the apoptosome leading to activation of the initiator caspase-9 followed by processing of procaspase-3 and its activation. Downstream of active caspase-3, cell death occurs. See text for details.

still is being noticed in many different murine and human cells, *e.g.,* macrophages, pancreatic islets, neurons, tumor cells, and vascular smooth muscle cells (see references in 11, 12, 14). In most, but not all, experimental systems, apoptosis by RNS is cGMP-independent with growing evidence that RNS signaling is not a simple, random process or mediated exclusively by diffusion. Cell demise by apoptosis (a) reveals a dependence on the NO concentration, (b) appears cell type-specific, (c) is determined by the cellular redox microenvironment, and (d) is affected by the pro- versus antiapoptotic balance of an individual cell. Therefore, the threshold for a proapoptotic triggering event of RNS is different from one cell to another and difficult to predict. Nevertheless, important targets for the proapoptotic behavior of RNS emerged and shaped principles of RNS action.

NO and mitochondria

NO may impair electron flux through the respiratory chain through interference at multiple sites. At low concentrations ($\leq$1 μM NO), NO reversibly inhibits cytochrome oxidase (complex IV), which may shift the electron transport chain to a more reduced state, a condition that favors O_2^- formation. O_2^- generation in the presence of NO may, under conditions when dismutation of O_2^- into hydrogen peroxide (H_2O_2) is saturated or impaired, provoke formation of peroxynitrite ($ONOO^-$), a situation that culminates in persistent inhibition of complex I (for references, 10, 68). One may envision that, at low concentrations, NO causes transient, reversible deenergization of mitochondria by affecting respiration, but not ATP generation. Prolonged exposure to NO, presumably via $ONOO^-$ formation, may impair ATP synthesis with the notion that a transient drop in ATP can provoke apoptosis, whereas a persistent ATP decrease results in necrosis. In a cell-free system containing mitochondria and nuclei, NO induced mitochondrial permeability transition and promoted apoptosis that was attenuated by permeability transition inhibitors (9). In macrophages, nitrosothiols caused caspase activation that is blocked by preventing opening of the mitochondrial permeability transition pore, whereas caspase activation by NONOates is much less sensitive to inhibitors of the mitochondrial permeability transition pore (8), thus making NO radical chemistry for this process unlikely. Along that line, RNS-evoked apoptosis is associated with increased $\Delta\psi_m$ (mitochondrial inner membrane potential) in macrophages, but a decreased one in HeLa or Jurkat cells (41), whereas inhibition of mitochondrial protein synthesis is associated with a greater susceptibility of cells to undergo RNS-dependent apoptosis (78). Although variations are noticed, there is accumulating evidence that mitochondria comprise a target for RNS and that inhibition of respiration may contribute to the proapoptotic effect of RNS by membrane potential reduction, transition pore opening, and release of cytochrome *c*. In addition, RNS may provoke an intracellular mitochondria-derived calcium increase that is blocked by cyclosporin A, which may contribute to deregulation of calcium and cell death, a situation with special interest to excitatory neuronal death (40). Interestingly, NO has also been reported to protect endothelial cells via a mechanism that involves mitochondrial membrane hyperpolarization, thereby interrupting the progression of apoptosis (27).

Caspase activation

Several studies emphasize a role of caspases, especially cytochrome *c*-dependent activation of caspase-3, in a cascade facilitating RNS-elicited cell demise. For example, RNS-induced neuronal cell death was accompanied by the cleavage of caspase substrates such as DEVD-AFC, VDVAD-AFC, or LEHD-AFC, which pointed to the activation of a caspase-3-like group (caspase-3 and -7), caspase-2, and caspase-9 (69, 108). Importantly, western analysis confirmed that pro-forms of caspases vanished, whereas cleaved fragments appeared with the further notion that blocking mitochondrial permeability transition often attenuated caspase activation (3). These effects have consistently been reproduced by chemically diverse NO donors at concentrations ranging from 10 μM to 1 mM (99). Mechanistically, it is proposed that down-regulation of inhibitors of apoptosis such as cIAP (cellular inhibitor of apoptosis) is associated functionally with caspase activation (89). Alternatively, from partially RNS-resistant thymocytes derived from caspase-1 knockout mice compared with wild-type littermates, the involvement of caspase-1 and thus an indirect, cytokine-evoked death pathway seem predictable (115). The importance of the cellular redox balance in caspase activation was demonstrated by the finding that RNS-elicited caspase activation was largely attenuated by incubating cells with iron sulfate to increase the intracellular iron content and thus to affect the chemical fate of RNS (52). Observing caspase activation under the influence of RNS should not be considered as a direct impact on the zymogen-active caspase transition but rather an indirect effect as a result of RNS-evoked signal transmission.

Accumulation of p53

In 1994, it became apparent that RNS, endogenously generated or exogenously supplied, evoked p53 accumulation in murine macrophages (66). Although basic observations have been reproduced in human cells, it is known that not all experimental systems demand p53 accumulation to provoke cell death (65; for references, see 12). Importantly, a p53 response should not be taken as proof that DNA damage is involved because NO generated DNA damage only inefficiently at the cellular level (75, 76). Studies with macrophages derived from inducible $NOS^{-/-}$ animals that failed to demonstrate nuclear p53 localization after *in vivo* bleomycin exposure supported a link between RNS and p53 (18).

There is unquestionable evidence that RNS-stabilized p53 is transcriptionally active based on observations that p53 is needed for RNS to up-regulate cell-cycle regulators or pro-apoptotic proteins such as $p21^{Waf1/Cip1}$ or Bax (45, 97), with the further observation that Akt attenuated p53-dependent transcriptional activation and suppressed RNS-elicited cell death (109). Experiments in thymocytes from p53 null mice or in mutant p53 human lymphoblastoid cells revealed that these cells were resistant or less sensitive to NO-induced apoptosis, underscoring the notion that p53 may transmit a pro-apoptotic RNS response (29, 57). Accumulated p53 showed phosphorylation at distinct residues, with serine-15 being the most prominent (49, 70). This fits current concepts proposing posttranslational modification of p53, particularly serine-15 phosphorylation, in transcriptional activation of p53. Studies

with p53 N-terminal mutations at serines 15, 20, 33, or 37 indicated multiple and functionally overlapping phosphorylation sites to control p53 activity in response to DNA damage. Recent studies excluded a role of either ataxia telangiectasia-mutated (ATM) or the ARF (alternate reading frame) tumor suppressor protein in accumulating p53 (103). Instead, a transient and reversible down-regulation of murine double minute (Mdm2) by RNS is associated with p53 accumulation. However, these findings were recently questioned by demonstrating that phosphorylation of p53 at serine-15 is ataxia-telangiectasia (ATM) and ATM- and Rad3-related (ATR)-dependent, but p38- and DNA-PK-independent based on studies in isogenic human cell lines and mouse embryonic fibroblasts from gene knockouts ($ATM^{-/-}$) (39).

A recent study confirmed p53 accumulation of a transcriptionally active, serine-15-phosphorylated p53 that revealed predominant nuclear localization (85). Although serine-15-phosphorylation, p53 still bound its negative regulator Mdm2 and polyubiquitination of p53 remained intact. Based on cell fractioning and heterokaryon analysis, it is suggested that RNS, in some analogy to leptomycin B, prevent nuclear-cytoplasmic shuttling of p53 that causes nuclear protein stabilization/activation. Serine-15 phosphorylation has been correlated with attenuated nuclear export, and it remains to be established whether this striking correlation accounts for a cause–effect relation (114). However, the assumption was corroborated by taking advantage of IMR32 human neuroblastoma cells (104). These cells express wtp53 that is mostly localized to the cytoplasm, and *S*-nitrosoglutathione-like leptomycin effectively provoked nuclear retention of p53. The authors conclude that RNS promote p53 nuclear retention and inhibit Mdm2-mediated p53 nuclear export with the notion that serine-15 phosphorylation of p53 requires the ATM-related ATR kinase. An ATR kinase dead mutant or caffeine, which blocks the kinase activity of ATR, effectively abolished the ability of RNS to cause p53 nuclear retention, concomitant with inhibition of p53 serine-15 phosphorylation. Based on luciferase assays and *in vitro* interaction studies, Dumaz and Meek (21) noticed that serine-15 modification of p53 alone did not dissociate p53 from Mdm2, although modification of serine-15 stimulates the transcriptional activity of the tumor suppressor. This situation is seen NIH 3T3 fibroblasts, as well as in human RKO cells, when RNS left the p53/Mdm2 complex intact, and at the same time p53-luciferase activity was enhanced (85).

As shown in Fig. 3, current concepts on RNS-evoked p53 stabilization suggest that activation may involve at least two distinct components. At early time points of RNS formation, steady-state levels of Mdm2 drop, which may account for p53 stabilization (103). With time progressing, Mdm2 increases above controls due to p53-elicited transactivation of the *mdm2* gene, and yet p53 remains stable, implying that it is refractory to the degradation-promoting effects of Mdm2. As outlined, two independent experimental approaches suggest that the reason for RNS-evoked p53 stabilization is impaired nuclear export (85, 104). With regard to the mechanism proposed, questions arise concerning the down-regulation of Mdm2, activation of ATR by RNS, and the potential molecular mechanism of how RNS blocks nuclear export. Considering that leptomycin B targets an active cysteine residue in CRM1 (chromosome region maintenance 1) that blocks formation of the export protein complex, one may suggest a similar mechanism for RNS, taking into account that RNS react with cysteine residues.

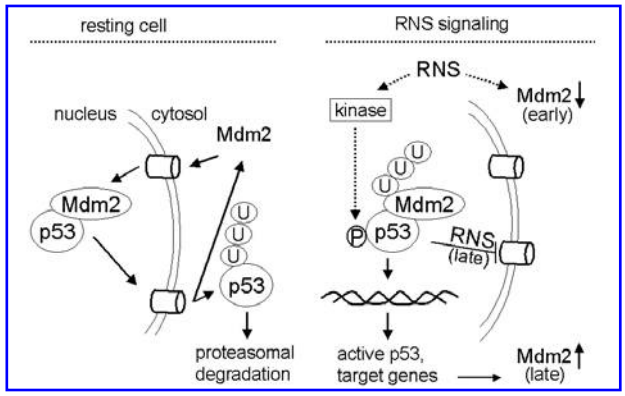

FIG. 3. p53 accumulation in RNS-stressed cells. In unstressed cells, p53 and Mdm2 shuttle between the nucleus and the cytosol with 26S-proteasomal degradation of p53. In RNS-stressed cells, p53 is trapped in the nucleus, where it is phosphorylated, ubiquitinated, and transcriptionally active, which is documented by transcriptional up-regulation of Mdm2. During the early phase of RNS exposure, Mdm2 is down-regulated, whereas its expression is up-regulated at later time points. Multiple kinases may (in)directly contribute to phosphorylation of p53. See text for further details.

Additional proapoptotic signaling components

Several cells respond to cytokines with inducible NOS expression, concomitant NO formation, and apoptotic cell death. Under some of these experimental conditions, mRNA and protein for CHOP [C/EBP homologous protein; also known as GADD153 (growth arrest and DNA-damage inducible gene)], a transcription factor known to respond to endoplasmic reticulum (ER) stress, were induced (31, 48, 75). CHOP induction preceded cytochrome *c* translocation, and a CHOP dominant-negative form prevented RNS-evoked apoptosis. As this observation was p53-independent, CHOP may account for a p53-independent death pathway that is initiated through ER stress because only agents that deplete ER Ca^{2+} activate CHOP. Conceptionally, RNS may target Ca^{2+} uptake pumps to provoke ER stress.

A great deal of studies on RNS-evoked cell death paid considerable attention to the thiol redox status either by using buthionine sulfoximine to deplete glutathione or by adding *N*-acetylcysteine (NAC) to increase intracellular glutathione (16, 32, 90, 100). As a rule of thumb, the sensitivity to RNS increased with GSH depletion and decreased under the impact of NAC. Although the level of glutathione in some cases dropped under RNS treatment, this did not necessarily turn out to be detrimental to cells as long as the overall reducing power of glutathione was preserved. This was confirmed in studies that looked at conditions of O_2^- and NO conformation (for references, see 12). Often, NO and O_2^- are simultaneously generated, which results in their diffusion-controlled interaction and thus redirects signaling properties of either RNS or O_2^-. This has been proven for mesangial cells (MC) where O_2^- formation attenuated RNS-initiated apoptosis. In contrast to MC, Hep G2 cells appeared resistant toward NO

donors, but displayed massive cell destruction following NO/O_2^- cogeneration. In association, a much stronger GSSG (oxidized glutathione) increase was noticed in Hep G2 cells than in MC. GSH depletion reversed cell protection in MC and enhanced cell damage in Hep G2 cells. NO/O_2^--mediated mesangial protection was associated with increased glutathione reductase activity and a marked GSH increase. It must be concluded that the NO/O_2^- sensitivity is found in a cell type-specific manner and is determined by the glutathione redox system (91).

In summary, RNS are capable of signaling proapoptotic components by following established roads proposed for the mitochondrial type of cell death with p53-dependent and -independent signaling pathways emerging.

ANTIAPOPTOTIC ACTIONS OF RNS (NO)

Although the antiapoptotic role of NO has been known for some time and described in several review articles (10, 12, 14), a simple, unifying concept is still missing. As outlined in Fig. 4, several parallel existing pathways are used to describe cell death by RNS, such as apoptosis versus necrosis. In contrast to cell injury, other pathways promoting cell protection under the influence of RNS are also summarized. These comprise immediate effects, such as (a) caspase inhibition by RNS, (b) protection via cGMP, and (c) radical–radical interferences, but also slow responses needed for (d) expression of cell death protective protein. Protective mechanisms are discussed in the following sections.

Caspase inhibition

In 1997, several independent studies reported *S*-nitrosation of caspases that resulted in loss of enzyme activity (20, 50, 58, 61, 64, 67, 74, 93). Although it was attractive to propose that RNS, while targeting the reactive cysteine residue inherent to all caspase-family members, would block apoptosis, it was and still is difficult to explain these results considering the proapoptotic, *i.e.,* caspase-activating, role of RNS. Many authors noticed attenuated caspase, *i.e.,* caspase-3 activity under the impact of RNS, but only a few studies clarified whether caspases actually had been processed and inactivation indeed resulted from posttranslational modification of the active-site cysteine (79) or whether RNS attenuated critical signaling steps in a pathway leading to caspase activation. *In vitro* caspase inhibition by NO donors was reversible with dithiothreitol and cleavage of S-NO bonds with Hg^{2+} implied *S*-nitosation (79). Based on structural similarities, it was not surprising that all caspases were sensitive and incorporated 1–2 moles of NO per mole of caspase. Using electrospray ionization mass spectrometry (ESI-MS) to analyze *S*-nitrosation of caspase-3 revealed poly-*S*-nitrosation. This became apparent by multiple relative mass increases of 30 ± 1 Da in both the p12 (small) and p17 (large) subunits of caspase-3, indicating single to triple *S*-nitrosation (112). Although *S*-nitrosation completely inhibited enzyme activity, this was not restricted to the active-site cysteine, because a significant portion of unmodified protein was enzymatically inactive, most likely due to *in vitro* oxidation of critical thiols. Moreover, *S*-nitroso bonds can be cleaved with release of NO and partial formation of protein mixed disulfides with glutathione, detected by a relative mass increase of 306 Da. Glutathionylation of nitrosylated caspase-3 by GSH can be interpreted as a NO-induced oxidation.

In addition to spectrum of posttranslational modification upon RNS exposure, not necessarily restricted to *S*-nitrosylation, the question of physiological relevance arises. Postulating *S*-nitrosylation or oxidation of caspases in intact cells demands that enzyme activity can be restored *in vitro* (breaking up the cells, measuring caspase activity in the cytosol) by excessive dithiothreitol. However, this is not the case, or was only marginally effective (52). In Jurkat cells, RNS indeed blocked apoptosis with the notion that caspases have not been processed to their active forms (113). This implies that RNS interfere at some point in the proapoptotic signaling cascade by not allowing processing, *i.e.,* activation of caspases, instead of interfering with the active site of a processed enzyme. As western analysis of cleaved versus noncleaved caspases is missing in many studies on caspase inhibition, some studies may have referred to caspase inhibition for conditions when caspases have not been properly processed. The emerging picture of blocking caspase processing instead of directly interfering with enzyme activity is supported by observations that oxidative stress reduced caspase activity only after the enzymes were activated, whereas oxidative stress was not inhibitory for unprocessed caspases (35, 82).

From experiments in Jurkat cells, it can be concluded that RNS interfered with apoptotic signaling by attenuating correct Apaf-1/caspase-9 apoptosome assembly via formation of an inactive ~1.4-MDa apoptosome complex, rather than allowing assembly of the active ~700-kDa complex (113). This observation is consistent with not observing processing of caspase-3, caspase-9, or the downstream caspase-8. In analogy, in hepatocytes, SNAP (*S*-nitroso-*N*-acetylpenicillamine) suppressed processing of caspase-8 in response to tumor necrosis factor-α/actinomycin D (59). Given that hepatocytes are type II cells (cells that require the mitochondrial amplification loop to execute apoptosis), it is unclear whether RNS inhibited receptor-mediated activation of caspase-8 at the death-inducing signaling complex (DISC) or caspase-3-mediated processing of caspase-8, downstream of cytochrome *c* release

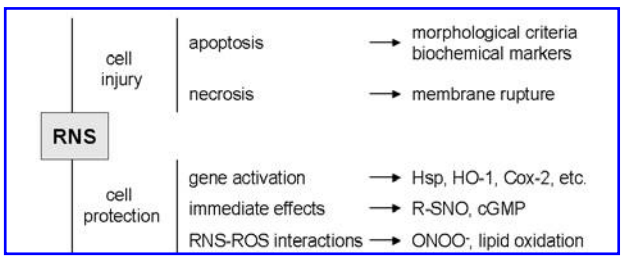

FIG. 4. RNS in provoking cell injury or signaling cell protection. Depending on the cellular context, RNS may provoke cell injury, *i.e.,* apoptosis versus necrosis, or initiate signaling pathways to prevent cell demise. Long-lasting effects may express protective proteins such as Hsps, heme oxygenase-1 (HO-1) or Cox-2. Immediate effects may promote *S*-nitros(yl)ation or production of cGMP. In addition, the interaction between RNS and ROS may produce $ONOO^-$ and/or alter lipid peroxidation. See text for further details.

and activation of the apoptosome. Modification of the proenzymes of caspase-3 or caspase-9 by RNS has been demonstrated (62, 98), although consequences for caspase processing remain undefined. That RNS blocked caspase-3 activity without attenuating cytochrome *c* release from mitochondria (52, 98, 113) strongly suggests some interference of RNS downstream of mitochondria and points to apoptosome formation as a rational target. Considering that apoptosome assembly is also a proposed target for antiapoptotic heat shock proteins (Hsps) (5, 81) makes this protein complex an interesting target for interfering with proapoptotic pathways.

cGMP in cellular protection

An antagonistic role of the NO-cGMP signaling system has been noticed in several experimental settings, although a detailed knowledge of mechanistic considerations still is missing (for references, see 11, 14). In most studies, the involvement of a cGMP signaling pathway was proven by applying lipophilic cGMP analogues to simulate protection or by using soluble guanylyl cyclase inhibitors to antagonize the NO effect. Among other cell types, examples are hepatocytes, neuronal PC12 cells, neurons, lymphocytes, eosinophiles, keratinocytes, or macrophages (11, 14, 95, 106, 110). Compatible with an interference in the proapoptotic signaling cascade, several studies pointed to attenuated cytochrome *c* release (56), decreased ceramide formation (19), inhibition of sphingomyelinases (4), blocked caspase activation (2, 50, 51) or decreased NO production (24). Direct targets that become phosphorylated to explain cGMP action are poorly defined. So far, activation of protein kinase B/Akt (60), expression of Bcl-2 (2) or the Bcl-2 binding protein BNIP3 (111), as well as activation of Akt with concomitant Bad phosphorylation that interferes with its mitochondrial localization (34), have been reported to explain cGMP-evoked protection. In addition, NO via formation of cGMP will elevate the levels of thioredoxin and thioredoxin peroxidase-1, which, in association with Mn-superoxide dismutase, reduces ROS levels to afford protection, with *S*-nitrosation of caspases playing a minimal role in NO-evoked protection of SH-SY5Y cells only (2). Along the line that cGMP may be protective, cyclic adenosine monophosphate (cAMP) shares the ability to inhibit cleavage of caspase-3, -7, and -9 in RNS-induced apoptotis in human osteoblasts (13). Assuming cell type-specific effects of the NO-cGMP signaling cascade (14), it will be most interesting to gain a detailed knowledge of potential substrates of a cGMP-dependent kinase phosphorylation event and to understand why the widely distributed cGMP system is not commonly active.

Expression of protective genes/proteins

Modulation of transcription factor activity by ROS/RNS is in line with observations that gene/protein expression is under the sphere NO/O_2^- action. As recently summarized, a number of potentially cell-protective proteins, *e.g.,* Hsps, heme oxygenase, cyclooxygenase-2 (Cox-2), Cu,Zn- and Mn-superoxide dismutase, and Bcl-2, are under the control of RNS (for references, see 11, 12, 14). Some proteins are associated with classical stress responses and may function indirectly by affecting the cellular redox balance. For other proteins, such as the 70-kDa family of Hsps a more direct antiapoptotic action appears reasonable. From *in vitro* studies, it is known that Hsp70 attenuates apoptosome formation (5, 81). In cellular studies, Hsp70 diminished RNS-evoked apoptosis in RAW 264.7 macrophages with the notion that p53 accumulation and cell-cycle arrest remained effective, cytochrome *c* translocation was reduced, and processing/activation of caspase-9 and -3 was attenuated (53). Along that line, Hsp70 obstructed activation of caspase-3 in chondrocytes (94). There is some analogy between protection afforded by overexpression of Hsp70 and Bcl-2. Bcl-2 has been shown to interrupt the apoptotic cascade at several steps. It inhibits the release of cytochrome *c* from mitochondria (86) and nuclear import of p53 (6) and protects Apaf-1$^{-/-}$ cells from apoptosis (37). Taking into account that Hsp70 has been reported to stabilize mitochondria (77), an action shared by Bcl-2, it can be speculated that Hsp70 attenuates propagation of mitochondria-derived apoptotic signals. Previous studies used heat treatment that up-regulated not only Hsp70, but also Bcl-2, thus making it difficult to draw conclusions on the action of Hsp70 on mitochondria (30). It may turn out that blocking apoptosome assembly as shown *in vitro* requires higher Hsp70 concentrations, whereas under cellular conditions lower Hsp70 expression may use distinct pathways to attenuate cell demise.

In some cells such as macrophages, expression and activation of Cox-2 conveyed protection toward RNS-evoked apoptosis (101). Expression of Cox-2 was achieved by treatment with either interferon-γ/lipopolysaccharide or nontoxic concentrations of NO donors. Formation of E-type prostanoids and, in turn, an intracellular cAMP increase accounted for blocked apoptosis. In further experiments, gene induction by cAMP in association with activation of cAMP-response element binding protein (CREB) was shown, and causation was established when oligonucleotides containing a cAMP-response element attenuated cAMP-evoked protection and reestablished proapoptotic parameters in macrophages. Protection toward apoptosis by cAMP-elevating maneuvers in association with Cox-2 expression has been shown in hepatocytes, neutrophils, thymocytes, MC, and neurons, proven to be effective toward diverse agonists (for references, see 101), and shown to abrogate p53-induced cell demise (36, 44). More recently, a role of RNS in activation of CREB and survival of neuronal cells (15) confirmed previous work in macrophages (101). Inspired by the observation that Cox-2 is overexpressed in colorectal adenomas and adenocarcinomas, and the notion that Cox-2 promotes cancer and inhibits apoptosis, it is now suggested that protection is associated with cAMP-mediated cIAP induction (72). Characteristic of IAP function, the activity of the central executioner caspase-3 is inhibited, providing a link between Cox-2 expression, cAMP formation, CREB activation, and up-regulation of an antiapoptotic protein.

Protection from apoptosis via expression of survival proteins may indicate a general stress response, facilitated not only by RNS. Thus, protective mechanisms are not necessarily specific against RNS intoxication, but rather initiate cross-tolerance to many apoptotic insults. Given the parallels between ROS and RNS, it seems inescapable that metabolic pathways exist to detoxify RNS, analogous to the systems detoxifying ROS. In this respect, the basis of microbial resistance toward RNS is becoming clear, and candidate gene products that may confer resistance have been grouped by presumptive mechanisms of action: (a) interference with pro-

duction or uptake of RNS, (b) conversion of RNS to less toxic forms, (c) mechanisms likely to involve repair of RNS-dependent lesions, and (d) NOxR-evoked resistance mechanisms (71). Assuming that counterparts of these gene products exist in humans will stimulate searches in that area, which at the end may help to understand the still existing and unexplained diversity of cellular responses under RNS delivery.

Radical–radical interferences

Early observations suggested that NO serves as a potent terminator of radical chain-propagating reactions (80). NO can be protective under conditions of oxidative stress resulting from O_2^-, H_2O_2, and alkyl peroxides by preventing heme oxidation, blocking Fenton-type chemistry, or attenuating lipid peroxidation (107). Kinetic analysis revealed that a simple radical–radical termination reaction does not account for inhibition of lipid oxidation by NO, because at least two molecules of NO are consumed per termination reaction (73). Studies using irradiation with UV-A light have shown that apoptosis or necrosis occurs as a result of formation of singlet oxygen, which reacts with unsaturated fatty acids to generate peroxyl radicals. These, in turn, initiate lipid peroxidation via radical chain reactions. NO scavenges lipid peroxyl radicals, thus attenuating apoptosis and necrosis and at the same time protecting phospholipids from oxidation (25, 92). A peroxyl radical-scavenging mechanism may account for inhibition of oxidized low-density lipoprotein-induced apoptosis by NO as well (54). Along that line, a radical interaction between O_2^- and NO protects against apoptosis as originally noticed in chondrocytes and MC (7, 83). In some analogy, ROS formation, most likely H_2O_2 generation in fibroblasts, attenuated apoptosis by favoring the reaction of NO with H_2O_2 over other targets. It can be concluded that, in some systems, the relative rate of O_2^- production will be a determinant of pro-apoptotic RNS actions and vice versa. One may envision that RNS are antiapoptotic in the presence of low levels of O_2^- formation, but detrimental if the intracellular redox balance is shifted toward strong oxidation (most likely associated with $ONOO^-$ formation), a situation that no longer can be handled by the reducing power of a cell (33, 91, 105). As a general concept, it appears that a very delicate balance of ROS versus RNS formation, interaction, and elimination contributes to regulate pro- versus antiapoptotic roles of RNS.

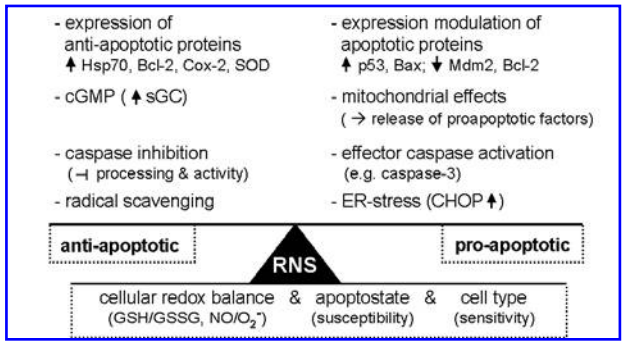

FIG. 5. RNS in affecting the balance of cell death and survival. Major signaling components (proteins, transducing pathways, cellular metabolic alterations) that determine, at least in part, the pro- versus antiapoptotic actions of RNS are shown. The activity of RNS in affecting cell demise is again under the modulatory impact of factors listed in the lower part of the figure (redox balance, apoptotic history, and cell type). The sum of these variables at the end will regulate cellular susceptibility toward RNS and dictate whether RNS are antiapoptotic or proapoptotic (→ activation; ⊣ inhibition; ↑ up-regulated; ↓ down-regulated). See text for further details.

CONCLUDING REMARKS

RNS often show conflicting actions in preventing or promoting apoptosis. Unfortunately, there is no simple explanation for this, and the picture of NO as "the good, the bad, and the ugly one" remains (see Fig. 5). Regulation of cell homeostasis by balancing proliferation versus death is important for a number of human diseases and again may refer to the dichotomous action of RNS (22, 47). In this context, the opposing reports on a role of RNS in modulating different diseases makes perfect sense. Our present gap of detailed knowledge concerning how RNS affect survival versus death explains the high scientific interest in these questions in the past, and probably will continue to attract researchers in the future, in a search for the holy grail to adjust RNS production as a therapeutic intervention for regulating, *e.g.,* apoptosis. The molecular recognition of decision-making events, such as the importance of relative ROS versus RNS formation, regulation of transcription factor activity, modulation of pro- versus antiapoptotic protein expression, concepts on posttranslational protein modification, and the signaling qualities of cyclic nucleotides, may add to a still growing list of variables that determine the cell fate toward RNS. Signaling qualities of RNS as either direct effectors or regulators of other signaling events allows the rationalization of individual pro- or antiapoptotic observations, but still fails to deliver a unifying and predictive concept on RNS action. Regulation of apoptosis occurs at multiple levels in three-dimensional signal transduction circuits, and many incoming signals, as well as intracellular set points, balance initiation of apoptosis or its inhibition. This type of apoptostate is cell-specific and decides the reactivity toward RNS. Our knowledge of the role of RNS in affecting cell demise will substantially increase when we understand how a RNS-sensitive cell can be shifted toward an insensitive one and vice versa. The plethora of variables and the inherent complexity of RNS biology hinder present efforts to define a simple role of RNS in apoptosis. Along the road, new information will appear and refine current concepts, and at the very end (nobody knows when it will be) we hope to have an answer on the precise role of RNS in apoptosis.

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ABBREVIATIONS

Apaf-1, apoptosis protease-activating factor 1; ATM, ataxia telangiectasia-mutated; ATR, ATM- and Rad3-related; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CHOP, C/EBP homologous protein (also known as GADD153); cIAP, cellular inhibitor of apoptosis; Cox-2, cyclooxygenase-2; CREB, cAMP-response element binding protein; ER, endoplasmic reticulum; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; Hsp, heat shock protein; MC, mesangial cells; Mdm2, murine double minute; NAC, *N*-acetylcysteine; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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