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Nitric oxide and cell signaling; modulation of redox tone and protein modification

Review Article

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Summary. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have an impact on many cellular processes, often serving as signal transducers in both physiological and pathological situations. These small molecules can act as ligands for receptors as is the case for nitric oxide and guanylate cyclase. However, they can also modify proteins, changing their function and establishing a baseline for other signals in a process that we have termed "redox tone." In this review, we discuss the different mechanisms of redox cell signaling, and give specific examples of RNS participation in cell signaling via classical and redox tone pathways.

Keywords: Nitric oxide – Redox cell signaling – Reactive nitrogen species – Protein modification – Redox tone

Introduction

Over the past few years, redox cell signaling has increasingly been accepted as an important cellular process, and its dysfunction may play a role in the pathogenesis of a number of diseases including atherosclerosis, cancer, diabetes, and ischemia-reperfusion injury (Buzard and Kasprzak, 2000; Fukagawa et al., 2000; Das, 2001; Droge, 2002). Droge (2002) aptly defined redox signaling as "a regulatory process in which the signal is delivered through redox chemistry." This primarily involves the post-translational modification of specific amino acid residues on signaling proteins by reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Redox signaling has many of the characteristics of the "classical" receptor binding initiated signaling cascades and also uses common second messengers such as calcium, cyclic nucleotides, or kinase pathways. However, the characteristic that defines redox signaling is that proteins within these pathways contain amino acids that, when modified by a redox reaction, change function. This simple definition raises important issues regarding the molecular mechanisms that endow specificity, localization and reversibility, and these aspects will be discussed in detail in this short overview.

Specificity, amplification and reversibility in redox cell signaling

Redox reactions involve the exchange of electrons with ROS or RNS and in many cases these molecules are highly reactive. Therefore, specificity to the signaling process is achieved through a number of mechanisms that are distinct from other signal transduction pathways. As will be seen proteins that transduce the signal from ROS/RNS must recognize the specific species and also couple the protein modification to a change in function. A great deal has been learned in conceptual terms about how a free radical can act as a signaling molecule from studies with nitric oxide (NO). Clearly, targeting of ROS/RNS to a specific amino acid (or prosthetic group) on a protein should be constrained in a similar fashion to the processes that allow kinases to phosphorylate specific amino acids. In other words, access to the site for modification and the reactivity of the target residue is determined by the surrounding amino acid sequence, transition metal content, and subcellular localization.

The coupling of the transducer molecule to an amplified second step is also essential in initiating the cascade of reactions that ultimately ends in a change in cell function.

Redox effectors often activate or inactivate target enzymes or proteins that are critical to cell processes. Reversibility of modification by ROS or RNS is a more complex issue to address. Some cases involving thiol modification are known to be reversible while others involving adduct formation with oxidized lipids or nitration reactions are chemical processes that are essentially irreversible. For example, S-nitrosylation and NO adduction to heme groups are both reversible reactions. In addition to the above characteristics, the generation of redox species is an energy requiring process. As with other signaling mechanisms, the cell must invest energy to produce the redox species or to sequester it until needed.

Recently, the mechanistic details of this broad class of signal transduction pathways have been defined. This review defines subcategories of these mechanisms and gives examples based on the known reactions of ROS or RNS. The two most extensively studied redox effectors are hydrogen peroxide and nitric oxide, and these have been reviewed in a number of recent articles (Rhee, 1999; Patel et al., 2000; Levonen et al., 2001; Brookes and Darley-Usmar, 2002; Droge, 2002). A special emphasis will be placed on NO-derived species since these appear capable of modulating signal transduction pathways by multiple mechanisms.

Classification of redox signaling pathways

There are two major categories of redox signaling mechanisms emerging from the current research in the field. The first of these is the classical receptor-mediated signaling pathway in which the ROS/RNS binds to a specific protein target. The second is a more complex modulation of the activity in which redox-dependent reactions control the overall activity of a pathway by reacting with amino acid residues that modulate protein function. We have termed the second concept "redox tone," in order to distinguish it from direct receptor activation (Brookes and Darley-Usmar, 2002).

The classical interaction of the ROS/RNS or its reaction product with cellular receptor has been defined in a number of important biological relevant examples. Such interactions may be direct, as occurs with NO binding to the heme group in soluble guanylate cyclase, resulting in the activation of a particular signal transduction pathway (Fig. 1A). The interaction may also be indirect. For example, ROS or RNS interaction with lipids results in the formation of oxidized or nitrated lipids, including hydroperoxides, isoprostanes, or nitrated lipids (LNO_2 and $LONO_2$) (Fig. 1B). The formation of the oxidized lipids

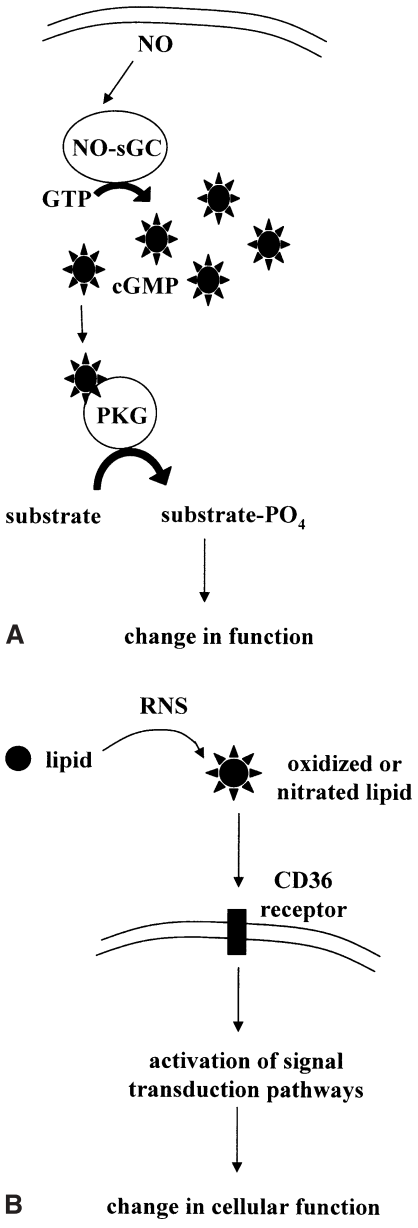


Fig. 1. Examples of direct and indirect RNS activation of classical signaling pathways. Panel **A**: NO interacts directly with a receptor (soluble guanylate cyclase), resulting in signal amplification (conversion of GTP to cGMP), and activation of protein kinases (*PKG*), which phosphorylate a variety of substrates and ultimately change cellular function. Panel **B**: RNS indirectly modulates a classical signaling pathway by modifying a lipid which then interacts with its specific receptor (*CD36*)

reflects changes occurring in the redox chemistry in pathological or physiological processes. These oxidized lipids then interact with specific receptors to elicit biological responses. They do not necessarily have to result in a covalent modification of the protein to elicit a response. Whether there are receptors that specifically recognize

oxidized lipids is an important and actively investigated area of research. In support of this concept, CD36, a receptor thought to be important in the development of the atherosclerosis, responds to oxidized lipids formed through non-enzymatic mechanisms (Terpstra et al., 1998). It has also been shown that nitrated lipids may possess bioactivity through eicosanoid receptors, or by acting as antagonists/competitive inhibitors of eicosanoid receptor-ligand interactions (O'Donnell et al., 1999). While the redox species itself does chemically modify specific amino acids on the receptor, it ultimately results in the stimulation of a particular receptor and therefore a change in cellular function.

The second mechanism of redox signaling we can classify is the establishment of cellular "redox tone." Redox tone is a concept analogous to vascular tone, where a redox effector acts on parts of a signaling pathway, creating a baseline that can be acted upon when the pathway is activated. This may involve the sensitization or desensitization of the signaling pathways by ROS or RNS. One example is the effect of peroxynitrite on prostacyclin synthesis. Peroxynitrite stimulates one part of the pathway by providing the "peroxide tone" for cyclooxygenase, while it inhibits prostacyclin synthase (Zou and Ullrich, 1996; Zou et al., 1999). Thus, peroxynitrite changes flux through the pathway, altering the final product and changing the cellular response (Fig. 2). The following sections will describe the interactions of NO and other RNS with key proteins involved in cell signaling.

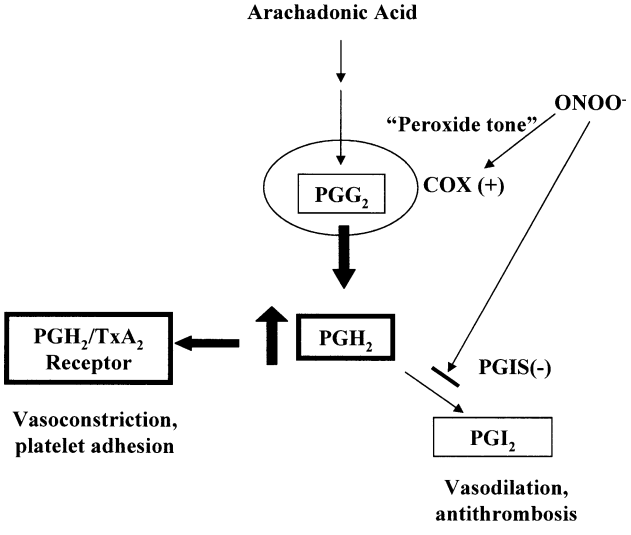


Fig. 2. Example of RNS establishment of redox tone. $ONOO^-$ provides peroxide tone that activates cyclooxygenase (*COX*), resulting in increased production of prostaglandin G_2 (*PGG*$_2$) and prostaglandin H_2 (*PGH*$_2$). $ONOO^-$ also nitrates and inactivates prostacyclin synthase (*PGIS*), exacerbating the buildup of proinflammatory PGH_2

Reactions of NO and RNS with heme and non-heme iron proteins

NO and RNS reactions with proteins depend upon the reactive species and the characteristics of the proteins themselves. NO itself binds to heme iron prosthetic groups as well as non-heme iron, zinc, and other transition metals. Other RNS such as peroxynitrite, N_2O_3, and nitroxyl anion (NO^-) react with proteins resulting in protein oxidation, nitration, or nitrosylation (Table 1).

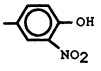
NO radical binds directly to the iron in heme-containing proteins with high affinity. NO reacts rapidly with ferrous heme (Kd $T > 1 \times 10^{-9}$) (Cooper, 1999), but may also react with ferric heme, as in the case of catalase (Brown, 1995; Cooper, 1999). The reactions of NO with heme are often competitive with oxygen and play a variety of roles including NO transport, NO metabolism/inactivation, and enzymatic activation or inactivation.

Nitric oxide synthase

The targeting of the nitric oxide synthase (NOS) by NO or RNS may be an important first step in the control of redox cell signaling by NO. Nitric oxide synthase (NOS) produces NO from arginine and is represented by three major isoforms, all of which are heme-containing proteins related to the cytochrome P450 family. NO production is regulated by substrate concentration, availability of a tetrahydrobiopterin cofactor, and for some isoforms, by intracellular calcium concentration (Andrew and Mayer, 1999). The NO produced also serves as a feedback inhibitor of NOS, though the mechanism of this inhibition is debated. One hypothesis is that the NO produced feedback inhibits the enzyme by interacting with the heme. In this scenario, the ability of enzyme to be inhibited by NO depends on the state of the iron. For example, NO inhibits the ferric form (Fe^{3+}) more readily than the ferrous form (Fe^{2+}) (Griscavage et al., 1995). Another hypothesis is that NO inhibits NOS via S-nitrosothiol formation, and this has been shown for enteric nNOS *in vitro* (Kurjak et al., 1999). The different isoforms of NOS may utilize different mechanisms of feedback inhibition, depending upon the isoform itself and/or the cellular redox state.

The inducible NOS isoform (iNOS) is generally considered the "high output" NO producer. iNOS is regulated at the transcriptional level, whereas other isoforms are constitutively expressed. Since the iNOS isoform generates

Table 1. Examples of protein modifications by RNS

Reactive species	Target	Modification
NO	Heme-Fe^{2+} (oxygenated)	Heme-Fe^{3+} and NO_3^-
	Heme-Fe^{2+} (deoxygenated)	Heme-Fe^{2+}-NO
	Other transition metals (Zn, Se, Fe–S clusters)	NO-metal adducts, or NO metabolism
NO^-	unknown	unknown
NO^+	RS^-	RSNO
N_2O_3 (forms NO^+ and NO_2^-)	RS^-	RSNO
$ONOO^-$	Cys, RSH	RSOH, RSO_2H
	Met, $RSCH_3$	$RSOCH_3$
	Tyr	nitro-Tyr 
	Trp	nitro-Trp
	His	nitro-His

higher levels of NO than the other isoforms, the feedback effect of the NO generated would be expected to be greater in the absence of other nearby NO targets. Griscavage et al. (1995) showed that iNOS was less sensitive than eNOS and nNOS to inhibition by NO, but that given the higher levels of NO produced by iNOS, feedback inhibition may still be physiologically relevant. However, Robinson et al. (2001) recently demonstrated that iNOS was not inhibited by the NO donor PAPA-nonoate in a murine epithelial cell line, but was inhibited by peroxynitrite. They propose that the mechanism of inhibition is tyrosine nitration. Another group reports that peroxynitrite inhibits NOS, particularly nNOS, and may destroy the heme-thiolate bond in the catalytic site (Pasquet et al., 1996). Thus, the mechanism of feedback inhibition by NO may be dependent upon the NOS isoform and the oxidative environment, and these will determine whether peroxynitrite will be formed from NO. Thus, the interaction of NO or peroxynitrite with NOS is not a classical receptor-ligand interaction, but rather serves as an example of the establishment of redox tone.

Soluble guanylate cyclase

Soluble guanylate cyclase was the first known physiological target of NO, and is an archetype for NO modulation of a protein that affects cell signaling. NO binds to the ferrous heme and releases the heme-ligating histidine, resulting in a heme Fe^{2+}-NO complex. A change in heme geometry then occurs causing a conformational change of the protein to an enzymatically active form (Denninger and Marletta, 1999). Activation of soluble guanylate cyclase by NO results in an increase in the second messenger cyclic GMP (cGMP), which then activates cGMP-dependent protein kinase (PKG), as well as phosphodiesterases, ion channels, and other important regulatory proteins (Denninger and Marletta, 1999; Lincoln et al., 2001). This results in relaxation of smooth muscle in the vasculature (Ignarro et al., 1987; Warner et al., 1994) Thus, NO interaction with its receptor soluble guanylate cyclase is an example of RNS participation in a classical signaling pathway.

Catalase

The binding and inhibition of catalase by NO provides a point of cross-talk between the H_2O_2 and NO-mediated redox signaling pathways. While several pathways can lead to the metabolism of H_2O_2, catalase has been shown to modulate redox cell signaling based upon experiments with over-expressed or targeted enzyme (Brar et al., 1999; Ushio-Fukai et al., 1999). NO binds to the ferric heme group of catalase and thereby inhibits enzymatic conversion of hydrogen peroxide to water (Brown, 1995; Cooper, 1999; Brunelli et al., 2001). However, the chemistry of NO binding to ferric heme is different than to ferrous heme. The dissociation rate of NO binding to the ferric heme of catalase has been found to be 5×10^{-7} M at pH 7 (Cooper, 1999). Though the on rate is only an order of magnitude different than observed with the ferrous heme of hemoglobin, $0.2 \times 10^7\,M^{-1}\,s^{-1}$ for catalase (Cooper, 1999) vs. $2 \times 10^7\,M^{-1}\,s^{-1}$ for hemoglobin (Cassoly and Gibson, 1975), the off rate is very high, $1\,s^{-1}$ for catalase (Cooper, 1999) vs. $<1.5 \times 10^{-10}\,s^{-1}$ for hemoglobin (Sharma and Ranney, 1978), resulting in a weaker interaction of NO with the heme of catalase. Nevertheless, the inhibition of catalase by NO is likely *in vivo* (Cooper, 1999), and the resulting increase in H_2O_2 concentration could then activate other ROS-sensitive pathways. In this case, NO establishes redox tone by interacting with and modulating the activity of catalase, which in turn alters the activity of other redox signaling pathways.

Aconitase/IRP

Some proteins targeted by NO contain iron, but do not contain heme. These proteins coordinate iron via iron sulfur clusters, which coordinate iron atoms by cysteine thiolate (cys-S^-) residues. One example of this is the bifunctional protein aconitase/iron-response protein (IRP). In the mitochondria, this protein functions as the citric acid cycle enzyme, aconitase. In the cytoplasm, however, its function is shifted during conditions of low iron to the IRP. The IRP is capable of binding to iron response elements (IREs) present on certain mRNAs of proteins critical for iron homeostasis, but only when the iron-sulfur cluster has been removed from the protein. The mechanism of aconitase inhibition by RNS has been debated in the literature. Some groups claim that NO itself is capable of inhibiting aconitase activity (Gardner et al., 1997). However, at physiological pH, peroxynitrite inactivates aconitase (Castro et al., 1994; Hausladen and Fridovich, 1994; Gardner et al., 1997). One possible mechanism by which peroxynitrite inhibits aconitase activity is by interacting with the labile Fe of the 4Fe–4S iron-sulfur cluster (Beinert et al., 1996). This would result in a 3Fe–4S iron-sulfur cluster that is catalytically inactive, but since peroxynitrite does not generate the apoprotein, it would not have IRP activity. However, another group has shown aconitase treated with peroxynitrite exhibits IRP activity under suitable reducing conditions (Bouton et al., 1997). A provocative hypothesis has been proposed that the NO may bind the Fe_a and this may lead to an autocatalytic S-nitrosation of one of the cysteines of the iron-sulfur center. Thus, the form of NO, NO radical or $ONOO^-$, that is responsible for targeting to aconitase under physiological and pathological conditions remains to be elucidated.

The interactions of RNS with heme and non-heme iron proteins change protein function and demonstrate both direct receptor-mediated redox cell signaling, as in the case of soluble guanylate cyclase, as well as modulatory establishment of redox tone, as with NOS, catalase, and aconitase/IRP.

Mitochondrial respiratory complexes

Other Fe–S containing proteins affected by NO that are important in cell function are the mitochondrial respiratory complexes. Interestingly, recent studies suggest that mitochondrial respiratory function is also coupled to the activation of MAP kinases (Levonen et al., 2001). Although this field is in its infancy the potential for mitochondrial proteins to contribute to signal transduction is likely to become an important field of investigation. At this stage considerably more is known about the interactions of ROS/RNS with mitochondrial electron transfer proteins, while little emphasis has been placed on signal transduction. Some of the key findings will be summarized here. Long-term exposure to NO results in damage to complex I, most likely by peroxynitrite or S-nitrosation and while mitochondrial respiration can continue by directly feeding reducing equivalents into complex II, the damage to complex I may change local oxygen radical production. This change in superoxide production in the presence of MnSOD could result in altered levels of hydrogen peroxide, providing another point of cross-talk between the NO and H_2O_2 signaling pathways (Brookes and Darley-Usmar, 2002). A recent review by Radi et al. (2002), eloquently describes the effects of NO and peroxynitrite on mitochondrial function.

The NO-cytochrome c oxidase signaling pathway

The primary site of interaction of NO with mitochondria is the cytochrome c oxidase (complex IV) of the mitochondrial respiratory chain. The cellular function of this interaction is still a matter of active debate. Nevertheless, the characteristics of the interaction satisfy many of the criteria of a signaling pathway. For example, cytochrome c oxidase inhibition by NO is controlled by the partitioning of NO into mitochondrial membranes (Shiva et al., 2001). Also, the enhanced reaction of NO with O_2 within the inner membrane of the mitochondrion and is proposed to be a key mechanism leading to reversal of NO binding to cytochrome c oxidase (Shiva et al., 2001).

Within the mitochondrial membranes, NO binds to the ferrous iron of heme a_3 in the binuclear site of cytochrome c oxidase (Giuffre et al., 2000). This reaction is likely physiologically relevant since NO binds with high affinity ($Kd = 1 \times 10^{-10}\,M$) (Cooper, 2002), and is very rapid with a rate constant of $1 \times 10^8\,M^{-1}\,s^{-1}$ (Blackmore et al., 1991). NO binding at this site inhibits the enzymatic binding of molecular oxygen and its subsequent reduction to water, and this inhibition is dependent on low oxygen tension (Shiva et al., 2001) as well as high electron pressure through the mitochondrial respiratory chain, represented by ferrocytochrome c concentration.

In addition to heme binding, NO interaction with cytochrome c oxidase may occur via other mechanisms depending upon the state of the protein, which in turn is determine by the cellular energy state. For example, when electron flux through the mitochondrial respiratory chain is low, ferricytochrome c concentration will also be low.

Under these conditions, NO still binds cytochrome c oxidase but the reaction is shifted from the heme a_3 to the Cu_B of the binuclear site (Giuffre et al., 2000). This interaction results in the oxidation of NO to nitrosonium ion (NO^+), followed by hydration to nitrite (the so called ''NO oxidase'' activity of cytochrome c oxidase) (Torres et al., 2000). This reaction may be an important mechanism in elimination of NO, removing it from its signaling role in the cell. The effects of NO removal may be significant in pathological conditions such as heart failure and diabetes, where low NO production is thought to contribute to disease evolution (Trochu et al., 2000).

Though cytochrome c oxidase is not part of a classical signaling pathway, NO interaction with cytochrome c oxidase may be important in the establishment of redox tone and cell signaling due to its effect on mitochondrial cytochrome c release, respiration, and H_2O_2 formation. The overall effect of RNS interaction with cytochrome c oxidase will be dependent upon the form of RNS, mitochondrial respiration, and oxygen tension.

Prostacyclin synthase

One protein that already has been identified as nitrated in atherosclerosis is prostacyclin synthase, the enzyme that converts PGH_2 to PGI_2 (prostacyclin). Prostacyclin synthase produces prostacyclin in endothelium to mediate vasodilation. Prostacyclin synthase is rapidly inactivated by submicromolar concentrations of $ONOO^-$ in endothelium (Zou and Ullrich, 1996; Zou et al., 1997; Zou et al., 1998). Prostacyclin synthase is particularly susceptible to nitration by $ONOO^-$ due to the presence of its heme-thiol group (Zou et al., 2000). Since prostacyclin synthase converts PGH_2 to prostacyclin, inactivation of this key enzyme leads to a buildup of PGH_2, which can then activate the PGH_2/TxA_2 receptor and promote vasoconstriction and platelet aggregation (Viner et al., 1996; Viner et al., 1999). Since $ONOO^-$ also provides peroxide tone necessary to activate cyclooxygenase (Landino et al., 1996), a sustained production of $ONOO^-$ in the vasculature can stimulate cyclooxygenase activity to produce PGH_2. Consequently, endogenous generation of $ONOO^-$ by endothelium can increase PGH_2 while decreasing the prostacyclin synthase product, prostacyclin, to promote the proinflammatory functions of the endothelium such as vasoconstriction and platelet adhesion as vasodilatory and antithrombotic functions will be impaired.

Cytochrome c

Cytochrome c is involved in cell signaling upon release from the mitochondrion and subsequent participation in the assembly of the apoptosome. Cytochrome c is also nitrated by peroxynitrite at a heme-vicinal tyrosine residue (Tyr-67). Upon nitration, a conformational change occurs which disrupts heme liganding, and ultimately causes changes in protein function such as an increase in peroxidatic activity, resistance to ascorbate reduction, and impaired mitochondrial respiratory function (Cassina et al., 2000). Furthermore, physiologically relevant concentrations of $ONOO^-$ cause cytochrome c nitration and release in intact mitochondria (Cassina and Radi, unpublished observation, see Cassina et al., 2000, suggesting a correlation between cytochrome c nitration and apoptosis. However, further study is needed to determine what role, if any, peroxynitrite modification of cytochrome c plays in apoptosis.

S-nitrosylation of cysteine residues and cell signaling

S-nitrosation, which has also been called nitrosylation to reflect its signal transduction role analogous to phosphorylation, is the reversible addition of NO to a cysteine residue. The mechanism of S-nitrosylation *in vivo* is controversial but three forms of NO have been proposed to be involved: NO^+, $ONOO^-$ via a nitrosonium-like species, and N_2O_3. Nitrosonium ion can be generated by the reduction of a transition metal (such as Cu^{2+} or Fe^{3+}) by NO. The resulting S-NO group is relatively stable, but the NO group may be released by transition metals (Singh et al., 1996), superoxide (Aleryani et al., 1998), or light. Peroxynitrite reacts very quickly with reduced protein thiols, often yielding an oxidation product, rather than a nitrosylated product (Alvarez et al., 1999). Higher oxides of NO, such as N_2O_3, can also S-nitrosylate protein cysteines (Keshive et al., 1996). However, the stoichiometry of the reaction is such that two molecules of NO and one molecule of O_2 are required for every S-nitrosothiol formed. This situation may be valid if NO and O_2 are preferentially sequestered into hydrophobic areas such as lipid membranes or hydrophobic protein interiors (Liu et al., 1998; Goss et al., 1999; Nedospasov et al., 2000; Rafikova et al., 2002).

Caspases

Members of the caspase family of aspartate-specific cysteine proteases have been shown to be inactivated by

S-nitrosylation at the conserved active site cysteine residue (Li et al., 1997). Caspase-3, the ''executioner'' of apoptosis, is inactivated by stable S-nitrosylation, with other cysteines reversibly modified as well (Rossig et al., 1999; Zech et al., 1999). The possibility that NO or other RNS provides a means for the cell to abort the process of apoptosis at early and late stages by inactivating caspase proteins makes them tremendously important in determining the fate of the cell, i.e. committing to apoptosis or not.

NMDA receptor

NO as a nitrosylating agent may have influence not only in apoptotic cell signaling, but in the establishment of redox tone. NO may also enhance disulfide bonding of vicinal sulfhydryl (thiol) groups of the redox sensitive proteins. One example is the disulfide bonding in the redox sensitive modulatory site of the N-methyl-D-aspartate (NMDA) receptor complex is enhanced by NO nitrosylation, thereby down-regulating its Ca^{2+} channel activity (Riedel, 2000). Thus, NO desensitizes the signaling pathway activated by NMDA and sets the redox tone, but does not participate directly as a signaling ligand for the receptor.

H-ras

Perhaps the best example of NO activating a classical signaling pathway via S-nitrosylation is the small guanine nucleotide binding protein H-ras. S-nitrosylation of a critical C118 residue causes guanine nucleotide exchange that activates H-ras (Lander et al., 1997). This results in the activation of a signaling pathway that includes ERK and NF-kappa B, and causes cellular proliferation and differentiation (Vojtek and Der, 1998) (Fig. 3). H_2O_2 also interacts with this pathway by activating Ras (Lander et al., 1995). The contribution of ROS or RNS to the activation of Ras will depend upon a number of factors, including the redox state of the cell (or subcellular region), the activation of the Ras pathway by other effectors (receptor tyrosine kinases), and the availability of competing targets for ROS/RNS.

Other forms of NO exist which can alter protein function are nitroxyl anion and nitrosonium cation, although the mechanisms of formation *in vivo* are still under investigation. Nitroxyl anion (NO^-) is a reduced form of nitric oxide that may be formed *in vivo* by the reversible reduction of NO by superoxide dismutase (Murphy and Sies, 1991). There is also preliminary evidence that NO^- is produced by NOS (Fukuto et al., 1992; Schmidt et al., 1996). NO itself may be formed from NO^- (Buyukafsar et al., 2001). For example, the copper-containing enzyme tyrosinase, which catalyses the hydroxylation of monophenols and the subsequent oxidation to a quinone, generated nitric oxide from Angeli's salt, but only in the presence of tyrosine (Buyukafsar et al., 2001), suggesting that quinone intermediates are involved in the conversion of NO^- to NO.

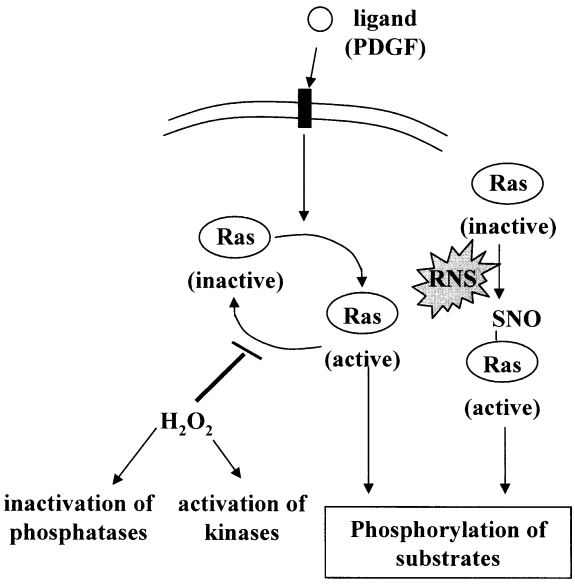


Fig. 3. Modulation of Ras activity by ROS and RNS. Ras is activated by the binding of a ligand (platelet-derived growth factor; *PDGF*) to its receptor. Turnover of Ras to the inactive form is inhibited by H_2O_2. Alternatively, Ras can be directly stimulated by RNS by S-nitrosylation of a critical cysteine residue (*SNO*)

NO^- is often formed experimentally by the decomposition of Angeli's salt ($Na_2N_2O_3$) in solution (Hughes, 1999). The decomposition of Angeli's salt to NO^- is an oxygen consuming process, and the reaction of NO^- and O_2 would be expected to yield peroxynitrite (Sharpe and Cooper, 1998; Miranda et al., 2001). However, the decomposition of Angeli's salt yields a product that has properties distinct from peroxynitrite (Miranda et al., 2001). There is evidence that NO^- has effects *in vivo* which are distinct from NO. NO^- is a positive cardiac inotropic agent in a canine model, and these effects were redox sensitive and independent of cGMP, indicating that NO^- acts independently of NO itself (Paolocci et al., 2001). Also, NO^- blocks glycine-independent desensitization of the NMDA receptor, an effect that is distinct from that mediated by S-nitrosylation (Colton et al., 2001). Thus, NO^- may be a physiologically relevant mediator of RNS targeting, and while its targets and mechanisms

are under intense investigation, they are likely to be different than those of NO.

In summary, there are numerous examples of RNS modulation of proteins encompassing a variety of mechanisms including interaction with heme iron, Fe–S clusters, cysteine and tyrosine residues, to name a few. While a small number of these modifications involve direct participation of RNS in a classical receptor-mediated signaling pathway, it is clear that RNS affect the activity of many other pathways via the establishment of redox tone.

Acknowledgements

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