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S-Nitrosylation of proteins

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Abstract. The transfer of a nitric oxide group to cysteine sulfhydryls on proteins, known as S-nitrosylation, is increasingly becoming recognized as a ubiquitous regulatory reaction comparable to phosphorylation. It represents a form of redox modulation in diverse tissues, including the brain. An increasing number of proteins have been found to undergo S-nitrosylation in vivo. These proteins are called S-nitrosothiols, and may play an important role in many processes ranging from signal transduction, DNA repair, host defense, and blood pressure control to ion channel regulation and neurotransmission. This review focuses on the importance of the S-nitrosylation reaction and describes some recently identified S-nitrosothiols in various fields of research.

Key words. Nitric oxide; nitrosothiols; S-nitrosylation; redox modulation; protein regulation.

Introduction

A multiplicity of biological functions has been ascribed to nitric oxide (NO). The observation that in many cases the NO signals were cGMP independent has made it clear that more than one NO activation pathway must be involved [1–3]. Direct regulation of protein function by NO, independent of the activation of the soluble guanylyl cyclase and subsequent production of cGMP [4–6] has been proposed as an alternative pathway by which NO may directly play a critical role in many processes such as blood pressure regulation, host defense, and neurotransmission [7].

Synthesis of nitrosothiols

Direct action of NO on proteins, independent of cGMP production, has meanwhile been shown to involve cysteine or tyrosine residues [8] and metals such as the heme-Fe^{2+} interaction [9, 10]. There is no precedent for a reaction of NO itself with thiol groups under physiological conditions, but the formation of NO quickly yields NO^+ equivalents upon interaction with oxygen and/or oxidative transition metals, and $ONOO^-$ (peroxynitrite) upon interaction with O_2^-. The transfer of a NO^+ equivalent on a free -SH group of a protein leads to the formation of a nitrosothiol (RSNO). This reaction is called S-nitrosylation or nitrosation. In the case of neighboring thiols, S-nitrosylation can promote subsequent disulfide formation. This post-translational modification can influence many protein functions. S-nitrosylation reactions may depend on catalytic amounts of transition metals, O_2, O_2^-, and pH.

Titration of free -SH groups by radioactive SH-modifying reagents [11] ultraviolet-visible spectrophotometry [12] and electrospray ionization-mass spectrometry [13] can be used to monitor the binding of NO to peptides (on precise amino acids). These techniques have demonstrated that cysteine residues are rapidly nitrosylated, while reactions with other amino acids occur at much slower rates. Cysteine residues are therefore the main target of S-nitrosylation. It should be noted, however, that the RS-NO bond is labile and therefore difficult to study and quantify [13].

Much attention has been paid to the chemistry and pharmacology of RSNOs. Numerous studies have concentrated on their synthesis, properties, reactions that lead to NO (NO^+) formation, and reactions whereby the NO group can be transferred to other thiol groups [14]. The main issue remaining is the possibility of such chemical reaction (S-nitrosylation) occurring in vivo and not only at acidic pH [15, 16]. Since it has been impossible until now to measure the free redox-modulated forms of NO, such as NO^+, in vivo, this issue has long been controversial.

Gow et al., [17] have recently proposed a different and novel reaction mechanism for the formation of S-nitrosothiols in vivo (under physiological conditions). They propose a mechanism whereby NO reacts directly with reduced thiol to produce a radical intermediate, R-S-N·-O-H. This intermediate then reduces an available electron acceptor to produce a RSNO. Under aerobic conditions, O_2 acts as the electron acceptor and is reduced to produce superoxide (O_2^-). Gow et al. [17] provide the following experimental evidence in support of their mechanism: cysteine accelerates the consumption of NO 2.5-fold under physiological conditions. The consumption of O_2 in the presence of NO and cysteine is also increased 2.4-fold. The reaction orders of NO and cysteine are second and first order, respectively. The second order of reaction for NO may result from interaction between NO and O_2^- to form peroxynitrite. In the presence of Cu,Zn-superoxide dismutase, the reaction of NO with cysteine generates hydrogen peroxide, indicating that the reaction generates O_2^-. Finally, the formation of RSNO is demonstrated in an anaerobic environment and, as predicted by the mechanism, is dependent on the presence of an electron acceptor. These results demonstrate that under physiological conditions, NO could react directly with thiols to form RSNO in the presence of an electron acceptor. Therefore, RSNOs can be formed in vivo under a wide variety of physiological and pathophysiological conditions.

NO versus RSNOs

NO is formed endogenously from the guanido nitrogen of L-arginine by the action of NO synthase (NOS), a NADPH-dependent enzyme (fig. 1). At least three isoforms of NOS have been characterized. Purified NOS isoforms have been isolated from brain tissue [18, 19] as well as from macrophages [20] and endothelial tissue [21]. The brain and endothelial forms of the enzyme are constitutively present and account for the role of NO in mediating rapid events, such as neurotransmission or vasodilation. These NOS isoforms are stimulated to produce NO by Ca^{2+}/calmodulin. The macrophage NOS is inducible and is generally expressed after the activation of the cells by cytokines.

The action of NO is determined largely by its membrane permeability and diffusivity; it has the highest diffusion coefficient of any biological molecule, being 1.4-fold higher than oxygen or carbon monoxide at 37 °C. The estimated diffusion distance of NO in tissue is 500 μm [22]. The physiological range of concentrations for active NO appears to be in the low micromolar range. In the brain (cerebellar slices), 70–100 nM of

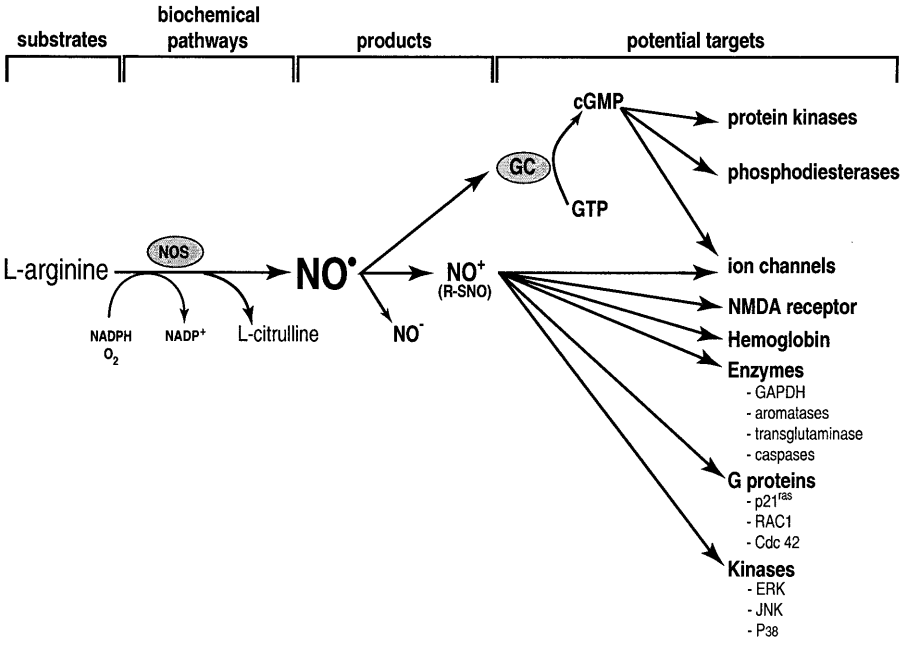

Figure 1. Biosynthesis pathway for NO and nitrosothiols. NO is synthesized from L-arginine by NO synthases (NOS). One NO-derived nitrosative compound can activate by S-nitrosylation an array of target proteins either directly or via the formation of intermediate nitrosothiols (RSNO).

NO was measured after brief electrical stimulation [23], whereas Malinski et al. [24] measured 2–4 μM NO in the cortex. In comparison with NO, RSNOs are often very stable compounds with half-lives of up to 40 min, as in the case of S-nitrosoalbumin [1]. These RSNOs could have regulatory functions, as shown for S-nitrosoglutathione and S-nitrosocysteine in the cerebellum [25, 26].

It is interesting to speculate on the possible physiological significance of the direct NO pathway, especially with regard to protein modulation. The NO free radical is a short-lived molecule with a half life that restricts not only its activity but also its spatial spread. In a cell, the NOS produces an NO radical which may then, through an as yet incompletely understood set of reactions (but possibly including the production of intermediate RSNOs like S-nitrosocysteine), result in the production of the more stable RSNOs. Thus, this pathway would retain the spatial restriction of NO, while providing a much longer lasting period of activity. RSNOs may therefore play an important role in NO storage within the body, given the ready pathway for the transfer of the NO group to a thiolate ion, as RSNOs are believed to decompose non-enzymatically to give again NO.

Specificity of S-nitrosylation

How is nitrosylation specificity achieved? It is not uncommon for several cysteine residues on a given protein to be candidates for nitrosylation. In the ryanodine receptor, out of a total of 364 cysteines, 84 provide free -SH groups, but only 12 are thought to undergo nitrosylation [27]. While the precise parameters governing accessibility by NO are unknown, the existence of a consensus nitrosylation acid–base motif has been postulated based on large database screenings [28]. The proposed motif is XYCZ, where X can be any of G, S, T, C, Y, N or Q; Y can be K, R, H, D or E; and Z can be D or E. The most important element of the sequence is believed to be the Asp/Glu residues following the cysteine. Despite this rather degenerate motif, in the cyclic nucleotide-gated channel, for example, only one cysteine residue, that identified by biochemical experiments as the NO target, possesses the required motif (i.e., Q, D, C, E) [29].

S-nitrosylation specificity may also be achieved through the subcellular localization of the NOSs which may be in proximity to potential targets. The effect of NO on cells depends on its local concentration, the redox status of its immediate environment, and the susceptibility of target sites for modification. Different degrees of accessibility to NO (RSNO) or different reaction rates with NO, as well as important functional differences in the -SH group being modified by NO might explain why and how specific S-nitrosylation of precise cysteine residues induces protein modulation.

Modulation of protein functions by S-nitrosylation

There are different possible explanations for the effects of -SH groups on protein function. The redox state of -SH groups on proteins can play an important role in determining protein structure and function, the -SH group being the most reactive group on proteins. The role of these free -SH groups in protein activity has been studied, in particular, in various ion channels, most recently in the slow delayed rectifier potassium channel [30], showing that the alteration of ion channel function depends mostly on the accessibility and the reactivity of -SH groups which can range by more than six orders of magnitude.

S-nitrosylation may represent an alternative pathway for protein modulation analogous to phosphorylation. Phosphorylation and nitrosylation indeed share many common features. Phosphorylation (or the transfer of a phosphoryl group) involves covalent attachment of a phosphate to either serine, threonine, or tyrosine, whereas nitrosylation (or the transfer of a NO group) involves the covalent attachment of NO to cysteines. The target proteins in both systems can be simple switches or components of more complex signaling circuits. They include enzymes, G proteins, transcription factors, transporters, and ion channels—all the machinery necessary for transmitting information from the cell surface to the nucleus [8, 28]. Representative NO targets are listed in figure 1. Phosphorylation has also been found to enhance a response under one experimental condition and to oppose it under another, as observed now for protein S-nitrosylation.

Examples of physiological RSNOs and their roles

The formation of a RSNO has now been shown to occur in vivo for an array of proteins [10, 31]. RSNOs like S-nitrosoalbumin, S-nitrosoglutathione (GSNO) or S-nitroso-L-cysteine have now been detected and quantified in vivo [32] and they may be responsible for some of the well-documented physiological processes that had been previously attributed to NO itself. GSNO, for example, has been measured in up to micromolar concentrations in human bronchial fluid [33].

RSNOs and hematology

Low molecular weight RSNOs are believed to play an integral role in a variety of different NO-dependent physiological processes, particularly in the vasculature. The physiological relevance of RSNOs has been confi-

rmed by the demonstration that the predominant redox form of NO in mammalian plasma is S-nitrosoalbumin, a substituted derivative of NO and serum albumin [1]. The total plasma RSNO concentration is about 1 μM, in contrast to the free concentration of NO which is approximately 3 nM [1]. S-nitrosoalbumin accounts for more than 85% of the plasma RSNOs pool. A number of RSNOs including GSNO have been shown to possess vasodilatory activity as well as the ability to inhibit platelet aggregation [34].

The high-affinity of NO binding to hemoglobin (Hb) has shaped our view of heme proteins and of small diffusible signaling molecules. Specifically, NO binds rapidly to the heme iron in Hb (k_{on} approximately 10^7 M^{-1} s^{-1}) and once bound, the NO activity is largely irretrievable (K_d approximately 10^{-5} s^{-1}); the binding is so tight as to be unaffected by O_2 or CO. However, these general principles do not consider the allosteric state of Hb or the nature of the allosteric effector, and they mostly derive from the functional behavior of fully nitrosylated Hb, whereas Hb is only partially nitrosylated in vivo. Stamler et al. [35] who studied blood flow regulation by Hb in the physiological oxygen gradient found that the binding of oxygen to heme irons in Hb promotes the binding of NO to a particular cysteine residue (Cysβ93), forming S-nitrosohemoglobin (SNO-Hb). Deoxygenation is accompanied by an allosteric transition in SNO-Hb [from the R (oxygenated) to the T (deoxygenated) structure] that releases the NO group. SNO-Hb contracts blood vessels and decreases cerebral perfusion in the R structure and relaxes vessels to improve blood flow in the T structure. Thus, by sensing the physiological oxygen gradient in tissues, Hb exploits conformation-associated changes in the position of Cysβ93 of SNO-Hb to bring local blood flow into line with oxygen requirements. SNO-Hb can therefore release NO on deoxygenation in the microcirculation. Gow and Stamler [36] have also studied the reactions between NO and Hb under physiological conditions and have shown that oxygen drives the conversion between the different nitrosylated-oxygenated forms of Hb. The yields of both SNO-Hb and methemoglobin are dependent on the NO/Hb ratio. These newly discovered reactions elucidate mechanisms underlying NO function in the respiratory cycle, and provide insight into the etiology of RSNOs, methemoglobin, and its related valence hybrids. Mechanistic reexamination of NO interactions with other heme proteins containing allosteric-site thiols may be necessary.

Pawloski et al. [37] have additionally shown that SNO-Hb inhibits platelet aggregation and that this mechanism is indeed cGMP independent. These authors suggest that the red blood cell has evolved a means to counteract platelet activation in small vessels and the proaggregatory effects of oxidative stress by forming SNO-Hb. Catani et al. [11] studied the importance of protein S-nitrosylation in blood clot formation. The plasma factor XIII (FXIII) is a transglutaminase which catalyzes the cross-linking of fibrin monomers during blood coagulation. These authors established that high concentrations of RSNOs have inhibitory effects on blood clot formation. Titration of the -SH groups of FXIII with [^{14}C]iodoacetamide (an SH-modifying reagent) demonstrated that FXIII is a target for S-nitrosylation on a highly reactive cysteine residue. Inhibition of FXIII activity by NO may represent an additional regulatory mechanism for the formation of a blood clot with physiopathological implications. These experimental results among many others strongly implicate the RSNOs as playing important roles in hematology.

RSNOs and intracellular signaling

Although free radicals have been traditionally implicated in cell injury, and associated with pathophysiological processes, recent data implicate them in cell signaling events. Free radicals are naturally oxygen-, nitrogen- and sulfur-derived species with an unpaired electron, such as superoxide, hydroxyl radical or nitric oxide. The NO-related species (such as NO^+) and reactive oxygen intermediates activate, for example, the MAP-kinases through upstream activation, most likely by direct activation of a low-molecular-weight G protein such as $p21^{ras}$, Rac1, or Cdc42. NO species are also able to trigger and activate directly some signaling events within the cell, such as ERK, JNK and P38 kinase, probably under conditions where cellular glutathione levels are depleted [38]. Critical signaling kinases such as ERKm p38 and JNK are activated by NO-related species and thus participate in NO signal transduction. Single S-nitrosylation of $p21^{ras}$ leads to redox regulation of the protein triggering guanine exchange and downstream signaling [39] and NO also inhibits c-jun N-terminal kinase 2 (JNK2) via S-nitrosylation.

delaTorre et al. [12] studied the differential effects of S-nitrosylation of the nuclear protein transcription factors p50 and c-jun on DNA binding. They obtained confirmatory evidence for RSNO bond formation by ultraviolet-visible spectrophotometry with the absorption maximum for S-NO bonds at approximately 320–360 nm. In the case of NF-kappa B p50, S-nitrosylation resulted in significantly decreased DNA binding. In contrast, S-nitrosylation did not alter c-jun DNA binding. The S-nitrosylating conditions themselves did not alter p50 or c-jun DNA binding. S-nitrosylation of transcription factors may be one mechanism by which NO may selectively regulate gene transcription.

RSNOs and ion channel regulation

Ion channel regulation has also been postulated to occur by direct nitrosylation, but specific cysteine targets have not yet been identified. These NO-sensitive channels currently include the *N*-methyl-D-aspartate receptor-channel complex [40], Ca^{2+}-activated K^+ channels [41], cyclic nucleotide-gated (CNG) channels [29, 42], Na^+ channels in baroreceptors [43], and cardiac Ca^{2+} release channels [27]. Only the CNG channels have been shown to be directly opened by nitrosylation; in other ion channels, it appears rather that the activation and inactivation parameters are being altered.

In a very interesting example of ion channel regulation, both direct and indirect effects of NO have been observed. Campbell et al. [44] studied the redox modulation of L-type calcium channels using the patch clamp technique. A dual mechanism of regulation by NO and RSNOs was observed. On the one hand, the NO inhibition of the channel was cGMP dependent, on the other, RSNOs, which donate NO^+, stimulated the ion channel. Similar activation of the ion channel by thiol oxidants, and reversal by thiol reductants, identified an allosteric thiol-containing 'redox switch' on the L-type calcium channel subunit complex by which NO/O_2^- and NO^+ transfer could exert effects opposite to those produced by NO. In summary, their results suggest that both an indirect (cGMP-dependent) and a direct (S-nitrosylation/oxidation) regulation of this ion channel exist and that the thiol redox state may be an important determinant of channel activity.

In the case of the native olfactory CNG channel, biochemical evidence points to a cysteine residue located on the intracellular face of the ion channel as a putative target site for S-nitrosylation, in the linking region between the sixth transmembrane domain and the cyclic nucleotide binding site [29, 42]. The cysteine residue of particular importance in NO activation is highly conserved among the cloned CNG channels. It is located in an intracellular loop of the channel protein (Cys460 in the case of the α subunit of the olfactory CNG channel). Direct CNG channel opening appeared to result from the S-nitrosylation of the thiol group of this amino acid residue.

RSNOs and host defense

RSNOs are also involved in host defense, helping to kill tumors and intracellular pathogens. For example, in a recent paper, Gobert et al. [45] found that murine macrophages use oxygen- and NO-dependent mechanisms to synthesize S-nitrosoalbumin and to kill extracellular trypanosomes. Reactive NO intermediates were indeed synthesized spontaneously in cultures of macrophages from *Trypanosoma brucei brucei*-infected mice by an inducible NOS. This was inhibited by the addition of nitro-L-arginine. These authors also studied the kinetics of the fixation of macrophage-derived NO on bovine serum albumin using an enzyme-linked immunoabsorbent assay and confirmed S-nitrosylation by the Saville reaction, using mercuric chloride.

In another study, Persichini et al. [46] found that cysteine nitrosylation inactivates the human immunodeficiency virus (HIV)-1 protease. HIV-1 protease action is modulated by the redox equilibrium of Cys67 and Cys95 regulatory residues. Thus HIV-1 protease inactivation via NO-mediated nitrosylation of Cys regulatory residue(s) may represent an interesting possible mechanism for inhibition of HIV-1 replication and an indication that S-nitrosylation modulates the catalytic activity of cysteine-containing enzymes.

RSNOs and apoptosis

Excitotoxicity and excess generation of NO are believed to be fundamental mechanisms in many acute and chronic neurodegenerative disorders. Cytotoxicity is thought to be the result of massive NO formation that is now established to initiate apoptosis. Disturbance of Ca^{2+} homeostasis and protein nitrosylation are key features in such conditions.

Apoptotic cell death as a result of inducible NOS activation comprises upregulation of the tumor suppressor p53, activation of caspases, chromatin condensation, and DNA fragmentation. The involvement of NO was established by blocking adverse effects by NOS inhibition. As the wide variety of NO 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 are not only cytotoxic but may also be cell protective. NO signaling during protection from apoptosis is in part understood by the requirement of gene transcription and protein synthesis. NO formation upregulates protective proteins such as heat shock proteins, cyclooxygenase-2, or heme oxygenase-1 which, in a cell specific manner may attenuate apoptotic cell death. NO shares with other reactive molecules, such as tumor necrosis factor-alpha, the unique ability to initiate and to block apoptosis, depending on multiple variables that are being elucidated. The cross talk between cell destructive and protective signaling pathways, and their activation or inhibition under the modulatory influence of NO, will determine the role of NO in apoptotic cell death [47].

Recently, a family of proteases collectively known as caspases has been implicated as a common executor of a variety of death signals. Tenneti et al. [49], Li et al. [50] and Haendeler et al. [48] have shown that each member of the caspase family, which participates in cytokine maturation and in apoptotic signal transduc-

tion and execution mechanisms, contains a critical cysteine residue in its active site and that S-nitrosylation of caspases on this particular cysteine residue decreases enzyme activity and is associated with protection from apoptosis. Their studies suggest that the cellular regulatory processes of NO to protect cells from apoptosis may be independent of their redox state and that low concentrations of NO inhibit the cellular suicide program in endothelial cells via S-nitrosylation of members of the caspase family.

Perspectives

The reversibility and the enzymatic control of RSNO dynamics is still largely unknown. In both prokaryotic and eukaryotic cells, uncharacterized enzymes are likely to detach NO from thiols [51, 52]. Reductases may be candidates for this SNOase activity, as thioredoxin and thioredoxin reductase cleave GSNO more rapidly than glutathione disulfide [53]. Interestingly, Hb catalyses the S-nitrosylation of its highly conserved Cysβ93 residue. Might heme-containing enzymes that support S-nitrosylation serve a similar role as protein kinases promoting phosphoryl group transfer in phosphorylation? The NO attached to a Hb thiol can be transferred to other substrate thiols such as glutathione [54]; a kinetic mechanism for nitrosylation-denitrosylation is therefore required [28].

Further work on redox modulation of proteins is required in order to better understand its physiological role(s). S-Nitrosylation as a new way of modulating proteins might have important implications for therapy, for example. Some RSNOs are already used therapeutically, especially GSNO. GSNO is used to inhibit platelet aggregation during coronary angioplasty [55] and to treat preeclampsia in pregnant women [56]. Its activity may be associated with its ability to inhibit platelet aggregation at dose levels that do not lower blood pressure, in contrast to other available NO donors. Further investigations on the biological activities of endogenous RSNOs and a better understanding of the processing and targeting of NO from the RSNO carriers [57] would probably open the possibility of developing tissue-specific NO donors targeted to a particular organ or cell type.

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