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Review

Regulation of Apoptosis by Nitrosative Stress

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Nitrosative stress can prevent or induce apoptosis. It occurs *via* S-nitrosylation by the interaction of nitric oxide (NO) with the biological thiols of proteins. Cellular redox potential and non-heme iron content determine S-nitrosylation. Apoptotic cell death is inhibited by S-nitrosylation of the redox-sensitive thiol in the catalytic site of caspase family proteases, which play an essential role in the apoptotic signal cascade. Nitrosative stress can also promote apoptosis by the activation of mitochondrial apoptotic pathways, such as the release of cytochrome c, an apoptosis-inducing factor, and endonuclease G from mitochondria, as well as the suppression of NF-κB activity. In this article we reviewed the mechanisms whereby S-nitrosylation and nitrosative stress regulate the apoptotic signal cascade.

Keywords: Nitric oxide, Apoptosis, Nitrosative stress, S-nitrosylation, Mitochondria

NO synthesis and its action mode

Nitric oxide (NO) is a short-lived, diffusible free radical that is produced from L-arginine in a reaction. It is catalyzed by one of the three isotypes of NO synthases (NOS)-endothelial NOS (eNOS), neuronal NOS (nNOS), or inducible NOS (iNOS) (Ignarro *et al*., 1987; Nathan, 1992). The functional role of NO can vary by cell type and enzyme isotype. The major function of eNOS is to regulate vascular tone. The main role for nNOS includes retrograde signaling across synapses. The primary inducible NOS isoform (iNOS) can be upregulated considerably *via* the activation of immune cells and many other tissues by the bacterial cell wall component lipopolysaccharide (LPS) and/or cytokines. At normal intracellular calcium levels, NO production by iNOS is limited only by the amount of enzyme, substrate, or co-factors that are present (Xie and Nathan, 1994; Gross and Wolin, 1995). In contrast, eNOS and nNOS are constitutively expressed and inactive at normal calcium concentrations, producing picomolar amounts of NO in response to transient increases in the cytosolic calcium level. However, chronic calcium elevation will cause persistent NO production (Ignarro *et al*., 1987; Nathan, 1992). NO interacts directly with several cellular target molecules before diffusing out of NO-producing cells. Since NO is relatively small and hydrophobic, it can easily pass through membranes. Because NO persists *in vivo* for a few seconds, it can diffuse several cell diameters from its site of synthesis (Lancaster, 1994). When co-cultured with red blood cells that scavenge biological NO, the formation of intracellular NO reaction products in the form of dinitrosyl iron complexes was dramatically suppressed in NO-producing hepatocytes (Nussler *et al*., 1993). This indicates that the transcellular diffusion of NO is more rapid than the rate of intracellular reaction with its target molecules within NO-generating cells. Consequently, the steady-state NO concentration that is experienced by a cell is determined by the number of NO-producing cells nearby (Beckman and Koppenol, 1996). NO that is produced within cells, therefore, interacts with biomolecules through both autocrine and paracrine modalities.

Biochemical reactivity of NO

NO decays to nitrite by a reaction with O_2 in an aqueous buffer, and its decay rate is largely determined by the NO concentration (Kerwin and Feldman, 1995). At maximum biological concentrations (1-5 μM), the half-life of NO *in vitro* is several minutes; it is far longer at more normal physiological concentrations (Ignarro *et al*., 1993; Kim *et al*., 1995).

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However, its biological half-life is less than 5 s (Ignarro *et al*., 1993). This suggests that *in vivo*, NO decays by a minimal reaction with oxygen, or through reaction pathways with other biological molecules (Kim *et al*., 1995). To put it simply, NO can react with molecular oxygen (O_2) to produce nitrite. However, this reaction is very slow at physiological concentrations of O_2. NO reacts rapidly with O_2^- to form the strong oxidant peroxynitrite ($ONOO^-$), a cytotoxic modulator (Beckman *et al*., 1990) and bactericidal agent (Zhu *et al*., 1991). NO also interacts with many heme-containing proteins. NO reacts with the heme-iron of hemoglobin and myoglobin to form nitrate. NO activates guanylate cyclase by binding to its heme-iron to generate cGMP from GTP, which plays a key role in vascular tone regulation and neurotransmission (Gruetter *et al*., 1980). The binding of NO to heme inhibits several heme enzymes, such as cytochrome oxidase, catalase, and cytochrome p450 (Kim *et al*., 1995). The reaction of NO with several biological components (such as metals, thiols, O_2, and O_2^-) produces a variety of secondary products that range from the innocuous oxidized components (NO_2^- and NO_3^-) to the reactive nitrogen intermediates, such as the nitrosonium equivalent (NO^+), peroxynitrite ($ONOO^-$), nitrosothiols (RSNO), nitroxyl anion (NO^-), dinitrogen trioxide (N_2O_3), and nitrogen dioxide (NO_2) (Beckman *et al*., 1996). These reactive nitrogen species, including NO, interact with biomolecules to cause lipid oxidation, protein modification (e.g. cysteine and tyrosine residue), DNA damage by base modification, and regulation of enzyme activity.

In nitrosation reactions, *N*-nitrosamines and *S*-nitrosothiols are formed by the addition of a nitrosonium equivalent (NO^+) to amine and thiol moieties, respectively (Gruetter *et al*., 1980). The activation of rodent macrophages with IFN-γ and/or LPS results in NO production by the iNOS gene expression and nitrosation of target molecules that are present in the culture medium (Gruetter *et al*., 1980; Zhu *et al*., 1991). Several iNOS-dependent reaction pathways have potential relevance in the nitrosation chemistry of biological systems. Acidified nitrite (HNO_2) is a nitrosating agent, but it is easily formed only in low pH environments, such as that of the stomach (Zhu *et al*., 1991). Dinitrosyl iron complexes from macrophages and hepatocytes that are activated by cytokines and LPS (Kim *et al*., 1995; Kim *et al*., 2000) may provide a mechanism for nitrosation, in particular, trans-*S*-nitrosation (Kim *et al*., 2000). Chemically synthesized dinitrosyl iron complexes induce the S-nitrosylation of caspase and albumin *in vitro* (Boese *et al*., 1995; Kim *et al*., 2000). Dinitrogen trioxide (N_2O_3) that is formed by the reaction of NO with molecular oxygen has a strong propensity to nitrosate, both amine and thiol moieties at physiological pH (Kharitonov *et al*., 1995).

NO induces S-nitrosylation through nitrosative stress

As clearly demonstrated, NO or related molecules can covalently modify cysteine residues in proteins through three chemical reactions S-nitrosylation, oxidation (RS-SR), and ADP-ribosylation. (Brune and Lapetina 1989; Stamler, 1994). These post-translational modifications serve in the regulation of cellular responses (Stamler, 1994; Stamler *et al*., 2001). Particularly, the redox-based S-nitrosylation of proteins or nonprotein thiols occurs both *in vitro* and *in vivo* (Brune and Lapetina, 1989; Kim *et al*., 1995). S-nitrosylation regulates the gene expression and cellular homeostasis through an alteration in protein function (Stamler, 1994; Kim *et al*., 1995; Chung *et al*., 2001). The concept of nitrosative stress has emerged from an understanding that nitrosylation can also reach hazardous levels. Under such conditions, nitrosylation may directly inhibit critical protein functions (Stamler, 1994), decrease redox potential (Chung *et al*., 2001), and/or promote deleterious oxidative modifications (Marshall *et al*., 2001). At the cellular level, nitrosative stress has been linked to the inhibition of cell growth and apoptosis, and thus may be widely implicated in the pathogenesis of many human diseases. In particular, the caspase proteolytic enzymes, key mediators for apoptotic cell death, possess a redox-sensitive cysteine residue in the catalytic site. NO could modify this enzyme by S-nitrosylation *in vitro* and *in vivo*, indicating that biological and chemical NO generation regulates apoptotic cell death.

Caspase is a critical mediator in the apoptotic signal cascade

Apoptosis, known as programmed cell death, is essential for the normal development of a multicellular organism, as well as the maintenance of tissue homeostasis (Steller, 1995). It is an active, energy-dependent process of cell shrinkage, plasma membrane blebbing, chromatin condensation, and DNA fragmentation. Apoptosis is biologically initiated by tightly controlled intracellular signaling events through the ligation of specific death receptors of the tumor necrosis factor receptor (TNF-R) family, such as tumor necrosis factor-alpha (TNF-α), CD95/Fas/Apo-1, and the receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Ligand binding of the trimerized receptor recruits intracellular adaptor molecules like FADD/MORT1 and TRADD into the death inducing signaling complex (DISC). It then recruits and activates the protease zymogen called procaspase-8 (Boldin *et al*., 1996). The activation of caspase-8 is known to involve the sequential activation of other caspases (Thornberry and Lazebnik, 1998). Caspases are constitutively expressed as inactive proenzymes in cells, and become activated by proteolytic processing when cells receive an apoptosis-inducing signal (Craen *et al*., 1999). Proteolytic cleavage results in the removal of an N-terminal prodomain and generation of small (p10) and large (p20) active subunits, which form an active tetramer $(p10/p20)_2$. Caspases are a family of cysteine proteases (consisting of 14 isoforms) which are the mammalian counterpart of *ced-3*, a protease that is required for programmed cell death in the

nematode *Caenorhabditis elegans* (Nicholson *et al*., 1995). Some members (including caspase-8, -9, and 10) contain a large prodomain and are initiators of the apoptotic signal cascade. Others, such as caspase-3, -6, and 7, possess a small prodomain and participate in the execution phase of apoptosis.

Mitochondria are central subcellular organelles in apoptotic signaling

There are two apoptotic signaling pathways that are mediated by the death receptor ligation and other proapoptotic stimuli. The activation of the cell surface death receptor (such as TNF-α receptor, Fas, and TRAIL receptor) recruits procaspase-8 to the DISC; caspase-8 then becomes autoactivated. The active caspase-8 can both directly activate downstream caspases, such as caspase-3, and cleave the cytochrome c-effluxing cytosolic factor Bid into two fragments (Luo *et al*., 1998; Li *et al*., 1998). The C-terminal p15 fragment translocates into mitochondria, and induces the release of cytochrome c and endonuclease G into the cytosol (Li *et al*., 2001).

On the other hand, receptor-independent inducers of apoptosis (such as the growth factor withdrawal, ultraviolet irradiation, and chemotherapeutic drugs) will provoke the release of mitochondrial cytochrome c and endonuclease G release without the activation of caspase-8 activation, probably through the induction of mitochondrial membrane damage. The redistribution of cytochrome c by treatment with apoptosis-inducing stimuli can be prevented by antiapoptotic oncogenes, such as Bcl-2 and Bcl-X_L (Yang *et al*., 1997). Many studies have revealed that cytosolic cytochrome c interacts with Apaf-1 and procaspase-9 and forms the "apoptosome" in the presence of dATP, which results in the activation of caspase-9 (Liu *et al*., 1996). The active caspase-9 leads to the activation of the downstream protease caspase-3, which is thought to assure the cleavage of target proteins that are required to complete the terminal events in apoptosis. For example, the cleavage of the DNA fragmentation factor (DFF) in humans (Liu *et al*., 1997), or the murine analogue inhibitor of caspase-activated DNase (ICAD) (Enari *et al*., 1998) by caspase-3-like caspases, leads to the release of activated CAD. It then translocates into the nucleus, which results in DNA degradation and apoptotic cell death (Sakahira *et al*., 1998).

Recent studies, however, have shown that as result of mitochondrial membrane damage that is induced by tBid or other toxic reagents, endonuclease G is released into the cytosol. It then translocates into nuclei to induce interchromosomal DNA fragmentation (Li *et al*., 2001). This endonuclease is found mostly in the intermembrane space with only a small fraction participating in the mitochondrial DNA replication in the matrix. The release of the mitochondrial endonuclease G represents a caspase-independent apoptotic pathway of mammalian cell death that is initiated from mitochondria. It will be interesting to see where on the evolutionary tree the endonuclease G (in different species) acquired this death-promoting character. Perhaps the endonuclease G is responsible for some of the caspase-independent cell deaths and DNA fragmentation that have been observed in plants, fungi, and protozoa.

Nitrosative stress inhibits caspase activity by S-nitrosylation

NO regulates either the induction of apoptosis in some cells, or the prevention of apoptosis in others (Kim *et al*., 1999). High levels of NO production are known to be cytotoxic through the suppression of ATP synthesis by the inhibition of mitochondrial aconitase and mitochondrial complexes I and II (Brown, 1999), induction of the p53 expression (Brockhaus and Brune, 1999), and the inhibition of the rate-limiting enzyme of DNA synthesis, ribonucleotide reductase (Kwon *et al*., 1991). The iNOS induction in macrophage cells by IFN-γ/LPS induces nitrosative stress by increasing the formation of S-nitrosylated proteins, which is a critical factor for the NO-mediated apoptotic cell death (Fisch *et al*., 2000). However, we, as well as others, have shown *in vitro* and *in vivo* that NO protects several cells (including hepatocytes, neuronal PC12 cells, and endothelial cells) from cytotoxic stimuli, such as TNF-α, Fas, serum deprivation, and oxidative stress (Kim *et al*., 1995; Kim *et al*., 1997; Kim *et al*., 1999; Kwon *et al*., 2001). Low level of endogenous NO production or NO from exogenous NO donors was previously shown to prevent apoptosis in human B lymphocytes (Mannick *et al*., 1994), ovarian follicles (Chun *et al*., 1995), neuronal cells (Kim *et al*., 1999), MCF-7 breast cancer cells (Kim *et al*., 1998), Jurkat T lymphocytes (Mannick *et al*., 1997), endothelial cells (Tzeng *et al*., 1997; Kwon *et al*., 2001), and hepatocytes (Kim *et al*., 1997; Kim *et al*., 1998). Several mechanisms for the antiapoptotic effect of NO have been proposed in addition to the S-nitrosylation of caspases (Chung *et al*., 2001). They include the up-regulation of cytoprotective gene products (such as heat shock protein 70) (Kim *et al*., 1997), heme oxygenase (Kim *et al*., 1995), Bcl-2 (Genaro *et al*., 1995), as well as increases in cGMP levels (Kim *et al*., 1997; Tzeng *et al*., 1997). All of the caspases contain a single cysteine at the catalytic site. This thiol is susceptible to redox modification and can be effectively modified by S-nitrosylation in NO-generating conditions (Chung *et al*., 2001). We previously showed that NO, either synthesized by iNOS or from exogenous NO donors, suppresses caspase activation/activity in cultured hepatocytes (Li *et al*., 1997), as well as *in vivo* (Kim *et al*., 1997; Kim *et al*., 2000). The antiapoptotic effect of NO was reversed by the addition of the NOS inhibitor N^G-monomethyl-L-arginine (NMMA), or the biological NO scavenger hemoglobin (Kim *et al*., 1997). Furthermore, we showed that seven recombinant members of the caspase family proteases were inhibited by redox-related S-nitrosylation (Li *et al*., 1997). This inhibition was reversed by the reduction of the nitrosylated caspases with the strong reducing agent dithiothreitol (Kim *et al*., 1997), which indicates that caspase-dependent apoptosis can be modulated

by a reversible S-nitrosylation of the active site cysteine of caspases (Kim *et al*., 1997; Kim *et al*., 2000). Stoichiometical studies revealed that the ratio of enzyme subunit to S-nitrosylation was 1 : 1 in caspase-3 and 8 following treatment with the NO-generating donor S-nitroso-N-acetylpenicillamine (SNAP) (Kim *et al*., 1997; Kim *et al*., 2000). This evidence suggests that one cysteine residue in the catalytic site is redox-sensitive against S-nitrosylation, although caspase-3 possesses more than one nitrosylated site (Stamler *et al*., 2001).

Nitrosative stress inhibits the mitochondrial apoptotic pathway

Mitochondria play a critical role in apoptosis in response to a variety of stimuli. These organelles release proteins into the cytosol that trigger caspase activation, and perform other functions relevant to apoptosis. Proteins that are released from mitochondria include cytochrome c, apoptosis-inducing factor (AIF), SMAC (Diablo), and endonuclease G. The mechanism by which these proteins escape from mitochondria is associated with the mitochondrial outer membrane damage by the interaction of an active caspase and Bid. Caspase-8 cleaves Bid at Asp^{59} into two fragments. The C-terminal Bid fragment (p15) translocates into the mitochondrial outer membrane, and induces the release of cytochrome c and endonuclease G (Li *et al*., 2001). The Caspase-8 inhibitor inhibits the mitochondrial cytochrome c release and apoptosis that is induced by death receptor activation (Kim *et al*., 2000). We also have shown that NO inhibited the caspase-8-dependent Bid cleavage and mitochondrial cytochrome c release *in vitro* and *in vivo* (Kim *et al*., 2000). The activity of caspase-8 can be suppressed by S-nitrosylation (Li *et al*., 1997). S-nitrosylated caspases inhibit the cleavage of Bid and Bcl-2, and block the release of the mitochondrial cytochrome c (Tzeng *et al*., 1997; Kim *et al*., 2000). These results suggest that the S-nitrosylation of caspases suppressed mitochondrial apoptotic events, such as the release of cytochrome c, AIF, SMAC, and endonuclease G. Therefore, the inhibition of the caspase activity by nitrosative stress can suppress a key step in the positive feed-forward and feed-back amplifications of the apoptotic signaling cascade by blocking the release of mitochondria-derived apoptotic modulators.

Non-heme iron enhances S-nitrosylation

NO has weak chemical reactivity with thiols at physiological neutral pH. Although NO alone does not interact readily with proteins or nucleic acids, some reactive nitrogen species (such as NO^+, N_2O_3, $ONOO^-$, and NO^-) engage in the nitrosation of sulfur-containing biological target molecules. The strong nitrosylating agent NO^+ can be generated by the reaction of NO with O_2 (Wink *et al*., 1993), iron-sulfur clusters (Kim *et al*., 2000), or other transition metals (Stamler, 1994). NO production in cells, including hepatocytes and macrophages, leads to the formation of dinitrosyl iron complexes (DNIC), as detected by electron paramagnetic resonance spectroscopy (Kim *et al*., 2000). DNIC carries out the S-nitrosylation of caspases through the formation of NO^+-like species (Kim *et al*., 2000). Synthetic DNIC nitrosylates the cysteine residue in the catalytic site of caspase-3 and inhibits the enzyme activity (Kim *et al*., 2000). Therefore, the capacity of NO to S-nitrosylate caspases may depend on the abundance of proteins that contain iron-sulfur clusters and the availability of other thiol targets, such as glutathione and free cysteine. Similarly, NO prevents apoptosis by the S-nitrosylation of caspases in iron-rich hepatocytes, but not in the iron-limited macrophage cell line RAW264.7 cells. However, the increase in non-heme iron by pretreatment with free iron is associated with greater DNIC formation and increases in the nitrosothiol formation following NO exposure. In addition, the conversion of heme to non-heme iron by NO-mediated induction of heme oxygenase-1 increased the formation of DNIC, and protected hepatocytes from oxidative stress-induced apoptosis (Stamler, 1994; Kim *et al*., 2000), probably through the S-nitrosylation of caspases (Kim *et al*., 1999; Kim *et al*., 2000). The deprivation of cellular iron by desferroxamine protected the neuronal PC12 cells from NO-induced apoptosis. This suggests that the NO-mediated apoptotic cell death is dependent on intracellular non-heme iron levels (Desole *et al*., 1998). This evidence indicates that the cellular content of non-heme iron, such as proteins that contain iron-sulfur clusters, is a critical factor for the nitrosative stress-mediated caspase S-nitrosylation and suppression of apoptosis.

Nitrosative stress induces apoptosis through caspase activation

NO generated from the NO donor, or synthesized by NOS, also induces cell death *via* apoptosis in a variety of different cell types. These include macrophages (Albina *et al*., 1993), thymocytes (Fehsel *et al*., 1995), pancreatic islets (Kaneto *et al*., 1995), certain neurons (Dawson *et al*., 1996; Choi, 2001), and tumor cells (Son and Kim, 1995). The factors that affect cell-specific sensitivity to nitrosative stress-mediated apoptosis can be associated with the redox state and levels of transition metal complexes within cells (Kim *et al*., 2000), as well as the expression of survival genes (Kim *et al*., 1995). Nitrosative stress-mediated apoptosis is associated with increases in the ratio of pro-apoptotic Bax to anti-apoptotic $Bcl\text{-}X_L$ by the overexpression of the cell death gene p53 (Messmer and Brune, 1996), or the activation of caspase through mitochondrial cytochrome c release (Umansky *et al*., 2001). This results in the induction of cytotoxicity against tumor cells and normal tissues. Nitrosative stress also suppresses the DNA-binding activity of NF-κB by S-nitrosylation (Marshall and Stamler, 2001). It can then down-regulate the expression of anti-apoptotic genes, such as superoxide dismutase, TRAF1, TRAF2, c-IAP1, and c-IAP2 (Wang *et al*., 1998; Lee and Collins, 2001). Therefore,

nitrosative stress can modulate the dual pathways that lead to pro- and anti-apoptosis, depending on the redox state and transition metal complexes within the cells.

Conclusion

S-nitrosylation by nitrosative stress can regulate cellular homeostasis in order to maintain the balance between the induction and prevention of apoptosis. Abnormal homeostasis is linked to many human diseases, such as cancer, which may be initiated by the suppression of apoptosis. On the other hand, hyperapoptosis may influence other disorders that are involved in vascular disease, neural disorders, and autoimmune diseases. Nitrosative stress nitrosylates the active site cysteine thiol of caspases, which are the central mediator of apoptosis in multiple biological and pathological processes. The inhibition of caspases by S-nitrosylation results in the suppression of apoptosis in several cell types. In other cell types, nitrosative stress activates mitochondria-dependent apoptotic signaling pathways that involve the activation of caspases and release of endonuclease G. Thus, nitrosative stress by NO generation, either from endogenous biological enzymes or exogenous NO donors, can serve as *in vivo* regulatory mechanisms of signaling pathways of apoptotic cell death.

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