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Membrane Transfer of S-nitrosothiols

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Abstract

The distinctive function of nitric oxide (NO) in biology is to transmit cellular signals through membranes and regulate cellular functions in adjacent cells. NO conveys signals as a second messenger from a cell where NO is generated to contiguous cells in two ways; one is as gaseous molecule by free diffusion resulting in an activation of soluble guanylate cyclase (NO/cGMP pathway), and another form is by binding with a molecule such as cysteine or protein thiol through S-nitrosylation (SNO pathway). Both pathways transmit much of the biological influence of NO from cell where other messenger molecules but NO are confined, through the plasma membrane to the adjacent cells. Since SNO pathway cannot utilize free-diffusion mechanism to get through the membrane as the molecular size is significantly larger than NO molecule, it utilizes amino acid transporter to convey signals as a form of S-nitrosylated cysteine (CysNO). Although S-nitrosylated glutathione (GSNO) is the molecule which act as a determinant of the total S-nitrosothiol level in cell, transnitrosylation reaction from GSNO to CysNO is an initial requirement to pass through signal through the membrane. Thus, multiplexed combination of these steps and the regulatory factors involved in this system conform and modify the outcome from stimulus-response coupling via the SNO pathway.

Keywords

nitrosylation; SNO; transnitrosylation; signal transduction; transmembrane

1. Introduction

Physiological cellular regulation is achieved through a highly complicated signal transduction system with a myriad of signal elements that allow adaptation to various environmental changes in well-governed stimulus-response mechanisms. Extracellular stimuli transduce signals to cell through the plasma membrane, mainly via a receptor or a transporter. Signal conversion via a receptor complex on the plasma membrane is a principle way that tissue responses are formed and transduced. In response to stimuli, cells react and produce secondary signals that can be transferred to the adjacent cells, resulting in tissue responses. However, contiguous cells can transmit signals via alternative receptor

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independent pathways. This form of signal transduction is best exemplified by the discovery of the physiological role of nitric oxide (NO) as the endothelium-derived relaxing factor (EDRF). Nitric oxide can travel through the plasma membranes as a second messenger and hence transduce stimuli and initiate responses to adjacent cells. The discovery of intercellular signal transduction by NO provided an entirely new principle for signaling in biological systems.

Due to its redox nature, NO is capable of wide range of biologically relevant chemical reactions. Among the various types of NO-mediated reactions with biological materials, nitrosylation is the major form of protein modification under physiological conditions. Protein nitrosylation invariably results in a signal transduction event irrespective of the target protein. The classical example is soluble guanylate cyclase (sGC), a primary target in NO/cGMP pathway, where NO activates sGC in part by nitrosylation on iron in heme structure [1; 2; 3]. *S*-nitrosylation, the covalent attachment of NO to the sulfur moiety of cysteine, is a redox sensitive post-translational, reversible modification of protein in response to outer stimuli [4]. *S*-Nitrosylation reactions are highly specific in terms of target cysteine residues and are dynamically regulated both in synthesis and degradation. In many ways, *S*-nitrosylation can be considered as a prototype of redox-regulated post-translational modification in physiological systems[4; 5]. This article is going to focus on *S*-nitrosothiols (SNOs) in cellular signaling, in particular small molecular weight SNOs, with some recent reports published in the 6th International Conference on the Biology, Chemistry, and Therapeutic Applications of Nitric Oxide (NO2010).

2. Unique nature of *S*-nitrosylation in signaling

Rather than free diffusion of NO from a NO synthase (NOS), *S*-nitrosylation allows for the compartmentalization of the source of NO (NOS) and the site of nitrosylation (Cysteine). Examples include the *S*-nitrosylation of Dexras where CAPON scaffolds nNOS and Dexras [6; 7], or of cPLA2 α by iNOS, which requires COX-2 to form a complex of the three proteins [8]. In addition, this compartmentalization allows for localized NOS to target proteins within a particular organelle, such as the formation of *S*-nitroso-proteins in the Golgi apparatus [9].

Protein *S*-nitrosylation has been revealed as a ubiquitous mechanism for NO-dependent coordination of physiological signaling. Several chemical properties of nitrosothiols make *S*-nitrosylation an intriguing alternative mechanism for regulation in cellular signaling by NO. Nitric Oxide is freely diffusible, however, it is readily oxidized in biological systems and thus its functionality as a second messenger is limited by these consumption reactions. The formation of *S*-nitrosothiol (SNO) preserves NO's bioavailability by protecting the NO moiety from oxidative consumption, while also limiting its diffusibility. Thus SNOs can be considered to extend the functionality of NO on a temporal basis while restricting it spatially. In addition, one must consider that *S*-nitrosylation of a protein alters the signaling pathway by providing a new binding partner to the SNO-substrate resulting in a novel direction [10; 11; 12; 13; 14].

One mechanism of protein *S*-nitrosylation appears to be the transfer of a nitroso group from one SNO to a target thiol, referred to as transnitrosylation. This reaction is slow but reversible ($k = 1-100 \text{ M}^{-1}\text{S}^{-1}$) [6] and may often take place between a low-mass SNO (e.g. GSNO, CysNO) and a protein thiol or vice versa. Low-mass SNOs have been shown to have a crucial role in the regulation of protein function by NO [15; 16; 17; 18], and to be a critical determinant of total cellular SNO [18]. The major regulatory enzyme of GSNO metabolism is GSNO reductase (GSNOR, also known as ADH3), which has been shown to have a great

deal of influence on the outcomes of nitrosative-stress both in health and disease[18; 19; 20; 21].

3. GSNO formation in physiological condition and abnormal metabolism in disease

In cells, SNO levels under steady-state conditions are determined by the balance between the formation and the decomposition of SNO: *S*-nitrosylation and *S*-denitrosylation. As low-mass SNOs (e.g. GSNO and CysNO) are determinants of cellular protein SNO levels [18], one can deduce that these molecules are pivotal in SNO-signal transduction. Hence, dysregulation of the production and the consumption of low-mass SNO may alter protein-SNO resulting in a failure of cellular signal transduction. Potential mechanisms involved in the formation of SNO from NO source have been demonstrated in *in vitro* chemical reactions [6; 22; 23; 24; 25; 26; 27]. The multiple mechanisms of *S*-nitrosylation include direct reaction and subsequent oxidation, direct nitrosation, thiyl radical formation and radical/radical recombination, and metal-catalyzed electron transfer. Each of these reactions is favored by different local conditions, which may affect the propensity to occur at specific cysteine residues. Many of these reactions have either been proposed or shown to occur in the enzymatic formation of SNO *in vivo* [28; 29; 30; 31; 32; 33; 34]. Furthermore, all isoforms of NOS have been shown to contribute to cellular SNO formation [18; 35; 36; 37].

At the recent meeting, the 6th International Conference on the Biology, Chemistry, and Therapeutic Applications of Nitric Oxide (NO2010), a new model on GSNO production was presented as a direct action that takes place on the inducible NOS (iNOS) dimer complex [38]. *N*-nitrosylation (N-NO) on biopterin occurs at an active iNOS complex as a result of NO production, becomes a source of NO for transnitrosylation to glutathione (GSH) by coming in to close proximity via a GSH-binding site. Furthermore, the nitrosative chemistry favors NO transfer from N-NO to S-NO [32], implying that GSNO formation on iNOS occurs through N-NO formation on BH₄.

There are a number of proteins that have been reported to have denitrosylating activity including thioredoxin reductase. However, GSNOR [19] has been shown to be crucial to regulate the total protein-SNO levels in inflammation [18], further demonstrating the role of low mass SNOs in signal transduction. Moreover, linkages between GSNOR activity change and human disease have been revealed [18; 20; 39; 40; 41; 42; 43]. In the NO2010 meeting, a new linkage was disclosed between decreased GSNOR activity and hepatocellular carcinoma (HCC) [44]. An increase in the intracellular concentration of GSNO, in response to decreased GSNOR activity during inflammation, leads to *S*-nitrosylation of the DNA repair enzyme, AGT, resulting in the accumulation of damaged DNA and HCC.

4. S-nitrosylation in intercellular signaling

One of the most distinctive facets of NO signaling is the formation of an intercellular signaling pathway following direct entry into the cell. As NO is a gaseous molecule, it has been implied that NO can be generated in one cell, diffuse through the plasma membrane and into an adjacent cell and activate the cGMP pathway. This pathway is the classical form of NO-signaling; as such it defines certain criteria for direct NO signaling. Namely that it is controlled by the level of NO production (dependent upon NOS isoforms); it is limited in its range as NO is reactive with a wide range of biomolecules; and targeting is controlled by the concentration of guanylate cyclase and NO turnover.

S-nitrosylation provides an alternative to this classical pathway. Translation of NO bioactivity to this alternative pathway introduces some key principles by which NO

signaling is altered. Firstly there are a whole range of new target proteins including kinases and transcription factors; secondly, effects can be observed far from the source of NO production, providing the potential for NO to act as an endocrine rather than a paracrine signal; finally, the time-scale of signaling is now controlled by the enzymatic processes of nitrosylation and denitrosylation and thus is dependent upon the local concentration and activity of regulatory proteins.

There is interplay between these very different signaling pathways as NO can be derived from SNO and SNO can play a role in the regulation of guanylate cyclase. As a hybrid system to transduce NO signals the membrane bound form of protein disulfide isomerase (mPDI) has been implicated as a signal converter [45; 46]. In this system, extracellular GSNO delivers a NO moiety to the surface of plasma membrane, where mPDI releases NO via a *S*-denitrosylation reaction through disulfide exchange. In this way, NO bioactivity diffuses into the cell resulting in the activation of cGMP pathway. This mechanism suggests that SNO molecules can transduce NO bioactivity to cGMP pathway. However, the physiological importance of mPDI and this pathway is still in debate [47].

5. Regulatory machineries on intercellular SNO transduction

Low-mass SNOs, e.g. GSNO and CysNO, are potential signal transducers in the SNO-pathway. Following synthesis in a cell GSH can be exported through its transporter to the extracellular space [48]. GSNO can also be exported in this way and thus, following intracellular formation, it can be secreted to the extracellular space. Extracellular GSH is readily degraded by γ -glutamyl transpeptidase (γ -GT) on the plasma membrane of many cells. This enzyme removes the glutamate residue from GSH while concomitantly transporting the cysteinylglycine into the cell. GSNO is also a substrate for γ -GT and this has been shown to be an import mechanism for *S*-nitrosocysteinylglycine (CG-SNO) in both the kidney and Nucleus Tractus Solitarii [49] [50]. As there is no known GSH importer on mammalian cell membrane [48], it can be assumed that neither GSH nor GSNO can reenter. Therefore, it appears that GSNO is predominantly an outflow transport system, and the use of γ -GT as an import system would be energetically costly. Obviously it is necessary for SNOs to enter as well as exit cells for them to operate as a viable intercellular communication system. Importantly, there are many cases where exogenous (S)NO-treatment causes alterations on cellular functions [13; 15; 16; 17; 51]. These issues raise the question of how the SNO-signal traverses the plasma membrane?

That GSNO is distributed heterogeneously in biological systems has been empirically shown. For example, the extracellular concentration of GSNO in normal brain tissue is around 6 to 8 μ M [52]; however, it is hardly detectable in cytosolic extracts in normal tissue [53]. One of the principle determinants of the total amount of SNO on protein is the activity of GSNO reductase (ADH III) [18]. Certainly the presence of a catabolic cytosolic enzyme, GSNOR with NADH as cofactor, and the fact that GSNO does not freely cross the plasma membrane will contribute to the heterogeneous distribution of SNO. Although, it has been shown that extracellular administration of GSNO can lead to activation of intracellular SNO signaling [16; 17; 18]. In contrast, CysNO metabolism in physiological systems has not been clearly elucidated to date, although it has been shown experimentally that exogenous L-CysNO administration leads to relatively larger cellular responses than GSNO. Treatment with GSNO or CysNO results in different accumulation of intracellular of SNO demonstrating that different mechanisms are involved in the uptake of these low mass SNOs [54].

Intracellular SNO accumulation was measured over time following a GSNO/CysNO treatment (Materials and Methods are in supplemental material). As shown in figure 1,

cytosolic accumulation of SNO within HEK293 cells was observed with a peak at 30 min following an addition of GSNO at 100 μ M in the culture medium (D-MEM). However, in HBSS rather than D-MEM, HEK293 cells show little accumulation of SNO in the cytosol. As shown in figure 1B, 100 μ M CysNO on HEK293 cells in HBSS showed speedy and vast accumulation in cells (over a thousand times higher than a GSNO exposure). Furthermore, SNO accumulation following CysNO treatment was similar in both D-MEM and HBSS. This difference indicates that L-CysNO can rapidly transfer SNO moiety into cells and maintain the level of SNO relatively stable manner, but that GSNO uptake is dependent upon the growth medium; indicating a difference in the transport mechanisms. It has been reported that CysNO utilizes L-AT (L-type Amino acid transporter) on the plasma membrane to travel into cell [55; 56; 57; 58] but GSNO does not. Furthermore, L-ATs are competitively regulated the transport of other amino acids [56]. In this regard it is significant that in HBSS, an amino acid free medium, there was a greater transport of L-CysNO derived SNO into HEK293 cells than in D-MEM, where various amino acids are included. For GSNO treatment on HEK293 cells an opposite pattern of SNO accumulation is seen; a treatment with GSNO in HBSS induced little accumulation although GSNO in D-MEM induced a significant amount of SNO acquisition.

D-MEM contains a significant quantity of cystine, which can be reduced to cysteine following the uptake into cell by cystine reductase. Following reduction, spontaneous secretion to extracellular space will undoubtedly occur [54]. Therefore, cystine in the culture medium can be converted to cysteine and become a target for transnitrosylation from GSNO to form CysNO. Thus GSNO exposure to cells in D-MEM, but not in HBSS, could transfer SNO efficiently into cell via the formation of CysNO. Alternatively, since γ -GT catalyzes GSNO to *S*-nitrosocysteinyglycine (CG-SNO), CG-SNO may transfer NO to cysteine to form CysNO. However, the lack of transport of SNO moieties to the intracellular space from GSNO in HBSS suggests that γ -GT cannot operate as a direct importer in HEK293 cells. The above pathways suggest that SNO accumulation in cell by an exogenous SNO-treatment is regulated by the following three factors: 1. CysNO transporter (L-ATs); 2. Amino acids contents in the culture medium; 3. Turn-over rate from cystine to cysteine by cystine reductase in cell (GSNO case). As a clarification of these effects on intracellular SNO accumulation, cysteine (300 μ M) was added into HBSS with GSNO to determine whether the addition increased the level of SNO accumulation following GSNO treatment. As shown in figure 2A, an addition of cysteine in HBSS dramatically increased the SNO level similar to that obtained by a GSNO treatment in D-MEM.

In figure 1 following an extracellular addition of GSNO/CysNO, intracellular SNO accumulation kinetics display a sharp increase followed by gradual decrease. The intracellular accumulation of SNO is dependent upon the balance between influx and decomposition rate of SNO. Therefore, this biphasic kinetic suggests the limiting factor in SNO accumulation is the influx rate through the transporter. L-AT is a transporter for neutral amino acids, the driving force to move these amino acids is the gradient between extracellular and intracellular concentration of the amino acids (e.g. CysNO). Since the enzymatic activities for SNO decomposition in cell (e.g. GSNOR) are almost constant, one can conclude that the influx rate is gradually slowed down over time (presumably by the lowering of the CysNO concentration gradient). Indeed, the concentration of CysNO in culture medium over cells decreases at a significantly faster rate than in the same culture medium without cells (Figure 2C). As an addition of cysteine to HBSS helps GSNO to transfer SNO into cell (Figure 2A), GSNO consumption was measured in HBSS over cells to determine this dependency upon transport. There was no change on GSNO concentration in HBSS medium even in the presence of HEK293 cells; however, cysteine addition clearly initiates consumption of GSNO from the medium (Figure 2D).

These data demonstrate that CysNO is the predominant form of SNO to enter HEK293 cells and that the primary mechanism of transport was likely the L-AT. In the case of GSNO, cysteine or cystine works as a carrier for SNO moiety into cells. These observations are in consistent with the previous reports [47; 54].

6. Future direction

Signal transduction in the cell requires several steps to achieve spatial and temporal regulation; initiation, such as strength, duration, direction, and specificity [4; 59]. Intercellular signal transduction mediated by a gaseous messenger molecule, NO, is a distinctive pathway where the messenger molecule itself travels through the border of cell by a free diffusion mechanism to convey stimulus-coupled signal to the adjacent cells. NO increases cGMP through the activation of sGC, namely NO/cGMP pathway. Since this pathway utilizes the free-diffusion mechanism, it is difficult to envisage how signal transduction by this pathway is regulated in terms of direction enforcement and duration control. Free diffusion driven by a concentration gradient utilizes no specific pathway to cross the membrane, thus it has little force to drive NO (signal) towards a specific direction. Moreover, as NO is prone to oxidation during transduction, it tends to lose biological activity as a messenger molecule.

On the contrary, the SNO pathway depends on low-mass SNOs and their specific transporters enforce the signaling direction. As SNO is a relatively stable molecule resistant to oxidation, the signaling function of SNO is predicted to last longer than NO gas. In addition, there are specific enzymes for SNO decomposition with specific localizations in the cell, such as GSNO reductase, thioredoxin in the cytosol, γ -GT on the plasma membrane. These enzymes regulate low-mass SNO and SNO-protein at the site and on the route during signal transduction. GSNO needs cysteine to signal NO through the plasma membrane as GSNO can be exported from cell but cannot enter due to mono-directional transport across the plasma membrane. In this case, cysteine and cysteinylglycine, a metabolite of GSNO by γ -GT, would be able to act as vehicles to transport SNO equivalents via L-ATs following transnitrosylation from GSNO. Furthermore, cystine can contribute as it can be recycled to cysteine by intracellular cystine reductase and the resulting cysteine is secreted to extracellular space to help GSNO-signaling. Thus, SNO signaling pathway consists of at least GSNO, CysNO, *S*-nitrosocysteinylglycine, L-ATs, cystine, and metabolizing enzymes such as GSNOR, γ -GT, thioredoxin, thioredoxin reductase, cystine reductase (Figure 3).

The physiological importance of the SNO pathway and its regulation have been revealed from studies in various diseases [43], such as septic shock [18], bronchial asthmas [20], neural development [60], myocardial infarction [39], liver carcinogenesis [44]. Although dysregulations of the SNO pathway have so far been limited to intracellular reactions, a role for corrupted intercellular signaling by SNO have also emerged, such as in the intestinal mucosal barrier within Crohn's disease [61]. GSNO is a potent inducer of mucosal barrier function in the intestine through increased expression of perijunctional F-actin and tight-junction-associated proteins. Lack of GSNO production from enteric glia cells causes intestinal barrier dysfunction and inflammation. Thus, SNO moieties originating from a glial cell transduce a signal to the intestinal mucosa to maintain mucous membrane integrity. This cascade can be blocked at any point ranging from NOS activity in the glial cell to the induction of F-actin and tight-junction-associated proteins in the mucosa. These intercellular SNO pathways represent novel targets to understand the pathophysiological roles of SNO and how disruption of SNO biology can interfere with physiological cascades.

The SNO pathway starts from NO production, leading to low-mass SNO formation and secretion, migration to contiguous cells, uptake into cells, and finishing with transnitrosylation of target proteins. As SNO has a greater stability compared to NO gas, and as there is directional transport of SNO moieties from one cell to adjacent cells, the SNO pathway has many advantages for intercellular signaling. It allows for target specificity, and for spatial and temporal regulatory mechanisms. Further understanding of this pathway will allow for the identification of a novel therapeutic targets and for a greater understanding of the role of NO in pathophysiology.

Materials and methods

Materials and Reagents

All materials were from Sigma-Aldrich unless otherwise indicated. HBSS supplemented with 12.6 mM Calcium and 10.0 mM Magnesium were purchased from Wako Pure Chemical (Osaka, Japan). CysNO and GSNO were synthesized as described [53] and used immediately.

Cell Culture

All of the cells were cultured at 37 °C in a 5% CO₂, humidified atmosphere. Cells were grown in Dulbecco's modified Eagle's medium (D-MEM High Glucose Sigma D6429) with 10% fetal bovine serum (HyClone), 100 units/ml penicillin, and 100 µg/ml streptomycin from Nacalai Tesque (Kyoto, Japan).

Detection of Protein-SNOs with Cu(I)/Cys Chemiluminescence method

Protein-SNOs were measured essentially as described [62], with minor modifications. Following a treatment in a 6-cm dish, cells were washed three times with ice-cold PBS(−) and the soluble contents were released from cells into hypotonic buffer (20 mM HEPES pH 7.6, 10 mM NaCl, 1.5 mM MgCl₂, without detergents and chelators) on ice for 5 min in the dark. Following a centrifugation at 18 kG for 10 min at 4 °C, supernatant was injected into the purge vessel contained 10 ml of reaction buffer (100 µM CuCl, 1 mM Cysteine in PBS(−) pH 7.4) where the temperature was maintained at 50 °C. Released NO gas in the purge vessel was carried out by pure helium gas flow into chemiluminescent detector (CLD88, EcoMedics, Switzerland). The detector signals were digitized and recorded for further analyses. Data was analyzed by PowerChrom software, normalized to a GSNO standard curve sequentially obtained from a series of experiment. With this system, NO₂, NO₃ up to 100 µM did not produce signals.

List of abbreviations

NO	Nitric oxide
NOS	Nitric oxide synthase
SNO	S-nitrosothiol
GSH	Glutathione, reduced
GSNO	S-nitrosylated glutathione
GSNOR	GSNO reductase
γ-GT	γ-glutamyl transpeptidase
Cys	Cysteine
CysNO	S-nitroso cysteine

Trx	Thioredoxin
TrxR	Thioredoxin Reductase
NADH	Nicotinaide adenine dinucleotide, reduced
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
D-MEM	Dulbecco's Modified Eagle Medium
HBSS	Hank's Balanced Salt Solution

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Figure 1.

A: HEK293 cells in a 6-cm dish were treated with 100 μ M GSNO in 4 ml of either D-MEM (without serum, filled circle) or HBSS (Hank's Balanced Salt Solution, empty circle). Total amount of SNO was measured in the cell lysate, and showed in value normalized by protein used ($n = 4\sim 7$ at each time point). GSNO-treated cells in HBSS showed little or no accumulation of SNO, although D-MEM-treated cells accumulated SNO with a peak at 30 min following a GSNO treatment. **B:** HEK293 cells in a 6-cm dish were treated with 100 μ M CysNO in 4 ml of either D-MEM (without serum, filled square) or HBSS (Hank's Balanced Salt Solution, filled circle). Total amount of SNO was measured in the cell lysate, and showed in value normalized by protein used ($n = 2\sim 3$ at each time point). A CysNO treatment increased SNO-level in cells at least 4 times higher than GSNO in D-MEM. Furthermore, in HBSS the total SNO level increased around 35 times higher than GSNO-treatment.

Figure 2.

A: HEK293 cells in a 6-cm dish were treated with 100 μ M GSNO. In HBSS, a GSNO treatment gave little accumulation of SNO (empty circle). An addition of L-cysteine at 0.3 mM in HBSS augments SNO level following a GSNO treatment (filled circle). This level is similar to the treatment with GSNO in D-MEM (filled square).

B: HEK293 cells in a 6-cm dish were treated with either GSNO, CysNO, or PROLI-NONOate at 100 μ M in 4 ml D-MEM (without serum). Total amount of SNO in cell lysate was measured at 15 min after the incubation (n = 2~6).

C, D: Changes of SNO-level in culture medium following a treatment with GSNO/CysNO. HEK293 cells in a 6-cm dish were treated with either 50 μ M CysNO (**C**) or 50 μ M GSNO (**D**) in HBSS (4 ml/dish). An addition of L-Cysteine in HBSS (filled square) increased GSNO consumption rate in medium with cells, compare to HBSS (empty square). However, L-Cysteine supplementation in HBSS decreased CysNO consumption with cells. (n = 3~5).

Figure 3.

Exogenous NO influences cellular functions through two pathways. NO-pathway mainly changes cGMP levels; however, SNO-pathway changes SNO levels. CysNO directly enter cell through an amino-acid transporter (LAT), but GSNO doesn't. However, GSNO may utilize cysteine to transduce SNO-signal into cells. SNO-pathways depend on the coordination with channels and intracellular metabolizing enzymes, besides the strength of extracellular SNO.