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Review

Nitric oxide (NO): an effector of apoptosis

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Abstract

It is appreciated that the production of nitric oxide (NO) from L-arginine metabolism is an essential determinate of the innate immune system, important for nonspecific host defense, as well as tumor and pathogen killing. Cytotoxicity as a result of a substantial NO-formation is established to initiate apoptosis, characterized by upregulation of the tumor suppressor p53, changes in the expression of pro- and anti-apoptotic Bcl-2 family members, cytochrome *c* relocation, activation of caspases, chromatin condensation, and DNA fragmentation. Proof for the involvement of NO was demonstrated by blocking adverse effects by NO-synthase inhibition. However, NO-toxicity is not a constant value and NO may achieve cell protection as well. In part this is understood by transcription and translation of protective proteins, such as cyclooxygenase-2. Alternatively, protection may result as a consequence of a diffusion controlled NO/O_2^- (superoxide) interaction that redirects the apoptotic initiating activity of NO towards protection. NO is endowed with the unique ability to initiate and to block apoptosis, depending on multiple variables that exist to be elucidated. The crosstalk between cell destructive and protective signaling pathways under the modulatory influence of NO will determine the impact of NO in apoptotic cell death and survival.

Keywords: nitric oxide; apoptosis; necrosis; p53; caspases; Bcl-2 family

Abbreviations: Cox-2, cyclooxygenase-2; GSNO, S-nitrosoglutathione; IFN-γ, interferon-γ; iNOS, inducible NOS; LPS, lipopolysaccharide; NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PARP, poly(ADPribose) polymerase

NO: history of a cytotoxic molecule

In the early and middle 1980s it was demonstrated in studies inspired by analysis of carcinogenesis that mammalian cells synthesize inorganic oxides of nitrogen. An independent line of research elucidated the biological basis of the cytostatic effect of activated macrophages which led to the finding that L-arginine was necessary to sustain macrophage-evoked cytotoxicity and that authentic NO closely mimicked the pattern of macrophage-derived death.[1] In the late 1980s it was discovered that NO is generated by NOS isoforms and constitutes a unique molecular messenger. Isoenzyme activity allowed to discriminate a low *versus* high output system for NO and a rough correspondence between toxic and homeostatic functions of the molecule. NOS inhibitors such as N^G-monomethyl-L-arginine (NMMA) were used to intervene in NO production, thus allowing to trace back individual actions to the NO-signaling system. Alternatively, NO-relapsing compounds, generally termed nitrovasodilators or NO donors, are valuable tools to study NO signal transduction. The biological activity of NO is classified by cGMP-dependent and cGMP-independent pathways, both attributed to physiology and pathology.

Studies concerning the role of NO in mammalian organisms has given a picture of complexity. In particular, iNOS turned out to be both friend and foe. On one side iNOS-generated NO conveys protection against many bacteria and parasites, fights several viral infections, and is a modulator of malignancies, while on the other side it may promote tumor angiogenesis and may facilitate tissue destruction and/or disease states.[2] Lessons emerging from iNOS 'knock-out' mice now provide evidence for a beneficial and/or detrimental role of NO.[2]

Several disease-related conditions are controlled by macrophages and it is now confirmed that human monocytes or macrophages from patients with a wide range of infectious or inflammatory diseases express iNOS.[3] Furthermore, the expression of iNOS in human infectious, autoimmune, and chronically inflammatory diseases as well as several other human disorders is well established.[4] It is becoming clear that NO, including iNOS-derived reactive nitrogen species, comprises both, regulatory and effector functions. The interaction of these signals may determine the destructive or protective role of NO during apoptotic cell death.

NO-evoked apoptosis: initial observations

In earlier reports from 1991–1993 predominantly cell lysis of fibroblasts or islet cells by NO donors, activated macrophages, or an active NOS has been documented.[5,6] First observations on NO-mediated apoptosis were reported independently by several groups in 1993,[7–9] while primary observation on the involvement of typical apoptotic alterations and signaling components such as accumulation of the tumor suppressor protein p53 appeared in 1994.[10,11] Evidence for NO-elicited apoptosis in macrophages was provided by chromatin condensation and internucleosomal DNA fragmentation. The involvement of NO was confirmed by preventing

cell death in L-arginine-restricted medium or the presence of the NOS-inhibitor NMMA, and more directly by exposing cells to authentic NO gas.[12] Following these initial documentations numerous reports confirmed the ability of NO to initiate apoptosis based on studies in murine and human cells such as β-cells, thymocytes, chondrocytes, mesangial cells, neurons (see Lipton; this issue), mast cells, vascular endothelial cells, smooth muscle cells, various tumor cells, and several more.[13,14]

Under conditions of apoptosis NO left intracellular NAD^+ as well as ATP unaltered and preserved membrane integrity, as determined by the absence of LDH (lactate dehydrogenase) release. Moreover, inhibitors of poly(ADP-ribose) polymerase, such as 3-aminobenzamide, were non effective.[24] These experiments ruled out an overlap of apoptotic and necrotic alteration in our studies. However, PARP activation followed by energy depletion has been associated with NO-mediated neurotoxicity[15] and islet cell death.[16] Albeit it seems unlikely that PARP activation represents a general pathway leading to NO-elicited apoptotic death because programmed cell death is an energy requiring process[17,18] and PARP seems fully dispensable for apoptosis.[19,20] One might speculate that in these systems NO evoked a necrotic rather than an apoptotic response which is fully compatible with our observations that ATP-depletion shifted NO-elicited apoptosis to necrosis.[21]

The p53 response, cytochrome *c* relocation, and caspase activation

One pivotal target that responds to DNA damage is the tumor suppressor p53. The cellular level of p53 is kept at a low level due to a short protein half-life but in response to a variety of stimuli it is increased steeply by stabilization. In turn, p53 functions as a cell cycle checkpoint or an initiator of apoptosis.

In RAW 264.7 macrophages, RINm5F, or mesangial cells we established a role of p53 during NO-mediated cell death.[10,22,23] Activation of iNOS in macrophages resulted in p53 accumulation at an early time point, clearly preceding DNA fragmentation, while p53 accumulation was attenuated by blocking NO formation. p53 accumulation was further substantiated by using NO donors with the notion that p53 accumulation can be correlated to NO-intoxication.[22,24] In addition, macrophages stably transfected with plasmids encoding p53 antisense RNA exhibited reduced p53 levels in response to S-nitrosoglutathion (GSNO) and revealed a marked reduction in DNA fragmentation, thus suggesting a functional role of p53 during NO-induced macrophage apoptosis. The ability of NO to promote a functional p53-response has been confirmed in murine and human systems[25–27] and been extended to the observation that p53 in turn down-regulated iNOS expression through inhibition of the iNOS promoter. In line, cancer cells expressing iNOS and wild-type p53 showed reduced tumor growth, whereas those with mutated p53 revealed accelerated growth associated with increased neovascularization. Thus, NO may promote cancer progression by providing a selective growth advantage to tumors with mutated p53.[28] In addition, NO-mediated inhibition of cell cycle proliferation in smooth muscle cells is associated with p21 induction[29] which in some cases is achieved by p53. However, experiments in p53 negative cells (U937 cells) substantiated p53-independent signaling pathways during NO-mediated apoptosis as well.[30]

The importance of mitochondria and cytochrome *c* release in apoptosis was suggested by studies with cell-free systems in which nuclear condensation and DNA fragmentation demanded cytochrome *c* that forms an essential part of the 'apoptosome'. NO potently and reversible deenergizes mitochondria and inhibits mitochondrial enzymes including aconitase and cytochrome *c* oxidase.[13,31] In addition, peroxynitrite and NO donors have been shown to induce mitochondrial pore transition and it has been suggested that NO promotes apoptosis via a direct effect on mitochondria.[32] However, these studies do not correlate permeability transition to other responses such as p53 accumulation or caspase activation. Moreover, a general link between NO action and changes in the mitochondrial membrane potential are excluded.[31] Therefore, experiments were carried out to determine whether the release of cytochrome *c* is evoked by NO in RAW 264.7 macrophages and to position the p53-signaling capacity relative to mitochondria. GSNO and spermine-NO caused a fast p53 accumulation followed by cytochrome *c* release and caspase activation. In p53 antisense expressing macrophages (RΔp53asn-11) p53 accumulation, cytochrome *c* relocation, and caspase activation were blocked in response to NO. In addition, ATP-depleted cells showed a shift from apoptosis towards necrosis upon NO addition, together with attenuated p53 accumulation and caspase activation.[21] We suggest that NO-mediated apoptosis in macrophages is entirely controlled by the mitochondrial pathway with the implication that p53 accumulation is upstream of cytochrome *c* release. This assumption is supported by the observation that thymocytes from p53 knockout-mice are protected against irradiation-induced apoptosis together with suppressed mitochondrial transmembrane depolarization.[33] Further, it has to be mentioned that the receptor tyrosine kinase KIT can suppress p53-mediated apoptosis by attenuating p53-induced $\Delta\Psi_m$ depolarization,[34] and that p53 is required to achieve early caspase activation in a cell free system.[35]

After establishing a role of p53 accumulation in pro-apoptotic signaling we searched for potential pathways that account for p53 accumulation. Therefore, we analyzed the involvement of mitogen-activated protein kinases (MAPK) in NO-evoked macrophage apoptosis.[36] We used GSNO to activate extracellular signal-regulated protein kinases 1/2 (ERK1/2), c-Jun N-terminal kinases/stress-activated protein kinases (JNK1/2), and p38 kinases and determined the role of MAPK signaling in the apoptotic transducing ability of GSNO. ERK1/2 became activated in response to NO and blocking the ERK1/2 pathway by PD 98059 enhanced GSNO-elicited apoptosis. p38 was activated, but inhibition of p38 left apoptosis unaltered. GSNO enhanced JNK1/2 activities between 2 and 8 h and antisense-depletion of JNK1/2 eliminated the pro-apoptotic action of low GSNO concentrations (250 μM), whereas apoptosis proceeded

independently of JNK1/2 at higher doses (500 μM GSNO). Decreased apoptosis by JNK1/2 depletion prevented p53 accumulation, which positions JNK1/2 upstream of the p53 response at low agonist concentrations. In this respect we follow the hypothesis that NO may inhibit the proteasome which in turn facilitates p53 accumulation, as inhibition of p53 degradation which normally is very rapid, will result in protein accumulation.[37] Regulation of MAPK and the proteasome may define pathways that explain p53 accumulation in response to NO.

As expected, we noticed activation of caspases in response to endogenously produced or exogenously added NO as demonstrated by PARP cleavage.[38] Caspase activation was preceded by p53 accumulation and caspase activation after cytokine treatment was blocked by NMMA. Caspase activation by NO donors was confirmed in human leukemia cells,[39] mesangial cells,[40] and neuronal excitotoxicity triggered by nitric oxide.[41] Although PARP cleavage in response to NO is proven, a cause-effect relationship of protein degradation for apoptosis must be questioned.[19,20] However, under conditions that provoked apoptosis, activation of caspases seem an inherent step in the death pathway and caspase inhibitors can be used to block apoptosis.[41,42] Demonstrating that a caspase-3-like or a pan-caspase inhibitor left cytochrome *c* and p53 accumulation unaltered implied that a caspase activity is not required for both events.[21]

In close analogy to the macrophage system the ability of NO to initiate cell death with the occurrence of apoptotic parameters was identified in vascular smooth muscle cells of murine and human origin.[43,44] In these cells apoptosis was characterized by p53 accumulation, decreased Bcl-2 expression, and caspase-3 activation with the further notion that inhibition of caspase-3 significantly prevented apoptosis. However, in human neoplastic lymphoid cells NO-induced apoptosis correlated with caspase-8 activation that in part was independent of the CD95 receptor/ligand system.[45] In summary, activation of p53, relocation of cytochrome *c*, and caspase activation are inherent features of NO-evoked apoptosis.

The Bcl-2 protein family

In several studies NO-evoked apoptosis and decreased expression of anti-apoptotic Bcl-2 family members were correlated, although any cause-effect relation remained unproven. In contrast, expression of the pro-apoptotic protein Bax increased during NO-mediated apoptosis, at least in macrophages and mesangial cells.[46] In analogy to many other systems the balance of anti- and pro-apoptotic proteins may tune the decision between life and death. To appreciate the protective principle of Bcl-2 under conditions of NO-exposure we stably transfected macrophages with human Bcl-2.[46] Bcl-2 transfectants showed protection against various NO donors and endogenously generated NO, although p53 accumulation remained unchanged. This led to the conclusion that Bcl-2 acts downstream of p53 but upstream to cytochrome *c* relocation that was attenuated. It was confirmed by others that enforced Bcl-2 or Bcl-x_L expression protected against NO-mediated cytotoxicity.[47–49] Extending experiments revealed that NO-mediated caspase activation was attenuated in the Bcl-2 transfectants.[38] Overall, our studies position Bcl-2 downstream of p53 but upstream of

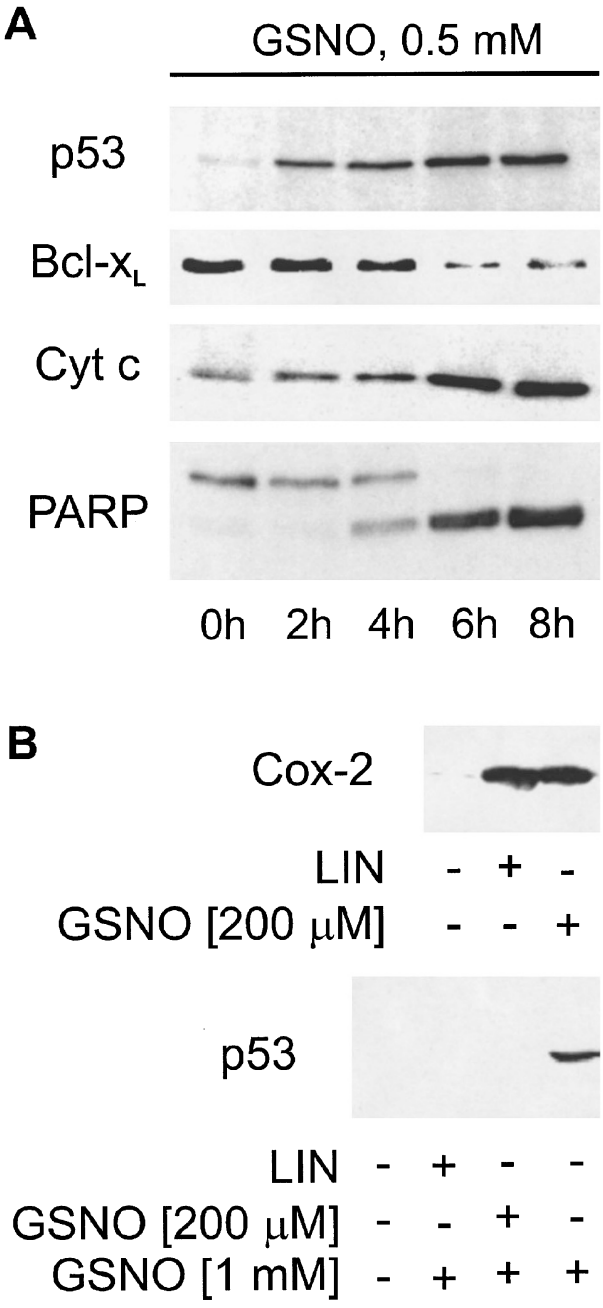

Figure 1 (**A**) Induction of various apoptotic alterations in RAW macrophages upon NO-stimulation. RAW 264.7 macrophages were incubated with 0.5 mM GSNO for 2–8 h. Expression of p53, down-regulation of Bcl-x_L, the appearance of cytosolic cytochrome *c*, and caspase-evoked PARP-cleavage were followed by Western blot analysis. For details, see the text. (**B**) Attenuation of apoptosis upon macrophage prestimulation. Accumulation of Cox-2 in RAW macrophages upon prestimulation with a non-toxic dose of GSNO (200 μM) or LIN (LPS, 10 μg/ml; IFN-γ, 10 U/ml; NMMA, 1 mM) for 15 h. p53 accumulation was analyzed in response to 1 mM GSNO during a 4 h incubation period with or without a 15 h lasting prestimulation period with 200 μM or LIN. Cox-2 expression and p53 accumulation are mutually exclusive. For details, see the text and references[75–77]

cytochrome *c* relocation and caspase activation. Figure 1 shows typical alterations upon NO addition and points to the anti-apoptotic role of low level NO prestimulation in association with Cox-2 expression.

Lessons from disease states

Bacterial LPS is a strong immunostimulator and can elicit a shock syndrome. In macrophages and other cell types LPS primarily induces the expression of an impressive number of genes and triggers the release of inflammatory cytokines as well as NO. LPS-induced apoptosis that occurs as a result of NO formation in macrophage cell lines and primary murine macrophages is well established.[13,14,50] The production of high levels of NO in various cells is associated with autocytotoxicity, suppression of tumorigenicity, and abrogation of metastasis which in part is explained by inducing apoptosis.[51] For example, macrophage-derived NO plays a role in apoptosis of splenocytes during the acute phase of experimental Trypanosoma cruzi infection, supporting the notion that immunosuppression and apoptosis are to be correlated under certain *in vivo* situations.[52] iNOS-generated NO evoked apoptosis in ascites hepatoma cells under *in vivo* conditions in tumor-bearing rats[53] and NO caused autolysis in tumor cells and lysed bystander cells under *in vitro* and *in vivo* conditions.[54] Moreover, macrophages from septic mice exhibited increased apoptosis that was blocked by NOS-inhibition.[55] Human intestinal epithelial cells responded to bacterial infection with apoptosis, that occurred at least in part as a result of NO formation.[56] Furthermore, one might speculate that apoptosis is associated with NO-evoked acute cardiac allograft rejection,[57] where iNOS predominately is localized in graft infiltrating immune cells[58] and that NO damages pulmonary smooth muscle cells via apoptosis thereby causing acute lung injury in response to cytokines.[59] In addition, vascular damage plays an important role in the pathogenesis of vasculitides that characterize rheumatic diseases, reperfusion disorders, as well as the adult respiratory distress syndrome. It appears that cytokine-mediated, neutrophil-dependent injury of human umbilical vein endothelial cells is associated with NO production.[60] A functional role of stimulated NO production was also established in TCR-triggered (TCR; T-cell receptor) apoptotic death of mature T lymphocytes.[61] Interestingly, TCR causes expression of the neuronal isoform of NOS to generate NO which may promote Fas ligand expression that in turn initiates apoptosis. In some analogy, a pro-apoptotic role of NO in close association with upregulation of Fas has been reported for β-cells.[62] Human pancreatic β-cells that do not constitutively express Fas, however became Fas positive after interleukin-1β exposure and concomitantly underwent Fas-mediated apoptosis. Fas expression is blocked by NOS inhibitors, while NO donors substitute in promoting Fas expression. It must be concluded that upregulation of Fas by NO contributes to β-cell death under conditions of insulin-dependent diabetes mellitus. In haemopoietic progenitor cells it has been observed that Fas, by causing iNOS expression, evoked apoptosis that in turn was attenuated by suppressing NO formation.[63]

Despite the knowledge that individual murine and human cells are susceptible to NO-mediated apoptosis, that iNOS expression often is associated with cytotoxicity in murine systems, and the information that NO is produced in humans in association with multiple disease states, reports on NO-evoked apoptosis in human studies are more discrete. Besides above mentioned studies which in part have been performed in human cells it appears that apoptosis, iNOS induction, and human cardiac allograft rejection and/or chronic heart failure are positively correlated. It is assumed that iNOS in macrophages or mycocytes is involved in regulating apoptosis[64,65] in close analogy to iNOS expression and apoptosis in human vascular smooth muscle cells.[44] Furthermore, the appearance of p53 and iNOS in apoptotic macrophages is established in human atherosclerotic lesions.[66] Moreover, a positive correlation between NO formation and apoptosis is also implied for articular cartilage samples from humans with osteoarthritis[67,68] or for the occurrence of human lupus nephritis.[69] Generally, it appears that NO is endowed with the capability to initiate apoptosis in cellular systems, co-cultures, and *in vivo*. Although primarily established for murine systems, a positive correlation between NO formation and apoptosis is becoming evident for some human disorders as well.

Protective principles in mesangial cells or macrophages

During our studies with rat mesangial cells we unexpectedly noticed that NO-mediated apoptotic cell death was antagonized by the simultaneous formation of superoxide (O_2^-).[23,70] Part of the signal transmission of both, NO and O_2^- may stem from their diffusion controlled interaction. We addressed the NO/O_2^--interaction by exposing cells to NO donors and O_2^- generating systems such as the redox cycler DMNQ (2,3-dimethoxy-1,4-naphtoquinone) or the hypoxanthine/xanthine oxidase system, thereby allowing a continuous radical formation. The balanced and simultaneous generation of NO and O_2^- turned out to be protective for mesangial cells, whereas the unopposed radical generation elicited apoptosis and in higher concentrations necrosis. Of particular importance is the simultaneous presence of both radicals. With either NO or O_2^- being offset, protection is less efficient. We conclude that signaling mechanisms as a consequence of the NO/O_2^--interaction redirect apoptotic initiating signals to harmless pathways as long as reduced glutathione is present.[71] Our studies are in analogy to *in vitro* experiments performed by Wink and colleagues.[72] They observed GSSG formation by incubating NO donors, O_2^-, and reduced glutathione and concluded NO-evoked nitrosative reactions to be quenched by the resultant oxidative stress. In some analogy NO attenuated O_2^--mediated toxicity in chondrocytes,[73] reduced stretch-induced programmed myocyte cell death that resulted from O_2^- formation, or abrogated toxicity of oxidized low-density lipoprotein in endothelial cells. These observations are in some agreement with a protective role of NO during ischemia-reperfusion, peroxide-induced toxicity, lipid-peroxidation, or myocardial injury.[13] As a general concept it appears that in some systems the balanced formation and interaction of biological radicals resembles a protective principle thereby eliminating harmful

reactions that operate as a consequence of unopposed radical generation.

By analyzing macrophage programmed cell death, we realized that desensitization towards NO-elicited apoptosis occurs upon preactivation with a combination of LPS and IFN-γ under conditions of blocked NOS or from prestimulation with a nondestructive dose of NO donors.[74] Apparently, induction of cyclooxygenase-2 (Cox-2) represents a critical regulator of macrophage apoptosis.[75] Resting macrophages do not express Cox-2, whereas LPS/IFN-γ/NMMA or a nontoxic dose of GSNO caused protein expression within 6–12 h. A protective role of Cox-2 was assured in Cox-2 overexpressing cells that was antagonized by the Cox-2 selective inhibitor NS-398 or was lost in antisense Cox-2 transfected macrophages. Obligatory for Cox-2 expression appears the activation of the nuclear transcription factor NF-κB (p50/p65-heterodimer formation). Degradation of I-κBα and activation of a luciferase reporter construct, containing four copies of the NF-κB-site derived from the murine Cox-2 promoter, confirmed NF-κB activation by NO. Furthermore, a NF-κB decoy approach attenuated not only cell protection but also restored DNA fragmentation and p53 accumulation in response to a high dose GSNO. These examinations provided evidence for an anti-apoptotic role of NO, transmitted by NF-κB activation (Figure 1B). Besides, NO also activated the transcription factor AP-1 that additionally was required for Cox-2 expression and protection.[76] Cox-2 may promote prostanoid formation thereby evoking an increase in cAMP that resembles a protective principle.[77] A protective and/or anti-apoptotic principle of Cox-2 has been confirmed for oxidative stress-evoked injury in cardiomyocytes[78] and is noted during the tumorigenic effect in colon epithelial cells.[79] Besides Cox-2, endorsed expression of CuZn-superoxide dismutase, heme oxygenase-1, heat shock protein 70 (see Li and Billiar; this issue), Mn-superoxide dismutase (MnSOD), or NO-elicited upregulation of Bcl-2 attenuates NO toxicity.[13] Consistent for these studies is the notion that cell survival demands the expression of protective proteins.[80,81] It remains to be established how these protective proteins circumvent cell death. Protection as a result of NO formation is also noted in association with soluble guanylyl, cyclase activation or as a result of protein thiol modification, i.e. inhibition of caspase processing (see Liu and Stamler, Dimmeler and Zeiher, Li and Billiar; this issue). Figure 2 summarizes main pro- and anti-apoptotic pathways that appear closely associated with NO delivery.

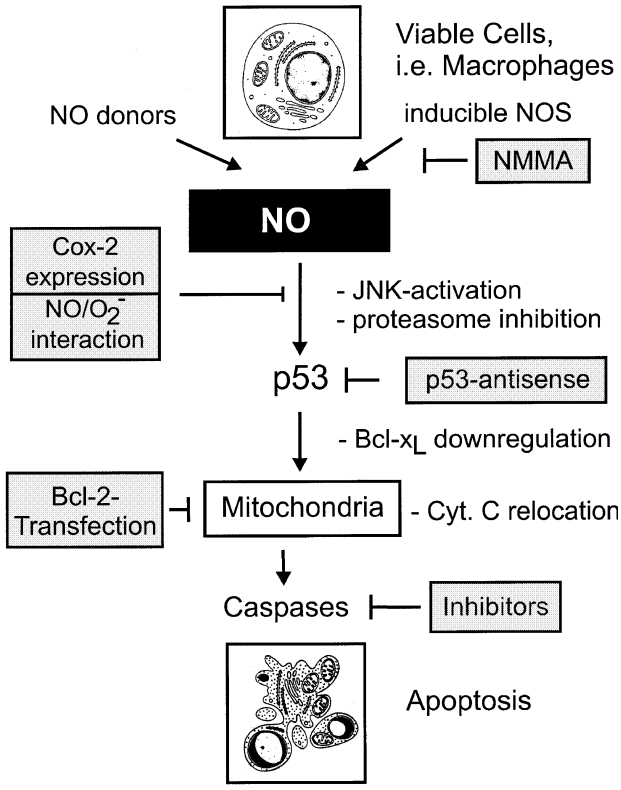

Figure 2 Signaling components that characterize NO-mediated apoptosis. Several signaling components of NO-initiated apoptotic cell death, as well as some possible antagonistic interventions are shown. Importantly, arrows do not imply direct cause-effect relations. Experimental evidence for the sequence of events mainly comes from our work with macrophages and mesangial cells. Inhibitory activities or defined pharmacological interventions are marked by boxes. Specific sites of intervention are indicated (⊥). For details, see the text

Concluding remarks

A large body of evidence suggests that NO either delivered by NO donors or generated by L-arginine metabolism initiates apoptotic cell death. This is proven in murine and human cellular systems, co-culture examinations, and *in vivo*. The toxicity of NO is influenced by the existing biological milieu, relative rates of NO formation, its redox state, the combination with oxygen, superoxide, as well as other biomolecules. In light of the numerous studies NO-evoked toxicity is defined by typical morphological and biochemical features of apoptosis. However, NO is no longer regarded a toxic molecule, only. Rather, NO made its way from an effector to a regulator of other signaling pathways. In line, not all cellular systems which express iNOS enter the death pathway. Antagonistic and/or protective principles exist. NO-derived protection can be divided into mechanisms that demand gene expression of protective proteins and into processes that are transmitted by thiol modification or cyclic GMP formation (see Liu and Stamler, Dimmeler and Zeiher, Li and Billiar, and Lipton; this issue).

It is mandatory to define the versatility of NO-signaling mechanisms in relation to their apoptotic inducing ability and to explore how NO-responsive targets serve both, sensory and regulatory roles in transducing a signal. The tuning of potentially protective and destructive NO-actions and the molecular recognition of these balances will be central to understand the role of NO during apoptosis and cell survival.

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