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Review

Nitric oxide and its role in apoptosis

Bernhard Brüne *, Andreas von Knethen, Katrin B. Sandau

University of Erlangen-Nürnberg, Faculty of Medicine, Department of Medicine IV-Experimental Division, Loschgestrasse 8, 91054 Erlangen, Germany

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Abstract

Nitric oxide ($NO^{\cdot}$), a potentially toxic molecule, has been implicated in a wide range of diverse (patho)physiological processes. It is appreciated that the production of $NO^{\cdot}$ from L-arginine is important for nonspecific host defense, helping to kill tumors and intracellular pathogens. Cytotoxicity as a result of a massive $NO^{\cdot}$-formation is now established to initiate apoptosis. Apoptotic cell death in RAW 264.7 macrophages and several other systems as a result of inducible NO-synthase activation comprises upregulation of the tumor suppressor p53, activation of caspases, chromatin condensation, and DNA fragmentation. The involvement of $NO^{\cdot}$ was established by blocking adverse effects by NO-synthase inhibition. Overexpression of the antiapoptotic protein Bcl-2 rescued cells from apoptosis by blocking signal propagation downstream of p53 and upstream of caspase activation. As the wide variety of $NO^{\cdot}$-effects is achieved through its interactions with targets via redox and additive chemistry, the biological milieu, as a result of internal and external stimuli, may modulate toxicity. Therefore, transducing pathways of $NO^{\cdot}$ are not only adopted to cytotoxicity but also refer to cell protection. $NO^{\cdot}$-signaling during protection from apoptosis is in part understood by the requirement of gene transcription and protein synthesis. $NO^{\cdot}$-formation causes upregulation of protective proteins such as heat shock proteins, cyclooxygenase-2, or heme oxygenase-1 which in a cell specific way may attenuate apoptotic cell death. Alternatively, protection may result as a consequence of a diffusion controlled $NO^{\cdot}/O_2^-$ (superoxide) interaction. The $NO^{\cdot}/O_2^-$-interaction redirects the apoptotic initiating activity of either $NO^{\cdot}$ or O_2^- towards protection as long as reduced glutathione compensates the resultant oxidative stress. Protective principles may further arise from cyclic GMP formation or thiol modification. $NO^{\cdot}$ shares with other toxic molecules such as tumor necrosis factor-α the unique ability to initiate and to block apoptosis, depending on multiple variables that are being elucidated. The crosstalk between cell destructive and protective signaling pathways, their activation or inhibition under the modulatory influence of $NO^{\cdot}$ will determine the role of $NO^{\cdot}$ in apoptotic cell death. © 1998 Elsevier Science B.V. All rights reserved.

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1. Apoptosis

The functional integrity of an organism demands regulatory mechanisms that control cell homeostasis which is achieved in part by affecting cell proliferation and cell death. The notion that cell proliferation and cell death follow programmed patterns is fundamental to recent developments in biology and medicine. In 1972, Kerr et al. marshaled morphological evidence to draw a clear distinction between the cell deaths that occur in both animal development and tissue homeostasis, as well as in some pathological states, and the pathological cell deaths that are noticed at the center of acute lesions (Kerr et al., 1972). Although the term 'apoptosis' was originally coined to describe defined morphological alterations, it is now generally used to describe the evolutionary conserved pathway of biochemical and molecular events leading to cell demise (Hale et al., 1996; Peter et al., 1997; Thompson, 1995; Leist and Nicotera, 1997; McConkey and Orrenius, 1997). Apoptosis comes from the Greek word *apo* (away from, with the implication of separation) and the root *ptosis* (to fall). Apoptosis literally means to fall away from, as leaves fall away from a tree. The term 'programmed cell death' adopted from developmental biology is now used as a synonym for apoptosis to appreciate genetic programs that regulate cell death (Vaux and Strasser, 1996). It is now generally accepted that animal cells have a built-in suicide, or death program, which largely became established through genetic studies in the nematode *Caenorhabditis elegans* that identified genes that seem dedicated to the

Abbreviations: NOS, nitric oxide synthase; LPS, lipopolysaccharide; IFN-γ, interferon-γ; Cox-2, cyclooxygenase-2

* Corresponding author. Tel.: +49-9131-856311; fax: +49-9131-859202; e-mail: mfm423@rzmail.uni-erlangen.de

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death program and its control, and then through the observations that some of these genes do have mammalian homologous (Jacobson et al., 1997). The elements of a core program controlling the proper executive phase of apoptosis seem to be constitutively expressed in virtually every cell. A cell will undergo apoptosis as a result of information received from its environment interpreted in the context of internal information, such as its cell type, state of maturity, developmental history, and state of differentiation (Steller, 1995). External informations triggering apoptosis are multiple and may be influenced by the appearance or disappearance of hormones, growth factors, cytokines, or intercellular and/or cell matrix interactions (Ruoslahti and Reed, 1994). Cells that die during normal development or tissue homeostasis usually shrink and condense, display surface alterations such as the exposure of phosphatidylserine that normally is confined to the cell interior, and cleave DNA into large and often small oligonucleosomal-sized (200 bp and multiples) fragments. During nuclear changes the organelles in the cytoplasm and the plasma membrane retain their integrity. The dead cell or their fragments (apoptotic bodies) are rapidly phagocytosed by neighboring cells or macrophages before lysis occurs, and thus prevent the induction of an inflammatory response. The efficiency of this process is rapid (cells often disappear in an hour or less) and therefore the contribution to cell death in tissues has often been underestimated (Savill, 1997). In contrast, during necrosis the cell and their organelles tend to swell and rupture thereby releasing cellular constituents that promote a secondary inflammatory response.

Since a particular external stimulus is only part of the information influencing the decision of self-destruction or survival, such stimuli are not, in general, exclusively involved in the control of apoptosis. Similarly, intracellular signals involved in the induction of apoptosis are often regulators of other cellular responses. Evidence is accumulating that multiple signaling pathways can intersect, a process known as crosstalk, and therefore a cell response to a given stimulus may alter significantly, not only between different cells but also within one cell population. Although the great variety of external signals that initiate apoptosis aim to multiple signaling systems that are involved, there is considerable evidence to suggest that transducing pathways converge to one, or very few, common final executive steps (Peter et al., 1997; Goldstein, 1997). Regulators such as the tumor suppressor p53, caspases, or the regulatory role of Bcl-2 family members are consistent with such convergence of activating or antagonistic pathways at the point where corresponding gene products act. Apoptosis can be triggered by a variety of extrinsic and intrinsic signals, which allows the elimination of cells that have been produced in excess, have developed improperly, or have sustained genetic damage (Thompson, 1995). Among the inducers of apoptosis the molecule nitric oxide has recently been recognized.

2. Nitric oxide ($NO^{\cdot}$): formation and signaling

The discovery that $NO^{\cdot}$ is a unique diffusible molecular messenger in the vascular and immune system motivated searches for $NO^{\cdot}$ biosynthesis and action throughout the body. $NO^{\cdot}$ is catalytically produced by different NO-synthase (NOS) isoforms in a reaction scheme, involving the five electron oxidation of the terminal guanido nitrogen of the amino acid L-arginine to form $NO^{\cdot}$ and stoichiometric amounts of citrulline (Mayer and Hemmens, 1997). The reaction demands oxygen and NADPH as cosubstrates, with numerous other redox cofactors including enzyme bound heme, reduced thiols, FAD, FMN, and tetrahydrobiopterin. The initial NOS nomenclature reflected the early observations that $NO^{\cdot}$ synthesis was characteristic upon immunoactivation of inflammatory cells (Nathan, 1992; Moncada et al., 1991). The corresponding enzyme therefore was designated 'inducible NO synthase' (iNOS, now known as NOS2). The iNOS was contrasted to a constitutive NO synthase activity 'cNOS' that was expressed in characteristic cell types (neuronal cells or endothelial cells). These NO synthase isoforms were termed nNOS (known as NOS1) and eNOS (known as NOS3). It is now known that the level of gene expression of both eNOS and cNOS may be induced and conversely, that iNOS may function as a 'constitutive' enzyme. However, a widely accepted nomenclature describes isoforms as nNOS, iNOS, and eNOS, reflecting the original tissue for the protein and cDNA isolates (Michel and Feron, 1997). For full enzymatic activity the level of intracellular calcium seem important. eNOS and nNOS may closely be modulated by transient changes in calcium, whereas iNOS appears to become fully activated even at low Ca^{2+} characteristic of resting cells. However, other factors such as intracellular localization of NO synthase isoenzymes, palmitoylation, and phosphorylation are recognized components that modulate enzyme activity (Nathan and Xie, 1994). Once activated, NO synthase isoforms not only produce $NO^{\cdot}$, the primary reaction product, but also those species resulting from oxidation, reduction, or adduction of $NO^{\cdot}$ in physiological milieus, thereby producing various nitrogen oxides, *S*-nitrosothiols, peroxynitrite ($ONOO^{-}$), and transition metal adducts (Stamler et al., 1992). The determinant of isoenzyme activity allows to approximate the classification as a low vs. high output system for endogenously generated $NO^{\cdot}$ and a rough correspondence between toxic and homeostatic functions of the molecule. NO synthase inhibitors such as N^{G}-monomethyl-L-arginine (NMMA), are commonly used to intervene pharmacologically in $NO^{\cdot}$ production, thus allowing to trace back individual actions to the NO-signaling system (Moncada et al., 1991).

Biological activity of $NO^{\cdot}$ is classified by cGMP-dependent and cGMP-independent pathways, both attributed to physiology and pathology (Stamler, 1994; Schmidt and Walter, 1994). NO is a key transducer of the vasodilator message from the endothelium to vascular cells, is a

constituent in central and peripheral neuronal transmission, and participates in the nonspecific immune defense. Activation of soluble guanylyl cyclase, formation of cGMP, and concomitant protein phosphorylation is considered the main physiological signaling pathway of NO. For our understanding of the cytostatic or cytotoxic signals, cGMP-independent reactions appear to be of greater importance.

To study $NO^{\cdot}$ signal transduction irrespective of NO synthase involvement, NO-releasing compounds are valuable tools (Butler et al., 1995). Nitric oxide releasing compounds, generally termed nitrovasodilators or NO donors preserve $NO^{\cdot}$ in their molecular structure and evoke biological activity after decomposition. These drugs exhibit considerable variations in their chemical structure, stability, and biological activity. Different bioavailability, at least in part, arises from differences in bioactivation and enzymatic vs. nonenzymatic NO release. Examples are organic nitrates, sodium nitroprusside, 3-morpholino-sydnonimine (SIN-1), *S*-nitrosothiols (i.e., *S*-nitrosoglutathione, GSNO or *S*-nitrosocysteine, CysNO), and compounds containing the $N(O)NO^{-}$ functional group (i.e., diethylamine–nitric oxide complex, DEA–NO or spermine–NO).

$NO^{\cdot}$ synthesis is part of the inflammatory response against pathogens, such as bacteria, viruses, and tumor cells (Kolb and Kolb-Bachofen, 1992; Nathan, 1997). Under conditions of massive $NO^{\cdot}$ formation the various regulatory, cytostatic, and/or toxic consequences of $NO^{\cdot}$ may play important roles in the pathophysiology of tissue or cell destruction. Toxic consequences as a result of $NO^{\cdot}$ generation seem predictable. However, during infection and inflammation $NO^{\cdot}$ generation appears to act both as a direct apoptotic inducer and as a regulator of other effectors. The ability of $NO^{\cdot}$ to function as a messenger molecule and to affect signaling pathways of adverse agonists allows to redirect cell destructive pathways into protection. Therefore, the role of $NO^{\cdot}$ during apoptosis is ambivalent and $NO^{\cdot}$ may function both, as an activator and inhibitor of the death program, depending on the biological milieu, i.e., the presence or absence of stimulatory or inhibitory cosignals.

In the following section we will describe established cellular responses that are closely associated with $NO^{\cdot}$-intoxication and will summarize some cell defensive mechanisms that are activated in response to $NO^{\cdot}$. The interaction of these signals may determine the destructive or protective role of $NO^{\cdot}$ during apoptotic cell death.

3. $NO^{\cdot}$: a toxic molecule

$NO^{\cdot}$, generated at a high level by activated macrophages is an important, often the major, cytotoxic molecule in the defense against tumor cells and pathogens. The ability of macrophages to kill tumor cells and bacteria in an L-arginine dependent fashion originally was noticed by Hibbs et al. (1987). They went on to demonstrate that nitric oxide gas was as toxic to tumors cells as activated macrophages. Although these initial observations date back to the late eighties (Stuehr and Nathan, 1989), the precise mechanisms underlying cellular toxicity caused by $NO^{\cdot}$ are still unclear. It is evident that $NO^{\cdot}$-mediated toxicity is a very complex process and may involve multiple pathways. To further complicate the picture, it has been observed that sensitivity to $NO^{\cdot}$ varies considerably from one cell type to another and that death may occur as a result of either necrosis or apoptosis (Bonfoco et al., 1995).

In earlier reports from 1991 to 1993 predominantly cell lysis of fibroblasts or islet cells by NO donors, activated macrophages, or an active intracellular NO synthase have been reported (Krönke et al., 1991; Bergmann et al., 1992; Duerksen-Hughes et al., 1992; Fehsel et al., 1993). Initial observations on NO-mediated apoptosis appeared independently by several groups in 1993 (Albina et al., 1993; Sarih et al., 1993; Xie et al., 1993), while primary observation on the involvement of typical apoptotic associated alterations and signaling components such as the accumulation of the tumor suppressor protein p53 were described in 1994 (Meßmer et al., 1994).

In very initial studies it was noted that $NO^{\cdot}$ targets naked DNA (Wink et al., 1991; Nguyen et al., 1992) and induces oxidative DNA damage in activated macrophages (DeRojas-Walker et al., 1995). In extension of these observation it is still believed that a $NO^{\cdot}$-damaged cellular DNA elicits a rapid stress response in mammalian cells. DNA damage involves attachment of poly(ADPribose) polymerase (PARP) to the strand breaks and extensive synthesis of short-lived polymers by the bound enzyme (Althaus and Richter, 1987; DeMurcia and Menessier-De Murcia, 1994). Although PARP has no direct role in DNA excision repair, the enzyme binds tightly to DNA strand breaks and sometimes interferes with repair if poly(ADP-ribose) synthesis is prevented. Massive PARP activation following extensive DNA damage leads to NAD^{+}, the ADP-ribose donor, depletion. In effort to resynthesize NAD^{+}, ATP becomes depleted which ultimately leads to cell death due to energy deprivation. Moreover, inhibition of mitochondrial respiration (thereby affecting ATP-synthesis) via destruction of Fe–S clusters has been noted (Henry et al., 1993) and been related to toxicity. With regard to $NO^{\cdot}$ signaling, PARP activation followed by energy depletion has been associated with neurotoxicity (Zhang et al., 1994) and $NO^{\cdot}$-mediated islet cell death (Heller et al., 1995) but it seems unlikely that PARP activation represents a general pathway leading to $NO^{\cdot}$-elicited death as (i) apoptotic cell death is an energy requiring process (Leist et al., 1997a; Eguchi et al., 1997), (ii) PARP seem fully dispensable for apoptosis (Leist et al., 1997b; Wang et al., 1997), (iii) glycolytic activity can compensate for decreased mitochondrial function (Leist et al., 1997a) thereby allowing cells to retain their ATP.

4. NO·-induced apoptosis

4.1. Initial observations

Working with peritoneal macrophages two groups reported NO·-dependent apoptotic cell death following immunological activation (Albina et al., 1993, Sarih et al., 1993). Evidence for apoptosis was provided by microscopic examination of chromatin condensation and by specific pattern of internucleosomal DNA fragmentation, i.e., DNA laddering detected by agarose gel electrophoresis. The involvement of NO· was confirmed by the preventive effects of cell activation in L-arginine-restricted medium or in the presence of the NO synthase inhibitor N^G-monomethyl-L-arginine, and more directly by exposing cells to authentic NO· gas leading to apoptosis. Corroboration came also from early reports on the involvement of endogenous NO· in apoptosis of cytokine-activated murine L929 transformed fibroblasts (Xie et al., 1993). Following these initial documentations numerous reports confirmed the ability of NO· to initiate apoptosis. This holds for macrophage cell lines (Meßmer et al., 1995; Zamora et al., 1997), β-cells or corresponding cell lines such as the RINm5F cells (Meßmer et al., 1994; Kaneto et al., 1995), thymocytes (Fehsel et al., 1995; Sandau and Brüne, 1996), chondrocytes (Blanco et al., 1995), mesangial cells (Mühl et al., 1996; Sandau et al., 1997a), neurons (Dawson et al., 1993; Lipton et al., 1993), mast cells (Kitajima et al., 1994), vascular endothelial cells (Lopez-Collazo et al., 1997), smooth muscle cells (Nishio et al., 1996), various tumor cells (Lorsbach et al., 1993), and several more. However, not all studies clearly discriminated between apoptotic and necrotic cell death and not all examinations addressed whether endogenously generated NO· would suffice to initiate the death program.

Induction of the iNOS in RAW 264.7 macrophages by a combination of lipopolysaccharide and interferon-γ produced typical morphological and biochemical alterations of apoptosis (Meßmer et al., 1995) such as chromatin condensation and DNA fragmentation, i.e., 'DNA-laddering'. These alterations were prevented by an inhibitor of NO synthase, NMMA, thereby proposing a link between NO· generation and induction of apoptosis. Moreover, we probed for apoptosis in RAW cells with several NO donors. With the use of spontaneously decomposing NO donors, known as NONOates, we showed that the total NO production (integrated concentration over time curves) accounted for the NO donor damaging ability (Meßmer and Brüne, 1996a). A 30-min exposure to the rapidly decomposing NO donor DEA–NO caused irreversible damage and apoptotic cell death after 6 to 8 h. For intermediate NO-releasers such as sodium nitroprusside, GSNO, and spermine–NO, removal of the NO-donating compound halted fragmentation to a certain degree, whereas the slowly decomposing DETA–NO complex (diethylenetriamine–nitric oxide complex) initiated fragmentation only after prolonged exposure. Comparable to the macrophage system, endogenously generated or exogenously supplied NO· promoted apoptosis in RINm5F cells (Ankarcrona et al., 1994).

NO·-mediated apoptotic signaling in macrophages left intracellular NAD^+ and ATP unaltered. Consistently, membrane integrity measured by LDH (lactate dehydrogenase)-release was preserved and inhibitors of poly(ADP-ribose) polymerase, such as 3-aminobenzamide, were non-effective (Meßmer and Brüne, 1996a). These experiments ruled an overlap of apoptotic and necrotic alteration in our studies out. In analogy to several other systems, protection was also observed in RAW cells in the presence of Zn^{2+}, an inhibitor for the Ca^{2+}, Mg^{2+}-dependent endonuclease that is thought to cleave DNA during apoptosis.

4.2. Apoptotic signals: p53 accumulation, caspase activation, and mitochondrial alterations

The tumor suppressor gene p53 has come to be known as a master guardian of the genome and a member of the DNA damage-response pathway (Oren, 1997; White, 1994; Wang and Harris, 1997; Almog and Rotter, 1997). p53 is able to induce growth arrest or apoptosis in DNA-damaged cells. Induction of a G1 arrest is dependent upon sequence-specific DNA binding and transcriptional activation of p53 target genes such as p21 (WAF1/Cip1). p21 is an inhibitor of cyclin dependent kinases and thereby blocks cell cycle progression (Liebermann et al., 1995; Shankland, 1997). Induction of apoptosis is less well understood but can be activated by both transactivation-dependent and transactivation-independent pathways. Activation of p53 is marked by increased protein levels, probably due to increased protein stability, i.e., prolonged protein half-life or decreased protein degradation. In our experiments with RAW 264.7 macrophages or RINm5F cells we established a role of p53 during NO·-mediated cell death (Meßmer et al., 1994, Meßmer and Brüne, 1996b). Activation of iNOS caused nitrite accumulation in the cell supernatant and resulted in p53 accumulation, clearly preceding DNA fragmentation. Apoptotic features, including accumulation of the tumor suppressor p53, were down-regulated by blocking iNOS. A role of NO· in promoting p53 accumulation was further substantiated by using NO donors. The level of the tumor suppressor increased in response to NO donors and effectively sensed NO·-intoxication at an early stage of cellular damage. NO· removal concomitantly allowed p53 to decline with only a small percentage of cells entering the apoptotic pathway. Apparently, apoptotic cell death correlated with the degree of p53 accumulation (Meßmer and Brüne, 1996a,b). In addition, RAW cells stably transfected with plasmids encoding p53 antisense RNA (Meßmer and Brüne, 1996b) exhibited reduced p53 levels in response to GSNO and revealed a marked reduction in DNA fragmentation. These observations suggest a functional role of the tumor suppressor p53 during NO-in-

duced apoptosis. The ability of $NO^{\cdot}$ to promote a functional p53-response has been confirmed in murine and human systems (Ho et al., 1996; Calmels et al., 1997; Zhao et al., 1997) and extended to the observation that p53 in turn down-regulates iNOS expression through inhibition of the iNOS promoter (Forrester et al., 1996). In addition, $NO^{\cdot}$-mediated inhibition of cell cycle proliferation at least in smooth muscle cells seems closely associated with induction of p21 (Ishida et al., 1997) which in some cases is known to resemble a p53-dependent mechanism. However, experiments in p53 negative cells (U937 cells) substantiated p53-independent signaling pathways to be operative during NO-mediated apoptosis as well (Brockhaus and Brüne, 1998).

Caspases, a family of cysteine proteases that specifically cleave a growing number of cellular substrates after Asp residues were first implicated in apoptosis by genetic analysis in the nematode *C. elegans* (Salvesen and Dixit, 1997; Nicholson and Thornberry, 1997; Cohen, 1997). Since the recognition that the cysteine protease CED-3 in *C. elegans* has sequence identity with the mammalian cysteine protease interleukin-1β-converting enzyme a family of at least 10 related proteases has been identified. The trivial name 'caspase' has been selected based on two catalytic properties where 'c' reflects a cysteine protease and 'aspase' refers to their ability to cleave after aspartic acid. Caspases are tentatively grouped into three subfamilies (Villa et al., 1997). First, the interleukin-β-converting enzyme/caspase-1 subfamily (caspase-1, caspase-4, and caspase-5), second the CPP32/caspase-3 subfamily (caspase-3, caspase-6, and caspase-7), while the third group gathers caspase-2, caspase-8, caspase-9, and caspase-10. The list of potential targets is steadily growing and presently subdivided into four groups: first, substrates that become activated as a result of their cleavage, second, substrates which are inactivated following their cleavage, third, structural proteins that alter their assembly/disassembly properties after being cleaved, and fourth, proteins that undergo cleavage with unknown consequences for apoptosis (Villa et al., 1997). The role of each caspase, their relationship(s) during activation, and the role of target cleavage are current subject of intense investigation. For propagating the apoptotic signal, activation of downstream caspases (i.e., caspase-3 or caspase-7) seems important and is considered the point of no return in the process leading to cell destruction. This has recently been highlighted by the fact that an active caspase is the prerequisite step in the sequence of events leading to DNase activation that cleaves DNA (Enari et al., 1998).

During $NO^{\cdot}$-mediated apoptosis we noticed cleavage of poly(ADP-ribose) polymerase (PARP) which is an established caspase-3 substrate (Meßmer et al., 1996d). PARP cleavage, i.e., caspase-3 activation was observed in response to endogenously produced or exogenously added NO donors. Caspase activation by cytokine treatment was blocked by NOS inhibition, thus proving the involvement of $NO^{\cdot}$. With regard to the time sequence of apoptotic events we noted that caspase activation and substrate cleavage was preceded by p53 accumulation. Our data implicate PARP as a proteolytic substrate during $NO^{\cdot}$-mediated apoptosis and point to caspase activation as a result of $NO^{\cdot}$ formation. Caspase activation by NO donors has been confirmed in human leukemia cells (Yabuki et al., 1997), mesangial cells (Sandau et al., 1998), and neuronal excitotoxicity triggered by nitric oxide (Leist et al., 1997c). Although PARP cleavage in response to $NO^{\cdot}$-intoxication is proven, a cause–effect relationship of PARP degradation for propagating the apoptotic signal must be questioned based on data from PARP knock-out animals (Leist et al., 1997b; Wang et al., 1997). We also noticed PARP cleavage under nonapoptotic conditions in U937 cells (Brockhaus and Brüne, 1998) which implies that PARP cleavage and apoptosis can be separated. Despite a questionable causative role of PARP during apoptosis, its cleavage resembles a convenient marker that identifies caspase activation. Under conditions that allowed $NO^{\cdot}$-mediated apoptosis, activation of caspases seems an inherent step in the death pathway and caspase inhibitors can be used to block apoptosis (Brockhaus and Brüne, 1998, Leist et al., 1997c, Meßmer et al., 1998).

It is known that nuclear apoptosis is preceded by the disruption of the mitochondrial transmembrane potential due to the opening of mitochondrial permeability transition (Petit et al., 1996). This applies to different cell types and to various apoptosis inducers. $NO^{\cdot}$ potently and reversible deenergize mitochondria and inhibit several mitochondrial enzymes including aconitase and cytochrome *c* oxidase (Szabo et al., 1996a; Balakirev et al., 1997; Burney et al., 1997). In addition, peroxynitrite and NO donors have been shown to induce mitochondrial permeability transition and it has been suggested that $NO^{\cdot}$ promotes apoptosis via a direct effect on mitochondria (Hortelano et al., 1997). However, these studies preclude investigations with endogenously produced $NO^{\cdot}$ and so far do not correlate permeability transition to other responses such as p53 accumulation or caspase activation. Moreover, a general link between $NO^{\cdot}$ action and changes in mitochondrial membrane potential are excluded (Burney et al., 1997).

4.3. Apoptotic alterations and antagonism: Bcl-2 family members

Cell death is controlled, in part, by a complex interplay between regulatory proteins. The prototypic regulator of mammalian cell death is the protooncogene *bcl-2* (Reed, 1994, Reed, 1997). The *bcl-2* gene was first discovered because of its involvement in t(14:18) chromosomal translocations found in follicular B-cell lymphomas where it contributes to neoplastic cell expansion. Constitutive expression of high Bcl-2 protein levels by transfection experiments has proven that Bcl-2 or related family members such as Bcl-x_L can protect many cell types from

apoptosis induced by a wide variety of adverse conditions and stimuli. This suggests that the protein controls a distal step in a signaling pathway leading to apoptotic cell death, although many questions regarding its biochemical mechanism of action remain unanswered (Gajewski and Thompson, 1996). In contrast, initiation of $NO^{\cdot}$-mediated apoptosis in some cases is associated with decreased Bcl-2 expression, but it remains to be established whether this is obligatory of initiation of apoptosis or represents a secondary phenomenon, only (Brockhaus and Brüne, 1998; Sandau and Brüne, unpublished observations, Haendeler et al., 1996). In contrast to a decreased Bcl-2 expression the level of the proapoptotic protein Bax is upregulated in close association with $NO^{\cdot}$-mediated apoptosis, at least in macrophages (Meßmer et al., 1996c). This may indicate that in analogy to many other systems the balance of anti- and proapoptotic proteins resembles a regulatory mechanism.

Initially, we stably transfected macrophages with human Bcl-2, the classical antiapoptotic protein (Meßmer et al., 1996c). Bcl-2 transfectants showed protection against various NO releasers and also appeared highly resistant towards endogenously generated $NO^{\cdot}$. Further to Bcl-2 inhibition we noticed that $NO^{\cdot}$-evoked p53 accumulation remained unchanged. This led to the conclusion that Bcl-2 acts downstream of p53. Protection against $NO^{\cdot}$-mediated cytotoxicity by Bcl-2 overexpression has been confirmed by other laboratories (Albina et al., 1996; Bonfoco et al., 1997; Melkova et al., 1997). In extending experiments we assessed that $NO^{\cdot}$-mediated caspase activation and concomitant PARP cleavage was attenuated in the Bcl-2 transfectants (Meßmer et al., 1996d). These data underscored the role of Bcl-2 as an efficient signal terminator during $NO^{\cdot}$-mediated apoptosis and further revealed that Bcl-2 interferes upstream of caspases, i.e., caspase-3 activation.

Main transducing steps that are known to participate during $NO^{\cdot}$-mediated apoptotic cell death are summarized in Fig. 1. Potential inhibitory mechanisms or antagonistic principles are indicated as well. Antagonistic pathways as a result of the $NO^{\cdot}/O_2^-$-interaction or stemming from cellular preactivation will be discussed in the following sections.

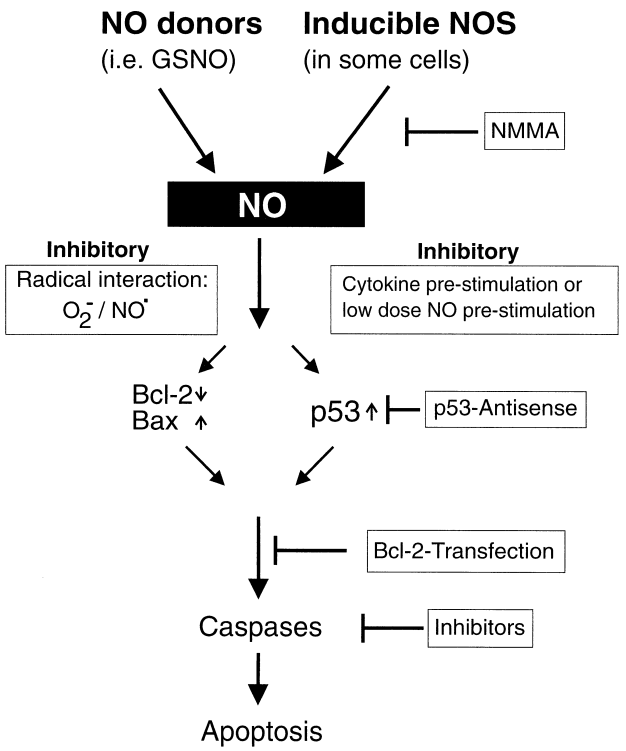

Fig. 1. Activating and inhibitory signaling components that characterize NO-mediated apoptosis. Some signaling components of $NO^{\cdot}$-initiated apoptotic cell death, as well as some possible antagonistic interventions, are shown. Importantly, the arrows do not imply direct cause–effect relations. Experimental evidence for the time-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.

4.4. Antagonistic pathways: the superoxide (O_2^-) / $NO^{\cdot}$-interaction

The production of superoxide (O_2^-) from NAD(P)H like oxidases, mitochondrial respiration, and the xanthine oxidase seems an inherent feature of oxygen-associated life. Despite the notion that O_2^- and related species such as the OH-radical are considered life threatening it is also established that O_2^- functions as a modulator of physiological signal transduction (Müller et al., 1997). Part of this regulatory role of O_2^- is achieved by gene transcription which is in part mediated by activation of the transcription factor NF-κB (Lander, 1997). A further regulatory component results from the diffusion controlled $NO^{\cdot}/O_2^-$-interaction (Pryor and Squadrito, 1995). The formation of both, $NO^{\cdot}$ and O_2^- is often associated with apoptotic and/or necrotic diseases under inflammatory conditions such as mesangioproliferative glomerulonephritis or rheumatoid arthritis. $NO^{\cdot}$- or O_2^--generation has been associated with apoptosis and in particular, the reaction product of the radical–radical interaction (peroxynitrite, $ONOO^-$) is highly reactive and considered to account in part for $NO^{\cdot}$-toxicity (Estevez et al., 1995; Szabo et al., 1996b). Most studies investigating the toxic role of $ONOO^-$ have used relatively high bolus concentrations due to the rapid decomposition of the molecule and further mathematically recalculated their doses to a flux rate of its formation. In our studies we addressed the $NO^{\cdot}/O_2^-$-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. This allowed the continuous formation of both radicals over an extended period. We established that a balanced and simultaneous generation of both radicals is nondestructive for mesangial cells or macrophages whereas the unopposed generation of either $NO^{\cdot}$ or O_2^- induces apoptosis

and in higher concentrations necrotic cell death (Sandau et al., 1997a; Sandau et al., 1997b; Brüne et al., 1997). While initiation of apoptosis was accompanied by increased p53 and Bax expression, caspase activation, and DNA fragmentation, these alterations were attenuated under conditions of $NO^{\cdot}/O_2^-$-coadministration. This was evident by using various NO donors with different decomposition kinetics in combination with two O_2^--generating systems, i.e., DMNQ vs. the xanthine/xanthine oxidase system. It is assumed that the breakdown of NO donors and thus the release of $NO^{\cdot}$ has to match the production of O_2^- with respect to time and concentration. It seems that signaling mechanisms as a consequence of the $NO^{\cdot}/O_2^-$-interaction redirect apoptotic initiating signals towards cell protection. For the $NO^{\cdot}/O_2^-$-mediated protection reduced glutathione (GSH) turned out to be essential (Sandau and Brüne, unpublished observations). Depletion of GSH by preincubation with BSO (buthionine sulfoximine) abrogated protection and converted protection into necrotic cell destruction. The assumption that the $NO^{\cdot}/O_2^-$-interaction would result in increased oxidative stress was substantiated by more exaggerated formation of GSSG (oxidized glutathione) under $NO^{\cdot}/O_2^-$-cogeneration compared to GSH oxidation as a result of either $NO^{\cdot}$ or O_2^- domination. The shift towards oxidative stress as a result of the $NO^{\cdot}/O_2^-$-interaction is in line with in vitro experiments performed by Wink et al. (Wink et al., 1993; Wink et al., 1997). They observed GSSG formation by incubating NO donors, O_2^-, and GSH and suggested $NO^{\cdot}$-mediated nitrosative reactions to be quenched by the resultant oxidative stress. In accordance with our system, protection was achieved by radical–radical interactions which acted as a chain breaker during apoptotic signaling as long as GSH is available in detoxifying the $NO^{\cdot}/O_2^-$-interaction products. In extension of these findings we observed that in close correlation with oxidative stress conditions, i.e., GSSG formation, some protective proteins such as Bcl-2 or heme oxygenase-1 were upregulated (Sandau et al., 1998). Although no cause–effect relation is established so far, it may turn out that oxidative conditions as a result of the $NO^{\cdot}/O_2^-$-interaction upregulate protective genes that attenuate $NO^{\cdot}$-mediated apoptosis.

Radical ($NO^{\cdot}$ and O_2^-) coformation that attenuated O_2^--mediated toxicity has also been established for chondrocytes (Blanco et al., 1995). As a general concept it appears that the balanced formation and interaction of physiologically relevant radicals resembles a protective principle in some systems thereby eliminating adverse reaction that are operating as a consequence of unopposed radical generation. These results and hypothetical considerations are in good agreement with several cellular- or organ-systems as well as animal studies where NO functions as a protective signal during ischemia-reperfusion, peroxide-induced toxicity, lipid-peroxidation, or myocardial injury (Oury et al., 1992; Rubbo et al., 1994; Wink et al., 1993).

4.5. Antagonistic pathways: Cellular preactivation

During macrophage activation with lipopolysaccharide and interferon-γ upregulation of iNOS, formation of large amounts of $NO^{\cdot}$, and concomitant cell death occurs. As the process can be intercepted by NO synthase inhibitors a functional role of $NO^{\cdot}$ is assumed. Preactivation of macrophages with a combination of lipopolysaccharide, interferon-γ under conditions of blocked NO synthase or stimulation with a low, nondestructive dose of NO donors (GSNO) conferred protection against high and thus apoptotic $NO^{\cdot}$-concentrations (Brüne et al., 1996). We found that induction of cyclooxygenase-2 during the preactivation period is a critical regulator of macrophage apoptosis (Von Knethen and Brüne, 1997; Von Knethen et al., 1998). Under resting conditions macrophages do not express cyclooxygenase-2, whereas lipopolysaccharide/interferon-γ/NMMA prestimulation for 12–15 h caused protein expression. In parallel, preactivation with a low, nontoxic dose of GSNO promoted protection and cyclooxygenase-2 up-regulation. To prove cyclooxygenase-2 involvement during protection RAW cells were stably transfected with a rat cycloxygenase-2 expression vector. Cyclooxygenase-2 overexpressing macrophages, that contain an activated phospholipase A_2 revealed protection against exogenously supplied $NO^{\cdot}$. Protection achieved by lipopolysaccharide/interferon-γ/NMMA prestimulation was reversed by the addition of the cyclooxygenase-2 selective inhibitor NS398 or by a stably transfected antisense cyclooxygenase-2 expression vector. A further signaling component during macrophage preactivation is the activation of the nuclear transcription factor NF-κB (von Knethen and Brüne, unpublished observations). NF-κB supershift analysis implied an active p50/p65-heterodimer following $NO^{\cdot}$ or lipopolysaccharide/interferon-γ/NMMA addition. Degradation of I-$\kappa B\alpha$ and activation of a luciferase reporter construct, containing four copies of the NF-κB-site derived from the murine cyclooxygenase-2 promoter confirmed NF-κB activation. Furthermore, a NF-κB decoy approach abrogated cyclooxygenase-2 expression and inducible protection after low dose GSNO, or lipopolysaccharide/interferon-γ/NMMA. Blocking NF-κB reversed protection and restored DNA fragmentation and p53 accumulation in response to high dose GSNO. These examinations provided evidence for an antiapoptotic role of NO which is transmitted by NF-κB activation. Experiments performed in microglial cells (BV-2 line) where lipopolysaccharide pretreatment protects against $NO^{\cdot}$ toxicity may aim in the same direction (Sugaya et al., 1997). Further support for $NO^{\cdot}$-inducible protection comes from chondrocytes, where low-dose GSNO enhances the tolerance to a second high concentration GSNO exposure (Turpaev et al., 1997).

With respect to a functional role of $NO^{\cdot}$ in attenuating cell death low dose $NO^{\cdot}$-induced autoprotection in association with suppression of $NO^{\cdot}$-mediated hepatocyte necrotic

cell death has been reported (Kim et al., 1995). With the use of tin–protoporphyrin IX, which is considered a potent inhibitor of heme oxygenase, it is assumed that $NO^{\cdot}$-mediated upregulation of heme oxygenase resembles the protective principle. Two other reports indicated that endorsed heat shock protein 70 expression can protect against $NO^{\cdot}$ damage (Kim et al., 1997a; Bellmann et al., 1996). The study of Kim et al. describes upregulation of HSP70 in response to exogenous or endogenous $NO^{\cdot}$ formation which in turn protects rat hepatocytes from tumor necrosis factor-α-induced apoptosis. An active role of HSP70 during protection was confirmed by antisense oligonucleotides directed against HSP70. Consistent with these studies showing a protective role of $NO^{\cdot}$ is the notion that cell survival is found as a result of protective associated protein expression.

In contrast to a protective principle which demanded transcriptional and translational activity, other groups noted cell protection in close association with $NO^{\cdot}$-mediated activation of soluble guanylyl cyclase. Although in some of these investigations a transducing role of cyclic GMP had been established, molecular targets and mechanistic insights into antagonistic pathways remain elusive. Cyclic GMP-mediated protection is reported for endothelial cells (Polte et al., 1997) and B lymphocytes (Genaro et al., 1995). In the case of B lymphocytes contradictionary, cyclic GMP-independent pathways in antagonizing apoptosis are also mentioned (Mannick et al., 1994).

4.6. The role of $NO^{\cdot}$ in modulating caspases and the Fas-system

Caspases are emerging as a new potential target of $NO^{\cdot}$. Caspase activation is inherent to the final executive phase of apoptosis and inhibition at this point appears as a rational pharmacological approach. Indeed, pharmacological inhibition of caspases blocks $NO^{\cdot}$-mediated apoptosis (Brockhaus and Brüne, 1998; Leist et al., 1997c). Inhibition of caspases, that contain a catalytically reactive cysteine moiety, by $NO^{\cdot}$-mediated *S*-nitrosation or oxidation emerges as a rational approach and now experimentally is proven by several groups (Dimmeler et al., 1997; Mohr et al., 1997; Kim et al., 1997b; Melino et al., 1997). However, most of these studies have been performed in cell extracts or with purified proteins and it appears that inhibition is largely reversed under stringent reducing conditions. This makes it difficult to extrapolate the mentioned results to cellular conditions and the question whether endogenously produced $NO^{\cdot}$ inhibits apoptosis due to a direct interaction with caspases remains unanswered, with the exception of the hepatocyte system where $NO^{\cdot}$ inhibited caspase activity by roughly 50% under cellular conditions (Kim et al., 1997b).

One has to consider that any interference of $NO^{\cdot}$ with the apoptotic signaling cascade upstream of caspase activation would result in attenuated caspase activity and not necessarily reflect direct enzyme inhibition.

$NO^{\cdot}$ signaling has recently also been proposed as a modulator of the Fas system. Fas, a member of the tumor necrosis factor receptor family is expressed in a wide variety of tissues including thymus, liver, heart, kidney, and lymphoid as well as nonlymphoid malignancies. Fas induces apoptosis when ligated by natural Fas ligand, which is found predominantly on activated T cells and natural killer cells (Nagata, 1997). Human pancreatic β cells that do not constitutively express Fas became strongly Fas positive after interleukin-1β exposure, and are then susceptible to Fas-mediated apoptosis (Stassi et al., 1997). Fas expression is blocked by NO synthase inhibitors, while NO donors substitute in promoting Fas expression. It must be concluded that $NO^{\cdot}$-mediated upregulation of Fas contributes to pancreatic β cell death under conditions of insulin-dependent diabetes mellitus where Fas ligand expressing T lymphocytes infiltrate the islets. An additional regulatory role of $NO^{\cdot}$ became apparent in examinations where $NO^{\cdot}$ inhibits Fas-induced apoptosis in human leukocytes and T cell clones (Mannick et al., 1997; Sciorati et al., 1997). Although both papers established an antagonism by basal NO synthase activity (iNOS vs. cNOS in either of the reports) the mechanisms differed with respect to its cyclic GMP-dependency. Assuming autocrine generation of $NO^{\cdot}$ as an early signaling event in the pathway that regulates apoptosis of immune competent cells allows further implications for the overall modulation of immune responses by $NO^{\cdot}$.

5. Conclusions

The toxicity of $NO^{\cdot}$ is influenced by the existing biological milieu. Relative rates of $NO^{\cdot}$ formation, its oxidation and reduction, the combination with oxygen, superoxide, and other biomolecules will determine the signaling pathway of nitric oxide. In some cellular systems activation of the iNOS generated sufficient amounts of $NO^{\cdot}$ to promote cell death that is defined by typical morphological and biochemical features which resemble apoptosis. Apoptosis in response to NO donors or endogenous $NO^{\cdot}$ generation is accompanied by an early accumulation of the tumor suppressor protein p53, caspase activation, and DNA fragmentation. These alterations are attenuated by Bcl-2 gene transfer, which blocked distal to p53 accumulation and proximal to caspase activation.

Not all cellular systems which show iNOS upregulation after cytokine treatment enter the death pathway which is exemplified for rat mesangial cells (Sandau et al., 1997a). Antagonistic and/or protective principles must exist (Fig. 2). An antagonistic principle becomes evident by a balanced rate of O_2^--production which redirects cell destruction to cell protection. Besides adverse effects of $NO^{\cdot}$ the

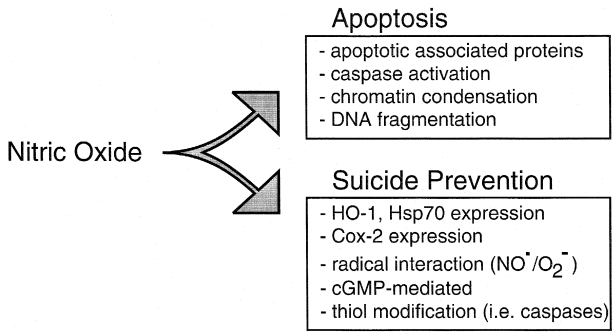

Fig. 2. The dual role of $NO^{\bullet}$ during apoptotic cell death. Main components that characterize $NO^{\bullet}$-mediated apoptosis are indicated in the upper part of the figure while the lower part indicates $NO^{\bullet}$-associated transducing pathways that signal cell protection. For details, see the text.

molecule also signals cell protection. $NO^{\bullet}$-derived protection can be divided into mechanisms that upregulate cell protective proteins such as heat shock proteins or cyclooxygenase-2 and into processes that are transmitted by thiol modification, i.e., caspase inactivation and elevated levels of cyclic GMP.

It will be interesting to define the versatility of $NO^{\bullet}$-signaling mechanisms in relation to their apoptotic inducing ability and to explore how $NO^{\bullet}$-responsive targets serve both, sensory and regulatory roles in transducing a signal. The switch from physiology to pathophysiology, the action of potentially protective and destructive $NO^{\bullet}$-species, and the molecular recognition of these balances will be central to the understanding of the $NO^{\bullet}$-action during apoptosis.

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