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Invited Review

Mechanisms Of Biological *S*-Nitrosation And Its Measurement

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Nitric oxide (NO) exhibits multiple biological actions through formation of various oxidized intermediates derived from NO. Among them, nitrosothiol adducts (RS-NOs) with the sulfhydryl moiety of proteins and amino acids appears to be an important species in view of its unique chemical reactivity. Understanding of the biologically relevant *S*-nitrosation mechanism is essential because RS-NOs seem to be critically involved in modulation of intracellular and intercellular signal transduction, including gene transcription, cell apoptosis, and oxidative stress. RS-NOs have been recently found to be formed efficiently via one-electron oxidation of NO catalyzed by ceruloplasmin, a major copper-containing protein in mammalian plasma. Ceruloplasmin is synthesized mainly by hepatocytes, but it is also expressed by other cells such as macrophages and astrocytes. Once RS-NOs are formed, they function as NO transporters in biological systems, the NO being transferred to different sulfhydryls of various biomolecules. This transfer may be mediated by transnitrosation reactions occurring chemically or enzymatically by a means of specific enzymes such as protein disulfide isomerase. The molecular mechanism of biological *S*-nitrosation is discussed as related to the important physiological and pathophysiological functions of RS-NOs. Also, RS-NO assays that are being successfully used for detection of biological *S*-nitrosation are briefly reviewed.

Keywords: nitric oxide; *S*-nitrosation; nitrosothiol; copper ion; transnitrosation; ceruloplasmin; peroxynitrite

INTRODUCTION

Many physiological events caused by nitric oxide (NO) are mediated through cyclic guanosine monophosphate (cGMP)-dependent pathways and involve NO-induced activation of soluble guanylate cyclase,[1–3] The formation of secondary nitrogen oxides that are generated from NO, leading to *S*-nitrosation and nitration of biomolecules, also appears to contribute to diverse physiological and pathophysiological phenomena through cGMP-independent pathways.[4–11]

NO-related oxidized intermediates may produce nitrosative additions to nucleophilic centers of biological molecules.[4,12–17] For example, sulfhydryl-containing molecules such as glutathione (GSH) are susceptible to *S*-nitrosation. Nitrosothiols (RS-NOs) thus formed seem to play an important role in NO-mediated signal-

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ing cascades, including down-regulation of *N*-methyl-D-aspartate (NMDA) receptors, production of nonadrenergic and noncholinergic responses, and modulation of gene transcription and apoptosis.[18–22] To obtain a better understanding of biologically relevant *S*-nitrosation, it is essential to identify and quantify the various RS-NOs generated in biological systems such as *S*-nitrosoglutathione (GS-NO) and protein-bound RS-NOs (e.g., *S*-nitrosoalbumin and *S*-nitrosohemoglobin). This article will highlight recent advances that may address the mechanism and biological implications of *S*-nitrosation caused by NO.

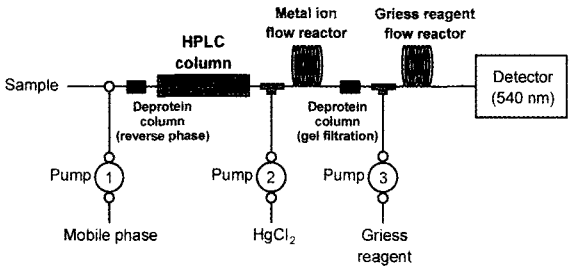

FIGURE 1 Flow diagram of the HPLC-based system using Hg^{2+}-Griess reagent flow reactor for RS-NO measurement. Various RS-NOs being eluted on the HPLC column are decomposed with Hg^{2+} and detected with Griess reagent after azo dye formation. Only *S*-nitrosated compounds give peaks in this system

DETECTION OF RS-NOs IN BIOLOGICAL SAMPLES

The traditional method for RS-NO quantification was first described by Saville.[23] It is based on the mercuric ion (Hg^{2+})-catalyzed release of NO^+ from RS-NO, followed by the Griess reagent assay to detect azo dye formation. However, Saville's method has serious limitations for determination of sensitivity and specificity of RS-NO determination in biological samples, because of the high background level of NO_2^- and various substances that interfere with Griess reagent reaction. To overcome these limitations, the original Saville method was modified by using high performance liquid chromatography (HPLC) coupled with a post-column flow reactor using Hg^{2+} and Griess reagent (Fig. 1).[24] This HPLC-based Hg^{2+}-Griess reagent assay is sufficiently sensitive and specific for RS-NO quantification in biological samples (detection limit: >5 nM); it can be also applied to measurement of protein-bound RS-NOs. Indeed, this method is useful for identifying the mechanism of *S*-nitrosation and characterizing *S*-nitrosated proteins, as described later in this article.

Although there are several methods for detection of RS-NOs described in the literature,[25,26] only a few can be successfully applied to quantitative measurement of RS-NOs in biological samples. Recently, chemiluminescence-based assays have been reported, and, most important, Moore's group successfully determined the total amount of RS-NOs in human plasma.[27] In their assay, RS-NO was decomposed in the reaction mixture of cuprous ion (Cu^+), iodine, and iodide to form NO, which was subsequently quantified by sensitive ozone-dependent chemiluminescence (detection limit: ≥5 nM). The normal level of RS-NO in the venous plasma of healthy human volunteers was thus estimated as 28 ± 7 nM. This seems quite reasonable, because we also found by using the above-mentioned HPLC-based assay a similar range of protein-bound RS-NOs in the plasma of a critically ill patient with severe pancreatitis and septicemia (unpublished data). The much higher values for RS-NOs in plasma reported by other groups are likely overestimations because of inaccurate RS-NO analysis with a lower specificity and/or artefactual RS-NO generation during sample processing.[28,29]

MECHANISM OF RS-NO FORMATION

NO is oxidized by molecular oxygen (O_2), and dinitrogen trioxide (N_2O_3), a strong nitrosating agent, is produced according to the following equations (eq. 1, 2):

$$2NO + O_2 \rightarrow 2NO_2 \cdots\cdots \text{eq. 1}$$

$$NO + NO_2 \rightarrow N_2O_3 \cdots\cdots \text{eq. 2}$$

Because NO *per se* does not react directly with sulfhydryl-containing compounds (RSH) to form RS-NO, metabolites formed during NO autooxidation such as N_2O_3 may be likely candidates for *S*-nitrosation.[12–15] However, the reaction (shown in eq. 1) of physiological NO concentrations (in the nanomolar to low micromolar range) with O_2 proceeds very slowly in solution at neutral pH under ambient conditions. Also, H_2O, which reacts with N_2O_3 to form HNO_2 in an aqueous milieu, competes in the reaction of N_2O_3 with RSH.[14] Therefore, *S*-nitrosation via this simple autooxidation of NO may not occur efficiently under physiological conditions, even if NO is produced in excess by inducible NO synthase (iNOS).

In contrast, peroxynitrite ($ONOO^-$), a potent nitrating and oxidizing agent formed by rapid reaction of NO and superoxide anion ($O_2^{\cdot-}$),[7–11] may contribute to *S*-nitrosation in biological systems.[30] This possibility is linked to many earlier studies showing that $ONOO^-$ induces activation of soluble guanylate cyclase, resulting in vasodilatation, inhibition of platelet aggregation and leukocyte adhesion, due to regeneration of NO from $ONOO^-$.[31–34] The mechanism of RS-NO formation mediated by $ONOO^-$ is, however, only poorly understood, because there are still inconsistent observations regarding thiol modification by $ONOO^-$ and NO regeneration from $ONOO^-$.[30,35–39]

It has been proposed that *S*-nitrosation is catalyzed by heavy metal ions through one-electron oxidation of NO. Vanin's group first reported the possible involvement of iron ion in RS-NO formation via production of a dinitrosyl iron-sulfur complex, which is often observed in cells and tissues expressing iNOS.[40] It was also suggested that hemoglobin may catalyze *S*-nitrosation; however, the detailed mechanism was not presented.[41] Although iron ion complexed with particular ligands in an NO redox reaction may contribute to *S*-nitrosation as suggested by Vanin, the efficiency and physiological relevance of these iron-dependent *S*-nitrosation reactions remain obscure.

However, two important recent reports indicated that at least free ferric ion (Fe^{3+}) is ineffective in *S*-nitrosation, and that cupric ion (Cu^{2+}) rather than iron is a potent catalyst in NO oxidation, producing NO^+or RS-NO in the presence of RSH according to the following reactions:[16,17]

$$Cu^{2+} + NO \rightarrow Cu^{+} + NO^{+} \cdots\cdots \text{eq. 3}$$

$$NO^{+} + RS^{\cdot} \rightarrow RS - NO \cdots\cdots \text{eq. 4}$$

Specifically, we found that Cu^{2+} is a potent catalyst of *S*-nitrosation for low molecular weight RSH such as GSH and *N*-acetyl-L-cysteine but that free $Cu^{2+}$is much less efficient than is the multicopper protein ceruloplasmin (CP) in cell culture systems.[16] In contrast, Stubauer et al. suggested from their cell-free in vitro assay that *S*-nitrosation catalyzed by Cu^{2+} occurs effectively only with sulfhydryls of proteins such as bovine serum albumin and hemoglobin, through direct reaction of NO with a stable Cu^{2+}-thiol complex formed in proteins without production of NO^+.[17] Their result is not entirely consistent with our finding, except that Cu^{2+} can be an important contributor in biological *S*-nitrosation. The *S*-nitrosation mechanism that we proposed for CP-dependent *S*-nitrosation is discussed in more detail below, with a focus on the unique catalytic action of CP as a multicopper oxidase.

CERULOPLASMIN FUNCTION AS AN NO OXIDASE, WITH FORMATION OF RS-NO

CP appears to efficiently catalyze one-electron oxidation of NO and thus functions as an NO oxidase. This multicopper enzyme, consisting of three domains, catalyzes one-electron oxidation of various elements and substances such as ferrous iron and organic amines.[42,43] Three different types of copper in CP, i.e., types 1, 2, and 3, are involved in electron transfer during such oxidation reactions.[44–49] The type 1 copper serves as an electron acceptor for substrates, and four

electrons are transferred from type 1 copper to the tricluster of types 2 and 3; O_2, as a final electron acceptor, is reduced to form H_2O. Therefore, similar to the ferroxidase and amine oxidase activity of CP, CP's NO oxidase activity, leading to efficient *S*-nitrosation, seems to occur via intramolecular electron transfer catalyzed by the multicopper components of CP, as shown schematically in Fig. 2. CP's activity in formation of RS-NO is remarkably attenuated when CP is treated with the potent oxidant $ONOO^-$,[16] which was previously demonstrated to destroy the copper ligands of CP, thus leading to Cu^{2+} release from the protein.[50] This result may also indicate that conformational integrity of the three types of copper in CP is of great importance for its *S*-nitrosation reaction.

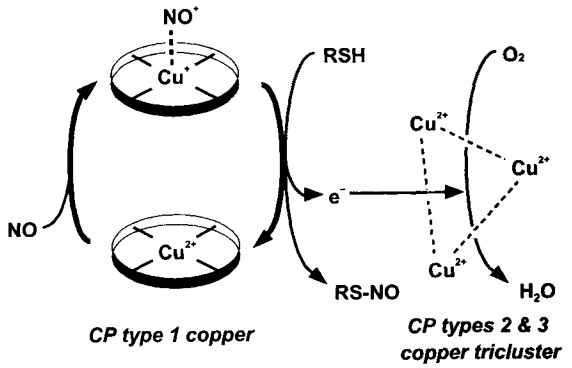

FIGURE 2 A possible reaction scheme for CP-catalyzed RS-NO formation

It has been known for a long time that reduced heavy metals such as Cu^+ decompose RS-NO to release free NO,[51–53] which is a reverse reaction of Cu^{2+}-catalyzed *S*-nitrosation. RS-NO readily decays in the presence of Cu^+; this decay is potentiated by use of reducing agents such as ascorbate and sulfhydryl-containing compounds. In fact, as described above, Moore's group used the Cu^+-dependent RS-NO decomposition in a chemiluminescence-based RS-NO assay, and they successfully quantified RS-NO content in biological samples.[27] CP-mediated RS-NO decomposition, however, occurs much less efficiently than does decomposition caused by free Cu^{2+} (or Cu^+). RS-NO synthesis thus predominates over its decay, possibly because of the relatively limited accessibility and reactivity of three types of liganded copper ions in CP.[16]

CP, which is highly up-regulated at the transcriptional level, exists abundantly in plasma (2–3 μM), as an acute-phase reactant in various inflammatory conditions, in liver cirrhosis, and in acute myocardial ischemia.[54,55] It is synthesized mainly by hepatocytes and secreted into the plasma.[16] Other cells such as macrophages also produce CP.[56,57] Recent reports indicate that CP is expressed in the central nervous system,[58–60] suggesting a neuroprotective effect of this protein. In fact, the hereditary disorder aceruloplasminemia is reported to be associated with retinal degeneration and neurological deficit.[61–63] More important, the antioxidant property of CP is well documented, although its mechanism is still unclear.[64–66] For example, an elevated level of CP in the lungs of patients with adult respiratory distress syndrome is suggested to contribute to antioxidant defense against neutrophil-induced lung injury.[67] Thus, the catalytic activity of CP in *S*-nitrosation is of considerable interest, in that the neuroprotective and antioxidant activities of CP may be linked to its promotion of RS-NO formation. RS-NOs are also thought to have a beneficial effect on neuronal cell injury caused by oxidative stress and even on excitotoxicity via stimulation of the NMDA receptor.[6,18,68,69]

FORMATION OF PROTEIN-BOUND RS-NO

Copper ion has the potential to nitrosate not only low molecular weight sulfhydryl-containing compounds but also protein-bound sulfhydryl-containing compounds. CP-catalyzed *S*-nitrosation, however, occurs most effectively with low molecular weight compounds such as GSH; it was not notable with protein-bound sulfhy-

dryl-containing compounds. This low efficiency of CP-catalyzed *S*-nitrosoprotein formation appears to be due to poor accessibility of the copper ions of CP to the sulfhydryls of the protein to be nitrosated. It is believed that free copper ion is not available in biological systems.[70] For example, about 95% of total plasma copper is found in CP, the rest of the copper being attached to albumin or amino acids. Hence, it is assumed that the copper ion may not directly induce *S*-nitrosoproteins in vivo. Rather, protein nitrosation may be induced indirectly through transnitrosation from low molecular weight RS-NOs formed after CP-dependent *S*-nitrosation. The possible mechanism for protein *S*-nitrosation via transnitrosation is illustrated schematically in Fig. 3. Of importance here is a recent finding that a cell-surface protein disulfide isomerase catalyzes transnitrosation, which promotes access of extracellular RS-NO to the intracellular environment.[71]

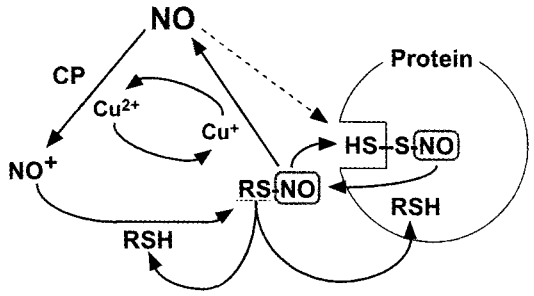

FIGURE 3 Schematic drawing of CP/Cu^{2+}-mediated *S*-nitrosation of low molecular weight thiol compounds (RSH). Low molecular weight RS-NO formed by CP/Cu^{2+}may transport NO to the protein sulfhydryl, leading to formation of *S*-nitrosated proteins. *S*-Nitrosation caused by NO via its autooxidation is indicated by dashed line. Transnitrosation occurring after CP-dependent *S*-nitrosation appears to be critically involved in steady-state formation of various RS-NOs in biological systems

It is intriguing that different efficiencies of *S*-nitrosation and transnitrosation are observed among various sulfhydryl-containing proteins. This difference is exemplified by much more efficient *S*-nitrosation of human α_1-protease inhibitor (α_1PI) than of albumin.[72,73] Our data show that α_1PI can be readily nitrosated by NO, not only via its autooxidation involving N_2O_3 formation but also via transnitrosation from low molecular weight RS-NO (GS-NO) (Fig. 4). Therefore, the efficiency and biological significance of protein *S*-nitrosation appear to depend largely on structural characteristics of the microenvironment surrounding the sulfhydryls of proteins, which may affect the accessibility of the nitrosative agents and the stability of the RS-NOs once generated.

α_1PI, also called α_1-antitrypsin, is the most abundant serine protease inhibitor found in human plasma.[74] Its physiological level ranges from 30 to 60 μM, which increases in the acute phase of many disorders including inflammatory diseases and infections.[75] Because α_1PI is a major endogenous inhibitor of neutrophil elastase, it is believed to play an important role in preventing tissue injury during inflammation. Its protease inhibitory activity is, however, readily reversed by reactive oxygen species (such as HOCl and ·OH generated under oxidative stress) because of oxidation of Met^{35} in α_1PI to form methionine sulfoxide.[76] α_1PI (53 kDa) is a glycoprotein having a single cysteine residue (Cys^{232}).[77] Although the biochemical role of the single sulfhydryl remains to be clarified, an earlier report suggested that mixed disulfide formation with sulfhydryl-containing compounds such as GSH modulates binding to neutrophil elastase.[78]

It is also important that *S*-nitroso-α_1PI (*S*-NO-α_1PI) exhibited multiple functions depending on the biochemical and pharmacological actions of NO conferred by *S*-nitrosation.[72,73] For example, *S*-NO-α_1PI not only retained original inhibitory activity against a series of serine proteases, e.g., neutrophil elastase and plasmin, but also gained thiol protease inhibitory activity. Of various thiol proteases targeted by *S*-NO-α_1PI, caspases, which are intracellular thiol proteases, are of great interest. In particular, caspase-3, a key factor in

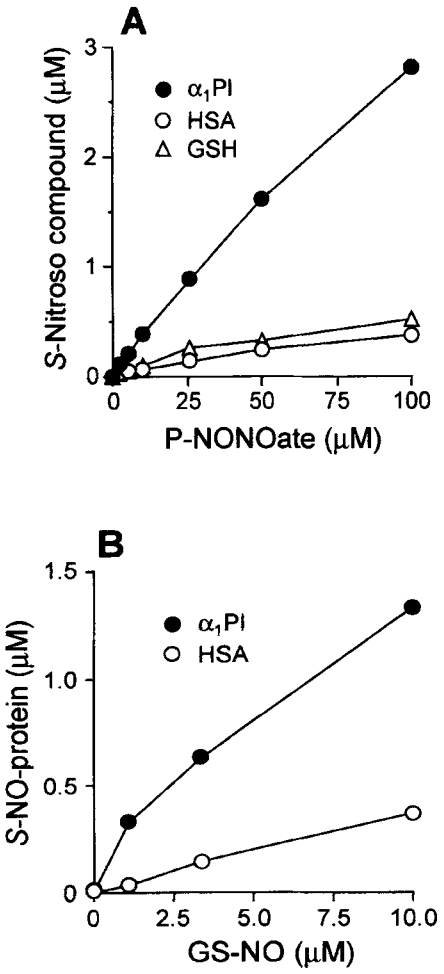

FIGURE 4 Different efficiencies of *S*-nitrosation by NO (autooxidation) (A) and transnitrosation (B) among various sulfhydryl-containing compounds. The sulfhydryl-containing proteins α_1PI (SH/molecule = 1.0) and human serum albumin (HSA; SH/molecule = 1.4) and GSH (10 μM proteinyl SH and GSH were used) were incubated with various concentrations of P-NONOate ($CH_3N[N(O)NO]^-(CH_2)_3NH_2^+CH_3$; $t_{1/2}$= 7.6 min) (A) or GS-NO (B) in chelex-treated 0.1 M potassium phosphate buffer (pH 7.4) for 30 min at 37°C. RS-NOs formed were quantified by the HPLC-based assay using Hg^{2+}-Griess reagent as described in Fig. 1. Data are mean values of duplicate experiments

cell apoptosis, was recently reported to be regulated by NO through *S*-nitrosation of cysteine at the active center of the enzyme.[20,22,79,80] Our preliminary data indicate that *S*-NO-α_1PI is the most potent caspase (apoptosis) inhibitor among a number of RS-NOs examined, including GS-NO, *S*-nitroso-*N*-acetyl penicillamine, and *S*-nitrosoalbumin (unpublished observation). *S*-NO-α_1PI also showed stronger antibacterial activity and a remarkable cytoprotective effect in hepatic ischemia-reperfusion injury in rats,[73,81] compared with other RS-NOs such as GSNO.[82–84] In the case of hepatic ischemia-reperfusion injury, *S*-NO-α_1PI seems to have protective functions via suppression of neutrophil infiltration, antiapoptotic effects, and induction of heme oxygenase-1.[71]These results suggest that not only *S*-nitrosation of α_1PI but also transfer of its nitroso moiety into the cells occur more efficiently compared with other RS-NOs, leading to the remarkable biochemical and pharmacological actions of *S*-NO-α_1PI.

CONCLUDING REMARKS

In the past decade, there has been a growing awareness of the biological effects of NO in the diverse fields of life science research. Improved understanding of the chemistry of NO has been gained during this time and is accelerating further, changing the cutting edge of NO research. From recent evidence, it is apparent that *S*-nitrosation has profound implications for many aspects of NO-related biological phenomena. This article briefly reviews the mechanisms of *S*-nitrosation that have been proposed, as well as RS-NO assays that have been introduced, which were successfully applied to quantification of RS-NO generated in biological systems. Although the physiological concentrations of RS-NO seem to be much lower (in the low nanomolar range) than those reported earlier, there is a potent catalytic pathway for in vivo RS-NO biosynthesis. Cu^{2+} or CP-dependent *S*-nitrosation is potentially of great interest, because CP is expressed by a wide range of cells and tissues and has various beneficial effects in preventing oxidative stress. Our ever-increasing

understanding of the complex redox reactions of NO will provide important clues to revealing the mechanisms of the diverse physiological and pathophysiological functions of NO.

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