Nitrogen Monoxide (NO) Storage and Transport by Dinitrosyl-Dithiol-Iron Complexes: Long-lived NO That Is Trafficked by Interacting Proteins

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Nitrogen monoxide (NO) markedly affects intracellular iron metabolism, and recent studies have shown that molecules traditionally involved in drug resistance, namely GST and MRP1 (multidrug resistance-associated protein 1), are critical molecular players in this process. This is mediated by interaction of these proteins with dinitrosyl-dithiol-iron complexes (Watts, R. N., Hawkins, C., Ponka, P., and Richardson, D. R. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 7670–7675; Lok, H. C., Suryo Rahmanto, Y., Hawkins, C. L., Kalinowski, D. S., Morrow, C. S., Townsend, A. J., Ponka, P., and Richardson, D. R. (2012) J. Biol. Chem. 287, 607–618). These complexes are bioavailable, have a markedly longer half-life compared with free NO, and form in cells after an interaction between iron, NO, and glutathione. The generation of dinitrosyl-dithiol-iron complexes acts as a common currency for NO transport and storage by MRP1 and GST P1-1, respectively. Understanding the biological trafficking mechanisms involved in the metabolism of NO is vital for elucidating its many roles in cellular signaling and cytotoxicity and for development of new therapeutic targets.

Nitric Oxide Mediates Many of Its Effects by Binding to Iron

Virtually all fields of biochemistry and physiology have been influenced by the discovery of nitrogen monoxide (NO) (1). NO is a small, unstable, potentially toxic, diatomic molecule that is produced by many mammalian cells (2, 3). It is well known that NO has a physiological role as a short-lived messenger and has two major functions in cells: regulation and cytotoxicity (2, 3). NO has a physiological role as a short-lived messenger and has been produced by many mammalian cells (2, 3). It is well known that NO is a small, unstable, potentially toxic, diatomic molecule that is produced by many mammalian cells (2, 3). It is well known that NO is a small, unstable, potentially toxic, diatomic molecule that is produced by many mammalian cells (2, 3).

Iron, an Obligate Requirement for Life

To understand the interaction of NO with iron, a brief overview of the basic mechanisms involved is important. Iron is crucial for the activity of many enzymes (2, 3). Extracellular iron is transported by transferrin (Tf) and is internalized after binding to Tf receptor 1 (TfR1) by receptor-mediated endocytosis (Fig. 1A) (2, 3). Ferric iron is released from Tf within the endosome after its acidification and is then reduced by an endosomal ferrireductase, e.g. STEAP3 (six-transmembrane epithelial antigen of the prostate 3) (11–13). This is then followed by transport of Fe^{2+} across the endosomal membrane by DMT1 (divalent metal transporter 1) (3). This nascent cytosolic iron then becomes part of the labile iron pool (LIP) that is utilized for metabolism or stored in ferritin (Fig. 1A) (2, 3). The nature of the LIP remains unclear and was thought to be composed of low-\(M_1\) complexes (14). However, work using reticulocytes demonstrated that this low-\(M_1\) iron was not an intermediate but instead had kinetic characteristics of an end product (15). These studies suggest that iron exiting the endosome may be bound to chaperone proteins and/or is transported by direct interactions between organelles (15). Other studies suggested a specific iron-delivering interaction of the Tf-containing endo-
Effect of NO on Cellular Iron Metabolism

Because of its high affinity for iron and rich coordination chemistry, NO has been shown to form complexes with a variety of important iron-containing proteins such as ribonucleotide reductase (22), [Fe-S]-containing proteins such as ferrochelatase (23), heme-containing proteins (24, 25), and ferritin (26). In fact, ferritin has been suggested to act as a store of NO (26).

NO strongly activates the RNA-binding activity of IRP1 and IRP2 and has a marked effect on iron metabolism (2, 3, 27, 28). In fact, the effect of NO on IRP1 RNA-binding activity occurs via two possible mechanisms: 1) a direct effect on the [4Fe-4S] cluster of the aconitase form of IRP1, leading to its disassembly, and/or 2) iron mobilization from cells, leading to iron depletion (27–30). The effect of NO on iron regulatory protein RNA-binding activity increases TfR1 expression and results in a slight increase in iron uptake from Tf (27). The discordance between the ability of NO to markedly increase TfR1 expression but only slightly increase iron uptake from Tf could be related to the ability of NO to reduce ATP synthesis, which is vital for iron uptake (31, 32).

The importance of cellular iron depletion by NO has recently been confirmed by Hickok et al. (33), who showed that NO suppresses tumor cell migration via up-regulation of the iron-regulated metastasis suppressor molecule, NDRG1 (N-Myc downstream regulated gene-1). NDRG1 is a cytosolic protein (34, 35) that is strongly up-regulated by cellular iron depletion via hypoxia-inducible factor-1α-dependent and -independent pathways (34–36). The effect of NO on the up-regulation of NDRG1 probably occurs through its ability to deplete the LIP with the subsequent mobilization of iron (28, 37–39). The importance of this iron pool in NO activity has been confirmed by studies showing that it provides iron for dinitrosyl-dithioliron complex (DNIC) generation (40).

Nitric Oxide-Iron Interactions: A Possible Mechanism by Which Activated Mφs Inhibit Tumor Target Cell Proliferation

The nonspecific effector component mediating resistance to tumor cells is activated Mφs (41). Mφ activation occurs after infection with mycobacteria and stimuli such as LPS and cytokines (42), which result in the synthesis of tumor necrosis factor–α (43) and NO (8).

Many of the effects of NO result from its tenacious binding to iron (3, 27, 28). Intracellular iron release via NO generated by Mφs mediates, at least in part, their cytotoxic effector activity against tumor cells (4). NO induces a loss of aconitase activity, complexes I and II in Mφs and co-cultured tumor target cells, and the formation of DNICs in both cell types (44). These species can be readily detected by EPR spectroscopy with a signal of g = 2.04 (45). Vanin (46) showed that they have the formula Fe(RS)2(NO)2, i.e. a DNIC. Physiologically relevant examples of DNICs include the dinitrosyl-diglutathionyl-iron complex (DNDCIC) and the dinitrosyl-dicysteinyl-iron complex (DNDCIC) (Fig. 1B). However, these mononuclear DNICs may not be the only species present and responsible for physiological/pathological reactions. In fact, a recent report showed that NO reacts with [2Fe-2S] clusters, forming dinuclear complexes (47).

Identification of DNIC formation in tumor cells links Mφ-induced inhibition of iron-containing enzymes with NO biosyn-
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thesis. As NO mediates tumor killing via its interaction with iron (2–4), it is important to reveal the nature of these interactions.

DNICs Are the Major Cellular Form of NO

DNICs are formed rapidly in cells upon exposure to NO, which binds iron from the intracellular LIP (40, 48). Later during this process, NO mobilizes iron from other sources, and this coincides with increasing cytotoxicity (48). It has been shown that DNICs are the largest NO-derived adduct in cells, greatly exceeding the production of S-nitrosothiols (48), which are another NO-stabilizing and signaling species (1).

The similarity of EPR spectra obtained at 77 K and at ambient temperature indicates that most cellular DNICs have a high 

\[ M \]

suggesting that they are bound to macromolecules (38, 40, 49).

Although low-

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DNICs such as DNDGICs and DNDCICs are likely to exist in cells in equilibrium with protein-bound complexes. In fact, low-

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DNICs could be a mobile NO-transporting component between proteins (50, 51). The role of DNICs in the storage and transport of NO is discussed below.

Role of DNICs in Cytotoxicity

Although the cytotoxic effects of NO are well known, the role of DNICs in this process is unclear. DNICs induce apoptosis in Jurkat cells even when the anti-apoptotic molecule Bcl-2 is overexpressed (52). However, recent studies by Vanin and co-workers (53) demonstrated that DNICs (derived from GSH or cysteine) did not show any apoptotic activity in HeLa cells, in contrast to the pro-apoptotic effects of S-nitrosoglutathione (GSNO). These authors concluded that DNICs were bound to membrane proteins, as protein-bound DNICs had been shown to limit the reaction of NO with superoxide and the formation of toxic peroxynitrite (54).

Other studies also do not agree the toxicity of DNICs, where the pro-apoptotic effect of NO on Mφs could be eliminated via its incorporation into DNICs (55). To add to the controversy, Pedersen et al. (56) showed that GSTs sequester toxic DNICs in cells. This property is important, as DNICs may cause irreversible inhibition of glutathione reductase (57, 58) and other biomolecules.

Collectively, it can be suggested that, at lower concentrations, DNICs may function as NO carriers and release it (59, 60), which can occur by spontaneous decomposition of DNICs in vitro (61), to acceptors with higher affinity for NO (e.g., the heme center of sGC). In fact, for DNICs to be a storage form of NO would imply that NO is liberated and/or transferred at some point (49, 60, 62). Although yet to be directly demonstrated, this could assist NO-dependent signaling in vivo. However, the cytotoxic effects of DNICs may occur at higher concentrations, as may be expected under conditions of chronic inflammation, where the mechanisms involved in their storage (e.g., GSTs) could potentially be overwhelmed.

Model of d-Glucose-dependent NO-mediated Iron Mobilization

A number of studies have examined the effect of NO on cellular iron mobilization (3, 32, 38, 39, 63). From these investigations, a model of d-glucose-dependent NO-mediated iron mobilization from cells was proposed (Fig. 2A) (3). These experiments demonstrated that d-glucose must be transported into cells and then metabolized to enable NO-mediated iron mobilization (39). In fact, only metabolizable sugars, and not those that cannot be metabolized or transported across the cell membrane, could promote iron mobilization (39).

These investigations indicated that the metabolism of glucose to generate NADPH via the pentose phosphate pathway was required to generate GSH (L-γ-glutamyl-l-cysteinylglycine), which was critical for NO-mediated iron release (Fig. 2A) (3, 32, 38, 39, 63). GSH is a major antioxidant in cells (64) and exists in reduced (GSH) and oxidized (GSSG) forms (65).

![Figure 2](image_url)
Interestingly, a specific inhibitor of GSH synthesis, l-buthionine (SR)-sulfoximine (BSO) (66), prevented NO-mediated iron release, an effect reversed by reconstitution of GSH (39). Moreover, the process of NO-mediated iron mobilization was shown to be dependent on ATP generation (39). However, the dependence on GSH for NO-mediated iron release was not observed when assessing the mobilization of iron via synthetic chelators (39). Hence, these two processes may be mediated by different mechanisms.

Considering these results in the context of the form of iron released by NO, it is known that NO enters cells, where it interacts with protein-bound iron or iron en route to ferritin (Fig. 2A) (3, 39). The high affinity of NO for iron results in a NO-iron complex, and GSH could act as a ligand to complete the coordination shell, forming a DNIC (Fig. 2A) (3). Studies were initiated to examine the mechanism responsible (38).

Ubiquitous GSH Transporter MRPI Mediates NO-mediated Iron and GSH Release

Considering that NO-mediated iron efflux from cells requires GSH (39), it was hypothesized that a DNIC composed of iron, GSH, and NO was released (3, 63). An active process was indicated due to the requirement for glucose metabolism, ATP, GSH, and an intact cell membrane (39, 63). Previous studies have described the role of MRPI (multidrug resistance-associated protein 1; ABCC1) in the transport of GSH conjugates (67, 68) and particularly complexes of antimony and arsenic (67, 68). Considering this together with the fact that MRPI is ubiquitously expressed (69) and requires ATP (70), this transporter was a candidate for exporting DNDGICs.

MRPI is a member of the ATP-binding cassette transporter superfamily (71, 72). It uses ATP hydrolysis to efflux various anticancer drugs and other organic anions, often as conjugates of GSH (67, 73).

Initial studies (38) examining the role of MRPI in NO-mediated iron release from cells investigated the MRPI-hyperexpressing human breast cancer cell line MCF7-VP and its relevant parental counterpart, MCF7-WT, which expresses only very low levels of MRPI (74). The MCF7-VP cell line demonstrated a 3–4-fold increase in NO-mediated $^{59}$Fe and GSH efflux compared with WT cells. Furthermore, the NO-mediated $^{59}$Fe and GSH efflux was prevented by the GSH-depleting agent BSO, which inhibits GSH synthesis (66). Classical MRPI inhibitors such as MK571, probenecid, and difloxacin inhibited NO-mediated $^{59}$Fe release (38). Moreover, EPR spectroscopy demonstrated that MRPI inhibitors increased the DNIC signal in cells after NO treatment, indicating inhibition of release of the complex from the cell via MRPI (38). More recent studies have shown that NO generated by several different sources, including transfection with iNOS or exposure to two exogenous NO generators (i.e. GSNO or spermine NONOate), leads to iron release from wild-type murine embryonic fibroblasts, but not from murine embryonic fibroblasts from MRPI knockout mice (37).

Considering the NO-mediated release of both iron and GSH from cells (38, 39), it is notable that GSH efflux is a key signal mediating apoptosis (75), and it is well known that iron efflux from cells using chelators results in antitumor activity (76). Hence, the dual action of NO resulting in iron and GSH mobilization could play a role in Mφ-mediated cytotoxicity against tumor cells.

It is notable that MRPI is responsible only for NO-mediated iron mobilization and does not play a role in iron release in the absence of NO. This was shown by studies in control cells (incubated without NO) in which BSO had no marked effect on iron mobilization (39, 77). In fact, in the absence of NO, Fpn1 mediates iron mobilization (19–21).

Potential Intermediary or Storage Role of DNICs by GST Enzymes

The GST enzymes form an integrated detoxification mechanism with MRPI that eliminates toxic exogenous (e.g. anticancer drugs) and endogenous agents as GSH conjugates (78). These enzymes catalyze the attack of GSH on compounds to form GSH conjugates. Human cytosolic GSTs are sorted into seven classes ($\alpha, \mu, \pi, \sigma, \theta, \omega,$ and $\xi$) (78).

Considering the coordinated role of GSTs and MRPI in detoxification (79–81) and that MRPI transports iron and GSH in a form consistent with DNICs (37, 38), it is of interest that the most abundant GST isoenzymes, $\alpha$ (GST A1-1), $\mu$ (GST M1-1), and $\pi$ (GST P1-1), bind DNDGICs with high affinity ($K_d = 10^{-9}$ to $10^{-10} \text{M}$) (82, 83). A crystal structure of the DNDGIC-GST P1-1 complex revealed that Tyr-7 in the active site of the enzyme coordinates via its phenolate group to iron, displacing one GSH ligand (82). Using EPR and bacterial cells, it was shown that binding of DNDGIC to human GST P1-1 was reversible, with the loss of the EPR signal occurring within 1 h (82). In tissue homogenates, the half-life ($t_{1/2}$) of the complex was markedly longer: 4.5 h for $\alpha$ and $\mu$ class GSTs and 8 h for GST $\pi$ (84).

Most studies examining DNIC-GST complexes have been performed on purified proteins or these proteins heterologously expressed in bacterial cells. In hepatocytes, DNDGIC binds to GST $\alpha$ (56), and it was hypothesized that NO removes iron from ferritin and Tf to form DNICs (56). However, NO does not directly remove Fe$^{3+}$ from Tf (31) or ferritin (63), and other sites of iron acquisition are likely (31, 63).

The interaction of DNDGICs with GSTs raises the question of their function and if there could be an interaction with MRPI considering the strongly associated role of these proteins in detoxification (79–81, 85). Of importance, studies in vitro showed that DNDGICs lead to inactivation of glutathione reductase (56). Thus, GSTs may act as a protective mechanism against high levels of DNICs. Alternatively or in combination with this latter function, GSTs may act as NO store and regulate the release of DNICs via MRPI.

Recent studies (37) have focused on the more abundant GST classes and have examined MCF7-VP cells (hyperexpressing MRPI) and MCF7-WT cells (expressing very low MRPI levels) stably transfected with vector alone or with GSTA1 (encoding GST A1-1), GSTM1 (encoding GST M1-1), or GSTP1 (encoding GST P1-1). MCF7-VP or MCF7-WT cells transfected with the empty vector alone (without a GST insert) do not highly express GST A1-1, GST M1-1, or GST P1-1. In contrast, cells transfected with GSTA1, GSTM1, and GSTP1 express very high...
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levels of these proteins and thus provide appropriate models for experimentation (37).

Only GST P1-1 Inhibits Cellular Iron Release from Cells Hyperexpressing MRP1

The GST-transfected cells and their relative vector-transfected controls described above were examined in terms of cellular $^{59}\text{Fe}$ mobilization in the presence and absence of NO (37). Intracellular iron pools were labeled by incubation with the physiological iron donor radiolabeled diferric Tf (viz. $[^{59}\text{Fe}_2]\text{Tf}$) (Fig. 1A). Reincubation of labeled cells with control medium alone did not result in any alteration in iron release when cells transfected with GST A1-1, GST M1-1, or GST P1-1 were compared with cells transfected with the vector alone (37). Reincubation of cells with GSNO led to a marked increase in iron release relative to those incubated with control medium. Importantly, only the GST P1-1-transfected cells showed a significant decrease in NO-mediated $^{59}\text{Fe}$ efflux relative to cells transfected with the empty vector control. A similar ability of GST P1-1 to reduce iron release was also observed in cells that were transfected with iNOS. Hence, NO provided extracellularly (e.g. as spermine NONOate) or intracellularly from iNOS led to similar results, with cells expressing GST P1-1 decreasing the release of cellular iron via MRP1 (37).

Assessment of the form of intracellular iron in NO-treated GST-transfected cell types using native (nondenaturing) fast pressure liquid chromatography demonstrated that an accumulation of iron occurred in fractions containing GST P1-1, but not GST A1-1 or GST M1-1 (37). Further studies using EPR spectroscopy demonstrated that, in cells expressing GST P1-1, but not GST A1-1 or GST M1-1, the DNIC signal was significantly more pronounced and was a different shape than in their vector-transfected counterparts (37). These observations are consistent with x-ray crystallography data demonstrating the different coordination sphere of the DNIC once bound to GST P1-1 (82). Thus, GST P1-1, but neither GST A1-1 nor GST M1-1, decreases iron release by MRP1 by binding DNICs and preventing their release from cells (Fig. 2B).

These latter results do not agree with those obtained by Pedersen et al. (56) using rat hepatocytes and liver homogenates, which suggested that DNICs bind to $\alpha$ class GSTs. The difference in results may be explained by the metabolic disparities between rat and human cells and/or the cell types studied (37, 56). It is notable that MCF7 cells naturally express low levels of GST P1-1, but not GST A1-1 or M1-1 (86). Thus, the biochemistry needed for the interaction between GST P1-1, MRP1, and DNICs may exist in these cells, whereas that for GST A1-1 or GST M1-1 may be absent.

Considering that the affinity of GST $\alpha$ for DNICs is very high and that it is present in hepatocytes at considerable concentrations (0.3 mM) (56), it could be that different cell types have different GST isoenzymes to protect against NO cytotoxicity. Hence, their roles in DNIC metabolism and interaction with MRP1 are important to investigate.

It is intriguing that although GST P1-1 is found in the cytosol, nucleus, and mitochondrion (87), it was recently identified to be associated with the intracellular surface of the plasma membrane (88). If this is validated, it could enable the DNIC bound to GST P1-1 to be brought into proximity to MRP1, allowing its efficient transport out of the cell.

Implications of MRP1-GST P1-1 Interaction in Understanding NO Biology

MRP1 is involved in the NO-mediated efflux of iron and GSH from cells in a form consistent with DNICs (Fig. 2B) (37, 38). Furthermore, GST P1-1 that binds DNICs could act as an intermediate form of NO for regulating biological processes, e.g. vascular tone (82). Considering this, it is of interest that GST polymorphisms correlate with preeclampsia, i.e. high blood pressure during pregnancy (89).

In addition to the significant role of diffusion, the ability of the cell to actively transport, store, and traffic NO augments the random process of diffusion-mediated NO delivery that is inefficient and non-targeted. Because GST enzymes and MRP1 form a well integrated system for removing a variety of toxic agents (79 – 81), these molecules could coordinate regulation of NO levels by binding and transporting DNICs (Fig. 2B) (37, 38). This has important consequences for NO signaling, NO-mediated apoptosis, and $M_b$-mediated cytotoxicity that is due, in part, to iron release from tumor targets.

Vital to the role of GST P1-1 and MRP1 in NO metabolism is the concept of DNICs as a useful currency for NO storage and transport. This is crucial due to the greater $t_{1/2}$ of NO when found complexed as a DNIC (33, 84, 90, 91). DNICs have been identified in tissues, sera, Mbs, and many cell types (44 – 46, 92). In addition, DNICs bind with GSTs to stabilize NO for hours ($t_{1/2} = 4.5 – 8$ h) (84), which exceeds the $t_{1/2}$ of “free NO,” i.e. 2 ms to 2 s (93). Moreover, DNICs appear to be transported across membranes to donate iron to cellular pools (91) and can nitrosylate targets (94, 95), illustrating their bioavailability.

Additional studies are needed to elucidate the regulation of three steps involved in NO metabolism and DNIC formation: 1) NO generation and the formation of DNICs (via iNOS), 2) NO storage and regulation (via GST P1-1), and 3) NO transport as DNICs (via MRP1). As increased intracellular iron reduces iNOS expression (96), we postulate a model of NO generation, storage, and transport that may be regulated by DNIC levels, and this requires validation.

Apart from DNICs, which form a bioavailable currency for NO, it is well known that $S$-nitrosylation also mediates stabilization and transport of NO. There is evidence that DNICs and $S$-nitrosylated molecules act to mediate the effects of NO and may coexist in equilibrium. In fact, it has been suggested that cellular DNICs also serve as a NO reservoir for protein $S$-nitrosothiol formation (97).

Possible Physiological Roles of DNICs and New Questions

The interaction between proteins that transport and store NO leads to crucial questions. For example, given DNICs are involved in the storage and transport of NO (37, 38), it would be worthy to investigate if other protein ligands bind DNICs. Moreover, it is relevant to discuss the potential biological functions of protein-bound DNICs. Indeed, although the role of NO in vasodilation is well established (98), there is evidence that DNICs also have an effect on blood pressure. Initially, the for-
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