Nitric Oxide Storage and Transport in Cells Are Mediated by Glutathione S-Transferase P1-1 and Multidrug Resistance Protein 1 via Dinitrosyl Iron Complexes*5

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Background: Nitrogen monoxide (NO) can target intracellular iron pools, leading to dinitrosyl iron complexes (DNICs).

Results: NO storage and transport are mediated by glutathione S-transferase P1-1 (GST P1-1) and multidrug resistance protein 1 (MRP1), respectively.

Conclusion: GST P1-1 and MRP1 form an integrated detoxification unit regulating storage and transport of DNICs.

Significance: These results have broad implications for understanding the transport, storage, and signaling roles of NO.

Nitrogen monoxide (NO) plays a role in the cytotoxic mechanisms of activated macrophages against tumor cells by inducing iron release. We showed that NO-mediated iron efflux from cells required glutathione (GSH) (Watts, R. N., and Richardson, D. R. (2001) J. Biol. Chem. 276, 4724–4732) and that the GSH-conjugate transporter, multidrug resistance-associated protein 1 (MRP1), mediates this release potentially as a dinitrosyl-dithiol iron complex (DNIC; Watts, R. N., Hawkins, C., Ponka, P., and Richardson, D. R. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 7670–7675). Recently, glutathione S-transferase P1-1 (GST P1-1) was shown to bind DNICs as dinitrosyl-dithiolated iron complexes. Considering this and that GSTs and MRP1 form an integrated detoxification unit with chemotherapeutics, we assessed whether these proteins coordinately regulate storage and transport of DNICs as long lived NO intermediates. Cells transfected with GSTP1 (but not GSTA1 or GSTM1) significantly decreased NO-mediated 59Fe release from cells. This NO-mediated 59Fe efflux and the effect of GST P1-1 on preventing this were observed with NO-generating agents and also in cells transfected with inducible nitric oxide synthase. Notably, 59Fe accumulated in cells within GST P1-1-containing fractions, indicating an alteration in intracellular 59Fe distribution. Furthermore, electron paramagnetic resonance studies showed that MCF7-VP cells transfected with GSTP1 contain significantly greater levels of a unique DNIC signal. These investigations indicate that GST P1-1 acts to sequester NO as DNICs, reducing their transport out of the cell by MRP1. Cell proliferation studies demonstrated the importance of the combined effect of GST P1-1 and MRP1 in protecting cells from the cytotoxic effects of NO. Thus, the DNIC storage function of GST P1-1 and ability of MRP1 to efflux DNICs are vital in protection against NO cytotoxicity.

Nitrogen monoxide (NO) is a short lived messenger molecule that plays multiple physiological roles (1). Many of these actions are mediated by NO binding iron in the heme group of soluble guanylate cyclase (1). The importance of iron in mediating the functions of NO is also apparent when examining its cytotoxic effects. The cytotoxic functions of NO are observed when it is produced in large amounts by activated macrophages (MØs)5 against pathogens and tumor cells (2). These effects are explained by the reactivity of NO with iron in iron-sulfur clusters, e.g. in the electron transport chain and other pools (1, 3). The high affinity of NO for Fe(II) results in the interaction of NO with iron-sulfur clusters in proteins, which leads to their degradation and the formation of dinitrosyl-dithiol iron complexes (DNICs) (4, 5). Landmark studies by Hibbs et al. (2) demonstrated that co-cultivation of tumor target cells with MØs results in the loss of cellular iron. Further, it is known that NO induces the formation of DNICs with the formula Fe(RS)2(NO)2 (6) in MØs and tumor cells (7, 8). These can be readily detected by electron paramagnetic resonance spectroscopy (EPR) and give a unique signal of g = 2.04 (8). Intriguingly, it has been recently suggested that DNICs are the most abundant cellular adducts after exposure to NO (9).

In previous studies, we examined the effect of NO on the iron metabolism of neoplastic cells and showed that it led to the

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The abbreviations used are: MØ, activated macrophage; BSO, L-buthionine-(S,R)-sulfoximine; DNIC, dinitrosyl-dithiol iron complex; GSNO, S-nitroso-glutathione; iNOS, inducible nitric oxide synthase; MEF, murine embryonic fibroblast; MRP1, multidrug resistance protein 1; SperNO, spermine NONOate; Tf, transferrin; VP, MCF7-VP cells; WT, MCF7-WT cells.

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Iron, GST P1-1, and Dinitrosyl Iron Complexes

efflux of intracellular iron (10, 11). Subsequently, we discovered that the glutathione (GSH) transporter, multidrug resistance protein 1 (MRP1), mediates NO-stimulated iron and GSH efflux from cells and that this process could be inhibited by l-buthionine-(S,R)-sulfoximine (BSO) (12), an inhibitor of GSH synthesis (13). Several recent investigations have suggested that DNICs such as dinitrosyl-diglutathionyl iron complexes can bind to glutathione S-transferase (GST) isoenzymes with high affinity (Kd = 10^-7 - 10^-10 M) (14, 15). In fact, a crystal structure of the DNIC-GST P1-1 complex (PDB ID: IZGN) revealed that Tyr-7 in the active site of the enzyme coordinates to iron in the DNIC, displacing one GSH ligand (14). Previous studies with toxic exogenous (e.g. anticancer drugs) and endogenous agents have shown that GST isoenzymes form an integrated detoxification unit with MRP1 that eliminates GSH conjugates (16, 17).

Considering the coordinated role of GSTs and MRP1 in detoxification processes (16-18) and our studies showing that GST is involved in NO-mediated iron and GSH efflux from cells (12), we have for the first time examined the hypothesis that GST isoenzymes form an integrated detoxification unit with MRP1 that eliminates GSH conjugates (16, 17).

**EXPERIMENTAL PROCEDURES**

**Tissue Culture**—Murine embryonic fibroblasts (MEFs) derived from MRP1 or GSTP1 wild-type or knock-out (KO) mice were gifts from Drs. P. Borst (NIK-IVL, Amsterdam, The Netherlands) and K. Tew (Medical University of South Carolina, Charleston, SC), respectively. MCF7-WT (WT) and MCF7-VP (VP) cells transfected with GSTA1, GSTM1, or GSTP1 or their empty vectors were described previously by us (16, 18). The HaCaT keratinocyte cell type was obtained from American Type Culture Collection (Manassas, VA).

**Protein Labeling**—Apo-transferrin (Apo-Tf, Sigma-Aldrich) was labeled with ⁵⁹Fe (PerkinElmer Life Sciences) to generate dimeric ⁵⁹Fe-Tf using established methods (12). ⁵⁹Fe was monitored using a γ-counter (PerkinElmer Life Sciences).

**Western Blot Analysis**—Western blot analysis was performed (19) using antibodies against GST A1-1, M1-1, and P1-1 (Calbiochem; 1:1000) and MRP1 (Alexis, San Diego, CA; 1:500).

**Efflux of ⁵⁹Fe, General Protocol**—Standard methods were used to examine the effect of NO and other agents on ⁵⁹Fe efflux from cells (10). Cells were labeled with ⁵⁹Fe-Tf ([Tf] = 0.75 μM) for 3 h at 37 °C. The cells were then washed four times on ice and reincubated at 37 °C in treatment medium as indicated. The supernatants and cell pellets were collected for ⁵⁹Fe measurement using the γ-counter above (10).

**Efflux of ⁵⁹Fe from MEFs**—Cells were prelabeled for 24 h with ⁵⁹Fe-Tf (0.75 μM), washed, and then reincubated for 30 min at 37 °C with medium containing the MRP1 inhibitor, MK571 (20 μM) (12). The cells were then reincubated for 6 h at 37 °C with MK571 (20 μM) in the presence or absence of the NO generator, S-nitroso-glutathione (GSNO; 0.5 mM). For concentration dependence studies, cells were prelabeled for 24 h at 37 °C with ⁵⁹Fe-Tf (0.75 μM), washed, and then incubated for 6 h at 37 °C with medium containing 0, 0.01, 0.05, 0.1, and 0.5 mM of the well characterized NO generators, GSNO or spermine NONOate (SperNO; both from Cayman Chemical, Ann Arbor, MI). As relevant negative controls, cells were also incubated with the same concentrations of the agents without the NO group, namely GSH and spermine (both from Sigma-Aldrich). Inducible Nitric Oxide Synthase (iNOS) Transient Transfection—The iNOS plasmid was a kind gift from Dr. Helen McCarthy (Queens University, Belfast, Ireland). MRP1 KO- and WT-MEFs or MCF7-VP cells stably transfected with GSTP1 or the empty vector were transiently transfected with the iNOS plasmid or this plasmid without the iNOS insert using Lipofectamine® 2000 (Invitrogen) using the manufacturer’s protocol. Then 24 h after transfection, cells were prelabeled for 24 h at 37 °C with ⁵⁹Fe-Tf (0.75 μM) and washed four times on ice, and the cells then reincubated for 24 h at 37 °C with control medium. Efflux of ⁵⁹Fe and nitrite (see below) was then determined.

**Determination of Nitrite Concentration**—Accumulation of nitrite in culture medium was used as an indicator of iNOS activity in cells. Nitrite concentration in the medium was measured using the Griess reagent via standard procedures (10, 11).

**siRNA Study**—siRNAs specific for GSTP1 (Ambion, Austin, TX) and the scrambled control siRNA (Ambion) were used for gene knockdown studies in HaCaT cells. Cells were transiently transfected with siRNA (100 nM) using Lipofectamine™ 2000 (Invitrogen). Then 72 h after transfection, the cells were assayed for ⁵⁹Fe efflux and GST P1-1 levels using Western blot.

**Determination of Intracellular ⁵⁹Fe Distribution**—Cells were incubated with ⁵⁹Fe-Tf (0.75 μM) and GSNO (0.5 mM) for 3 h at 37 °C. The cells were washed four times on ice and lysed. The lysates were centrifuged at 16,500 × g for 45 min at 4 °C, and the cytosolic fraction was analyzed to assess intracellular ⁵⁹Fe distribution by native fast protein liquid chromatography (FPLC) (19).

**Cell Proliferation**—Proliferation was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide assay and validated by viable cell counts (12).

**EPR Spectroscopy**—This technique was performed using a Bruker EMX spectrometer with 100-kHz modulation, as described (12).

**Statistics**—Results are expressed as mean ± S.D. (number of experiments). Data were compared using Student’s t test. Results were considered statistically significant when p < 0.05. Statistical analysis of EPR studies was done by repeated measures one-way analysis of variance with Newman-Keuls post hoc test.

**RESULTS**

MRP1 Mediates NO-induced ⁵⁹Fe Efflux from Cells—Our previous studies demonstrated that hyper-expression of MRP1 in VP cells led to a marked increase in NO-mediated efflux of ⁵⁹Fe and GSH when compared with their WT counterparts.
(12). Considering the potential for GST isoenzymes and MRP1 to form an integrated system for DNIC transport, further studies were performed to investigate the role of MRP1 in NO-mediated $^{59}$Fe transport using MEFs from wild-type mice (WT-MEFs, which express MRP1) and MRP1 knock-out mice (MRP1 KO-MEFs). Cells were labeled for 24 h at 37 °C with $^{59}$Fe-Tf (0.75 μM), washed on ice, and then incubated for 30 min at 37 °C with either GSNO (0.5 mM) or GSH (0.5 mM) with or without MK571 (20 μM) for 6 h at 37 °C. The inset shows MRP1 protein expression in WT- and MRP1 KO-MEFs (typical of 3 experiments). B and C, WT- and MRP1 KO-MEFs were labeled with $^{59}$Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and then incubated with control medium containing either GSNO (0.01, 0.05, 0.1, and 0.5 mM) (B) or SperNO (0.01, 0.05, 0.1, and 0.5 mM) (C) for 6 h at 37 °C. D, WT- and MRP1 KO-MEFs cells were transfected with either iNOS or the relevant empty vector, labeled with $^{59}$Fe-Tf as described for B and C, and then reincubated with control medium for 24 h at 37 °C. The activity of iNOS was assessed by nitrite accumulation in the incubation medium (see “Results”). Results are mean ± S.D. (3 experiments). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**FIGURE 1.** MRP1 mediates NO-stimulated $^{59}$Fe release. A, WT- and MRP1 KO-MEFs were labeled with $^{59}$Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and then incubated with control medium with or without MK571 (20 μM) for 30 min at 37 °C. Cells were then reincubated with control medium containing either GSNO (0.5 mM) or GSH (0.5 mM) with or without MK571 (20 μM) for 6 h at 37 °C. The inset shows MRP1 protein expression in WT- and MRP1 KO-MEFs (typical of 3 experiments). B and C, WT- and MRP1 KO-MEFs were labeled with $^{59}$Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and then incubated with control medium containing either GSNO (0.01, 0.05, 0.1, and 0.5 mM) (B) or SperNO (0.01, 0.05, 0.1, and 0.5 mM) (C) for 6 h at 37 °C. D, WT- and MRP1 KO-MEFs cells were transfected with either iNOS or the relevant empty vector, labeled with $^{59}$Fe-Tf as described for B and C, and then reincubated with control medium for 24 h at 37 °C. The activity of iNOS was assessed by nitrite accumulation in the incubation medium (see “Results”). Results are mean ± S.D. (3 experiments). *, p < 0.05; **, p < 0.01; ***, p < 0.001.
KO-MEFs relative to WT-MEFs. Two chemically different NO generators were compared to examine the possibility that the effect observed was only relevant for NO generated by GSNO.

In these experiments, cells were labeled with $^{59}$Fe-Tf (0.75 μM) for 24 h at 37 °C, washed, and then reincubated for 6 h at 37 °C in the presence of control medium or the NO generators (0.01–0.5 mm). As observed in Fig. 1A, NO-mediated $^{59}$Fe release was only identified in WT cells, there being a significant ($p < 0.05$) increase in $^{59}$Fe release at 0.05 mm with either agent relative to MRP1 KO-MEFs (Fig. 1, B and C). Efflux of $^{59}$Fe from WT-MEFs continued to significantly increase relative to MRP1 KO-MEFs as a function of concentration for both NO generators up to 0.5 mm, where the release reached 35–36% of the total cellular $^{59}$Fe. In contrast, GSNO or SperNO did not have any effect on $^{59}$Fe release from MRP1 KO-MEFs relative to the control medium (Fig. 1, B and C). Incubation with the relevant control agents without the NO group, namely GSH or spermine, led to no effect on $^{59}$Fe efflux relative to the control (data not shown), as observed in our previous studies (10–12). These results demonstrated that even very low concentrations of the NO donor markedly increased $^{59}$Fe efflux from cells that express MRP1 relative to those that do not. Furthermore, considering that GSNO is a physiologically relevant NO donor (21, 22) and that the nitrite levels generated by this agent (10) are similar to, or much less, than those identified in vivo (23), it is apparent the NO levels used herein are within the physiological range (1).

iNOS Only Increases $^{59}$Fe Release from Cells Expressing MRP1—Considering the NO-mediated $^{59}$Fe release from cells described above using exogenous NO-generating agents, the effect of intracellular NO generation via iNOS was then assessed. The MRP1 KO- and WT-MEFs were transfected with iNOS or the relevant empty vector, and the cells were prelabeled with $^{59}$Fe-Tf as described for Fig. 1, B and C, and reincubated in the presence of control medium for 24 h at 37 °C. Under these conditions, iNOS induced a significant ($p < 0.001$) increase in $^{59}$Fe release from WT-MEFs, whereas no significant ($p > 0.05$) alteration in $^{59}$Fe release from MRP1 KO-MEFs occurred relative to empty vector-transfected cells (Fig. 1D). Nitric oxide generation was monitored by the detection of nitrite, which was 8-fold greater in cells transfected with iNOS (8.0 ± 0.4 μM, n = 3) relative to those transfected with the empty vector (1.0 ± 0.1 μM, n = 3). Collectively, these data demonstrated the importance of MRP1 in NO-mediated $^{59}$Fe efflux.

MC7-F7-WT and MC7-F7-VP Cells Stably Transfected with GSTs as Models to Test Interaction between MRP1 and GSTs—To initially test the role of MRP1 and GSTs in NO and iron metabolism, a cell model that hyper-expressed one or both of these proteins was required. Based on our previous use of the VP and WT cell types to assess the role of MRP1 in NO-mediated $^{59}$Fe release (12), we used these two cell types stably transfected with three major classes of human cytosolic GST isoenzymes (α, μ, and π), namely: GST A1-1, GST M1-1, or GST P1-1 (Fig. 2) (16). In this study, these transfected cell lines are denoted as: VPα, VPμ, VPπ, WTα, WTμ, and WTπ, respectively. The vector used to transfect cells with GSTP1 (encoding GST P1-1) is denoted as “VP Vector,” whereas that used to transfect GSTA1 (encoding GST A1-1) or GSTM1 (encoding GST M1-1) is denoted as “VP Vector.” In all studies, the cell type transfected with the relevant empty vector was used as the control.

GST P1-1 Inhibits $^{59}$Fe Release from Cells Hyper-expressing MRP1—Although GSTs bind DNICs (14, 15), their role in iron and NO metabolism in intact mammalian cells has never been assessed, particularly in the context of their relationship with MRP1. Considering that GSTs bind DNICs (14, 15), MC7-F7-VP cells transfected with GSTA1, GSTM1, or GSTP1 (VPα, VPμ, or VPπ) and their relevant controls (VP Vector or VP Vector*) (Fig. 2) were examined in terms of cellular $^{59}$Fe mobilization with or without an NO generator (Fig. 3A).

Reincubation of the various $^{59}$Fe-labeled VP cell types with control media alone (without NO) did not result in any significant ($p > 0.05$) alteration in $^{59}$Fe release from all cell lines relative to the VP Vector or VP Vector* controls, with 5–8% of $^{59}$Fe being released (Fig. 3A). In contrast, GSNO (0.5 mM) induced a marked and significant ($p < 0.001$) increase in $^{59}$Fe release from all cell types relative to cells incubated with control media (Fig. 3A). The addition of GSNO had no effect on cell viability over 3 h as judged by phase contrast microscopy and Trypan blue staining, as shown in our previous investigations (11). However, VPπ cells showed a significant ($p < 0.01$) decrease in GSNO-mediated $^{59}$Fe efflux relative to VP Vector cells, whereas no effect on $^{59}$Fe efflux was observed with VPα or VPμ cells relative to the VP Vector* (Fig. 3A).

The efflux of $^{59}$Fe from VPπ cells in the presence of elevated GST P1-1 was then examined in time course studies with cells...
being labeled for 3 h at 37 °C with $^{59}$Fe-Tf (0.75 μM) followed by washing and reincubation with or without GSNO (0.5 mM) for 1–24 h (Fig. 3B). Again, VP $\pi$ cells significantly ($p < 0.05$) reduced GSNO-mediated $^{59}$Fe release between 2 and 24 h when compared with VP Vector cells (Fig. 3B). There was no significant effect of GST P1-1 on $^{59}$Fe release from VP $\pi$ cells incubated with control medium in the absence of exogenous NO for all incubation times relative to VP Vector cells (Fig. 3B). This indicates that the effect of GST P1-1 on cellular $^{59}$Fe release is restricted to NO-mediated iron efflux.

To examine whether the effect of GST P1-1 on $^{59}$Fe mobilization could potentially be due to decreased generalized membrane permeability of the VP $\pi$ cells relative to VP Vector cells, the cells were labeled with $^{59}$Fe-Tf (0.75 μM) for 3 h at 37 °C, washed, and then reincubated with the structurally distinct NO generators (GSNO or SperNO; 0.5 mM), their control compounds (GSH or spermine, respectively), or a variety of iron chelators at 25 μM (i.e. desferrioxamine, Exjade®, or di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT)) for 3 h at 37 °C. D, VP Vector and VP $\pi$ cells transfected with either iNOS or vector were labeled with $^{59}$Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and then incubated with control medium for 24 h at 37 °C. Activity of iNOS was assessed by nitrite accumulation in the incubation medium (see “Results”). E, HaCaT cells treated with either si-GSTP1 (siRNA) or scrambled siRNA (Scrambled) were labeled, washed, and treated as in A. Western analysis was then done to assess GST P1-1 expression (see inset that is typical of 3 experiments). Results are mean ± S.D. (3 experiments). *, $p < 0.05$; **, $p < 0.01$; $$$, $p < 0.001$. 

FIGURE 3. Only GST P1-1, but not GST A1-1 or M1-1, significantly inhibits NO-mediated $^{59}$Fe release from MRP1 hyper-expressing VP cells. A, cells were labeled with $^{59}$Fe-Tf (0.75 μM) for 3 h at 37 °C, washed, and then reincubated for 3 h at 37 °C with or without GSNO (0.5 mM) at 37 °C. B, cells were labeled and washed as in A and then reincubated with or without GSNO (0.5 mM) for up to 24 h at 37 °C. Significance values compare VP $\pi$ and VP Vector cells treated with GSNO at the relevant time points. C, cells were labeled and washed as in A and then reincubated with control medium or medium containing GSNO, SperNO, GSH, or spermine (Sper) (all at 0.5 mM) or the iron chelators desferrioxamine (DFO), Exjade®, or di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) (all at 25 μM) for 3 h at 37 °C. D, VP Vector and VP $\pi$ cells transfected with either iNOS or vector were labeled with $^{59}$Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and then incubated with control medium for 24 h at 37 °C. Activity of iNOS was assessed by nitrite accumulation in the incubation medium (see “Results”).
cells relative to VP Vector cells (Fig. 3C). This indicates that the effect of GST P1-1 at inhibiting $^{59}$Fe release was not due to a general difference in membrane permeability between the cell types, but was related to the interaction between NO and iron. Moreover, the inhibitory effect of GST P1-1 at preventing NO-mediated $^{59}$Fe mobilization could not be observed using diverse chelators that form iron complexes that differ greatly in structure from DNICS (24). This can be explained by the specificity of DNIC binding to the stereochmically defined site within GST P1-1 (14).

**GST P1-1 Expression in VP Cells Prevents $^{59}$Fe Efflux Mediated by Transient Transfection of iNOS—Considering that NO-mediated $^{59}$Fe efflux was only decreased in cells transfected with GST P1-1 (i.e. VP cells) after incubation with exogenous NO generators (Fig. 3A), studies then progressed to assess whether a similar effect could be observed when these cells were transfected with iNOS. In these experiments, VP and VP $\pi$ cells were transfected with iNOS. In these experiments, VP and VP $\pi$ cells were transfected with inOS or the empty vector alone, and the cells were then reincubated with medium alone for 24 h at 37 °C (Fig. 3D). Examining VP or VP $\pi$ control cells (not transfected with iNOS or its relative empty vector) or those transfected with the empty vector alone, there was no significant difference in $^{59}$Fe mobilization between these cell types, which was $\sim$7–11% of the total cell $^{59}$Fe. However, transfection of VP Vector cells with iNOS significantly ($p < 0.01$) increased $^{59}$Fe release to $29 \pm 2$% of the total relative to these cells transfected with the empty vector (Fig. 3D). In contrast, transfection of VP $\pi$ cells with iNOS led to $14 \pm 1$% of total cell $^{59}$Fe being released, which was significantly ($p < 0.05$) less than that observed when VP Vector cells were transfected with the same plasmid. Again, measurement of nitrite levels was used to monitor the generation of NO by iNOS. In these experiments, nitrite was present at levels 7-fold greater (7.0 ± 1.0 $\mu$M, n = 3) in cells transfected with iNOS relative to the empty vector (1.0 ± 0.1 $\mu$M, n = 3). In summary, the results in Fig. 3, A–D, indicate that GST P1-1 prevents $^{59}$Fe efflux mediated by exogenous NO donors or intracellular iNOS.

**Knockdown of GST P1-1 in HaCaT Keratinocytes Increases GSNO-mediated $^{59}$Fe Efflux—**To examine NO-mediated $^{59}$Fe release from cells expressing endogenous GST P1-1, HaCaT cells were used, which express high levels of GST P1-1 (25) and MRP1 (26). We transiently transfected siRNA specific for GSTP1 relative to a scrambled siRNA control to assess the effect of decreased expression of this protein on GSNO-mediated $^{59}$Fe release (Fig. 3E). A marked and significant ($p < 0.001$) decrease in GST P1-1 expression in siRNA-treated cells relative to control (scrambled siRNA) treated cells (Fig. 3E, inset) led to a significant ($p < 0.001$) increase in GSNO-mediated $^{59}$Fe mobilization (Fig. 3E). These results support the studies above showing that GST P1-1 prevents NO-mediated $^{59}$Fe efflux from cells.

**Non-denaturing FPLC Demonstrates $^{59}$Fe Accumulation in Cell Fractions Containing GST P1-1—**Collectively, the studies above demonstrated that GST P1-1 prevented $^{59}$Fe release from cells, and this was probably due to an intracellular accumulation of $^{59}$Fe as the DNIC-GST P1-1 complex (14). To examine this further, the intracellular distribution of $^{59}$Fe was assessed using non-denaturing FPLC (Fig. 4 and supplemental Fig. S1, A and B). VP $\pi$, VP $\alpha$, or VP $\mu$ cells and their relevant vector controls were labeled with $^{59}$Fe-Tf (0.75 $\mu$M) in the presence of GSNO (0.5 mM) for 3 h at 37 °C prior to analysis by FPLC. Significantly ($p < 0.001$) increased intracellular $^{59}$Fe was found in fractions 20–22 (Fig. 4, shaded area), which contained GST P1-1 in VP cells relative to VP Vector cells. This suggested that the altered cellular $^{59}$Fe distribution was due to the formation of a $^{59}$Fe-DNIC-GST P1-1 complex (14), and this conclusion was supported by the functional studies in Fig. 3.

**Intracellular $^{59}$Fe Distribution in VP Cells relative to VP Vector $^*$ cells was then assessed (supplemental Fig. S1, A and B).** The distribution of $^{59}$Fe in VP $\pi$ and VP $\mu$ cells was different from that found in VP $\pi$ cells, probably because these are different clones. However, although GST A1-1 and GST M1-1 were also detected by Western blot, and GST P1-1 expression was observed in fractions 20, 21, and 22 only. Results are typical of 3 experiments.

**FIGURE 4. Increased intracellular $^{59}$Fe accumulation is identified in a peak that contains GST P1-1 in VP $\pi$ cells relative to VP Vector cells after fractionation of the cytosol using non-denaturing FPLC.** VP cells (transfected with vector alone or GSTP1) were incubated with $^{59}$Fe-Tf (0.75 $\mu$M) for 3 h at 37 °C with GSNO (0.5 mM). The cells were then washed and lysed, and the cytosolic fraction was analyzed by FPLC. The fractions were then assessed by Western blot, and GST P1-1 expression was observed in fractions 20, 21, and 22 only. Results are typical of 3 experiments.

**EPR at 77 K Demonstrates Formation of a DNIC-GST P1-1 Complex in MCF7-WT Cells—**To further elucidate the altered $^{59}$Fe distribution in NO-treated VP $\pi$ cells observed in Fig. 4, EPR (7, 12) at 77 K was used to assess the formation of cellular DNICS after exposure to GSNO (0.5 mM) for 3 h at 37 °C. As a control, we first examined the EPR signal of a synthetically prepared (15) low $M_r$ DNIC, containing GSH (dinitrosyl-diglutathionyl iron complex), which gives the characteristic spectrum of this complex with $g = 2.04$ (15, 27) (Fig. 5A, panel i). Incubation of WT cells transfected with the empty vector alone (WT Vector) for 3 h at 37 °C with control medium gave no EPR signal (Fig. 5A, panel ii). In contrast, incubation of WT Vector cells with GSNO gave a clear EPR signal (Fig. 5A, panel iii) that was similar to the synthetic DNIC standard (Fig. 5A, panel i). Hence, these data indicate DNIC formation in cells incubated with an exogenous NO generator (7, 12). As expected, solutions of GSNO or GSH alone (0.5 mM) in the absence of cells gave no significant EPR signals (Fig. S2A, panels i and ii). Interestingly, in WT cells transfected with GSTP1 (WT $\pi$) and incubated with
GSNO, a different EPR line shape was observed (Fig. 5A, panel iv, circled area) when compared with WT Vector cells (Fig. 5A, panel iii). These results suggest changes in the coordination sphere of the complex due to the binding of different ligands (6, 12). Thus, these results are consistent with the formation of a chemically distinct complex in the presence of GST P1-1.

Over 3 experiments, there was no significant difference in the intensity of the EPR signal between WT Vector or WT π cells incubated with GSNO (Fig. 5A, panel v). In addition, no EPR signal was obtained when WT π cells were incubated with control medium alone (supplemental Fig. S2B, panel i).

**EPR at 77 K**

EPR at 77 K Shows That GST P1-1 Expression Increases Intensity of the EPR DNIC Signal in VP Cells—Studies then assessed EPR spectra of VP cells at 77 K (Fig. 5A, panels vi–viii) for comparison with the spectra of WT cells (Fig. 5A, panels ii–iv). As found in WT cells (Fig. 5A, panel ii), incubation of VP Vector
cells in control medium gave no EPR signal at 77 K (Fig. 5A, panel vi), and this was also observed with VP cells incubated in control medium (supplemental Fig. S2B, panel ii).

However, incubation of VP Vector cells with GSNO led to an EPR signal (Fig. 5A, panel vii) that was similar in line shape, but significantly less (p < 0.001) intense, than that in WT Vector cells (Fig. 5A, panel iii). The lower intensity EPR signal in the VP Vector cells relative to WT Vector cells incubated with GSNO could be explained by the hyper-expression of MRP1 in VP cells (Fig. 2). This leads to marked NO-induced iron and GSH efflux from the cell via MRP1 (12), and thus, a less intense DNIC EPR (Fig. 2). This leads to marked NO-induced iron and GSH efflux in the VP cells (Fig. 5A, panel iv), which shows that 59Fe accumulated in the GST P1-1-containing VP Vector cells relative to WT Vector cells incubated with GSNO (0.5 mM) for 3 h at 37 °C (Fig. 5A, panel iv). Furthermore, considering these results together with: (1) x-ray crystallography studies that showed that GST P1-1 forms a distinctive DNIC through ligation to Tyr-7 displacing one GSH ligand (14); 2) the significant increase in the EPR signal intensity at 77 K comparing VP cells transfected with GST P1-1 relative to the empty vector (Fig. 5A, panel v); and 3) FPLC experiments that directly demonstrated 59Fe accumulation in cell fractions containing GST P1-1 (Fig. 4), it can be concluded that GST P1-1 acted effectively to bind DNICS.

In further support of the results above, GSTP1 knock-out (GST P1-1 KO) MEFs displayed a marked (p < 0.001) decrease in the EPR-DNIC signal at 77 K (supplemental Fig. S2C, panel iv) relative to WT-MEFs (supplemental Fig. S2C, panel ii), confirming the role of GST P1-1 in DNIC binding (14). Clearly, a weak DNIC EPR signal was still observed in GSTP1 knock-out cells as other molecules apart from GST P1-1 (e.g. iron-sulfur cluster-containing proteins) are also known to form DNICS (1, 28, 29). Of relevance, WT-MEFs and GST P1-1 KO-MEFs incubated with control medium alone did not display a DNIC signal (supplemental Fig. S2C, panels i and ii).

Unlike GST P1-1, GST A1-1 and GST M1-1 Do Not Alter EPR Spectra—Unlike VP cells, no alteration in EPR line shape or a significant increase in signal intensity was found at 77 K when comparing VP Vector* cells (Fig. 5B, panel i) with VPVector (Fig. 5B, panel ii) or VPmu (Fig. 5B, panel iii) cells after incubation with GSNO (0.5 mM) for 3 h at 37 °C (Fig. 5B, panel iv). This supports our functional studies, where GST A1-1 or M1-1 had no significant effect on cellular 59Fe release (Fig. 3A), nor did these proteins bind intracellular 59Fe, as indicated by native FPLC (supplemental Fig. S1, A and B). As found for the VP Vector cell type incubated with control media (Fig. 5A, panel vi), VP Vector* cells treated in the same way gave no EPR signal, and this was also observed for VPα and VPμ cells incubated with control media (supplemental Fig. S2D, panels i–iii).

MRP1 Inhibitor, MK571, Increases EPR DNIC Signal in VP Vector Cells—The role of GST P1-1 in preventing NO-mediated 59Fe release via MRP1 (Fig. 3, A–D) was further examined using the MRP1 inhibitor, MK571 (12, 20). Preincubation of VP Vector cells with MK571 for 30 min prior to and during the 3 h at 37 °C incubation with GSNO (0.5 mM) led to a significant (p < 0.05) increase in the EPR signal intensity (cf. Fig. 6, panels i and ii), demonstrating cellular accumulation of DNICS, due to the inhibition of MRP1 transport activity. The addition of MK571 to VP cells incubated with GSNO also increased the signal relative to GSNO-treated VP Vector cells (cf. Fig. 6, panels iv and v), although this was not a significant increase (Fig. 6, panel iii). This lack of a marked increase in the signal may be attributed to the larger amount of 59Fe-NO already trapped within the cell by GST P1-1, saturating the compartment.
Effect of GSTs on Proliferation of MCF7-WT and -VP Cells in the Presence of NO—The relationship between GST P1-1 and MRPI in the storage and transport of DNICs may be part of a novel detoxification system, protecting cells from endogenous NO generated by nitric oxide synthase or from exogenous NO generated by MOs (2). Considering this, we assessed the effect of GSNO (0.02–10 mM) over 72 h on proliferation of WT and VP cells transfected with each GST isoform (Fig. 7A, panel i). Transfection of WT cells with GSTA1, GSTM1, or GSTP1 (WTα, WTμ, WTπ cells) had no significant effect on the IC50 value of GSNO relative to the WT Vector cells. This indicated that the hyper-expression of any of these GSTs alone does not protect cells from GSNO in the absence of high MRPI levels. However, all VP cell types were significantly (p < 0.001) more resistant to the cytotoxic effect of GSNO when compared with the WT cells (Fig. 7A, panel i), supporting a role for MRPI in the NO detoxification process. Transfection of VP cells with GSTA1 (VPα) or GSTM1 (VPμ) had no effect on the cytotoxic activity of GSNO, whereas transfection with GSTP1 (VPπ) led to a significant (p < 0.001) increase in the IC50, indicating greater resistance to GSNO (Fig. 7A, panel i). Together, these results indicate that the presence of both GST P1-1 and MRPI is necessary for maximum resistance to the cytotoxic effects of GSNO. Furthermore, it demonstrates the functional relationships of these two proteins in GSNO metabolism and reflects synergistic partnership roles in DNIC storage and transport. Hence, as found for cytotoxic chemotherapeutics (17, 18, 20), GST P1-1 and MRPI act together to suppress the cytotoxic activity of NO.

Throughout our studies, we used two chemically different NO donors (namely GSNO and SperNO; see Figs. 1, B and C, and 2C and supplemental Fig. S3) and both have demonstrated basically equivalent effects, namely, they induce NO-mediated cellular iron release via MRPI (Fig. 1, B and C); both have the same effect via GST P1 on preventing cellular iron release (Fig. 2C); and both NO generators lead to the same EPR spectra in cells (supplemental Fig. S3). Moreover, our studies with these NO generators have also been confirmed by transfecting cells with iNOS (Figs. 1D and 3D). Hence, in our system, SperNO and GSNO are acting in a similar way. In fact, we also showed that GST P1-1 hyper-expressing VP cells were more resistant than their empty vector-transfected VP counterparts to the effects of SperNO, as shown for GSNO (Fig. 7A, panel i).

Studies were then focused on the effect of the GSH inhibitor, BSO (Fig. 7A, panel ii); MRPI inhibitors MK571 and sulfinpyrazone (12, 20) (Fig. 7B, panels i and ii); and GSH inhibitors ethacrynic acid and dicumarol (20, 30) (Fig. 7C, panels i and ii) on the cytotoxic activity of GSNO in WT π and VP π cells. For all inhibitors, the most marked effects of sensitizing cells to the cytotoxic activity of NO were observed in cells hyper-expressing both MRPI and GST P1-1. This implies that disruption of the partnership between these proteins results in problems in handling and trafficking NO, leading to greater cytotoxic activity.

DISCUSSION

This study is the first to show that an integrated NO storage and transport mechanism exists within mammalian cells that utilizes a cooperative interaction between GST P1-1 and MRPI. The evidence for this mechanism includes the following: 1) GST P1-1, but not GST A1-1 or GST M1-1, acts to prevent NO-mediated 59Fe release from MRPI-hyper-expressing VP cells (Fig. 3, A–D); 2) Prevention of 59Fe efflux occurs via the binding of 59Fe to GST P1-1, probably as a DNIC (14), as shown by concomitant 59Fe accumulation in cellular fractions containing GST P1-1 (but not GST A1-1 or GST M1-1; Fig. 4 and supplemental Fig. S1, A and B); 3) Iron accumulation leads to a unique and much stronger DNIC EPR signal in VP cells transfected with GSTP1 (Fig. 5A, panel viii) relative to VP Vector cells (Fig. 5A, panel vii), but does not occur in those cells transfected with GSTA1 (Fig. 5B, panel ii) or GSTM1 (Fig. 5B, panel iii) relative to VP Vector* cells (Fig. 5B, panel i); 4) Cells expressing both MRPI and GST P1-1 are significantly more resistant to the cytotoxic effects of GSNO than cells expressing GST P1-1 or MRPI alone or cells expressing MRPI and other GST isoforms (Fig. 7A, panel i); and 5) For all MRPI and GST inhibitors (Fig. 7A, panel ii, and B and C), the most marked effects of sensitizing cells to GSNO were observed in cells hyper-expressing both MRPI and GST P1-1, demonstrating that disturbance of the relationship between these proteins
leads to greater NO-mediated cytotoxicity. Collectively, these data indicate a unique role of GST P1-1 in both NO and iron metabolism and also the cooperative relationship between GST P1-1 and MRP1.

We previously showed that GSH was essential for NO-mediated iron mobilization from cells (10) and that the GSH transporter, MRP1, was involved in NO-mediated efflux of iron and GSH from cells in a form consistent with DNICs (12). Further, GST enzymes bind DNICs with very high affinity and could act as a storage form of NO for regulating intracellular processes (14, 27). Most of these previous studies examining DNIC-GST complexes have been performed on purified proteins (14, 27).
and none of these have examined the cooperative role of GSTs and MRP1 on intracellular iron trafficking and release in mammalian cells. However, EPR studies by Pedersen et al. (27) using cultured rat hepatocytes and liver homogenates showed that DNICs bind to α class GSTs. This is inconsistent with our current work, where GST A1-1 hyper-expression did not influence NO-mediated 59Fe release (Fig. 3A), nor did it bind intracellular 59Fe (supplemental Fig. S1A). Further, GST A1-1 did not significantly increase or lead to a unique EPR signal relative to VP Vector* cells (Fig. 5B, panels i and ii). The discordance likely relates to the metabolic differences between rat and human cells and the cell types studied as GSTs demonstrate tissue-specific distributions and functions (27). Consistent with this idea, it is worth discussing that MCF7 cells naturally express GST P1-1 rather than GST A1-1 or M1-1 (31). Thus, the metabolic machinery necessary for the interaction between GST P1-1 and MRP1 and the handling of DNICs could be present in these cells, whereas that for GST A1-1 or M1-1 may not exist.

The GST enzymes have long been associated with the multi-drug resistance of tumor cells, and the cooperative relationship of MRP1 and GST P1-1 in drug resistance is well documented (16–18). In addition, it has been shown that GST expression is not always sufficient to confer protection from cellular toxins (20), and in some cell types, GSTs must be co-expressed with MRP1 to optimally protect cells from anticancer agents (16, 32). Until this investigation, the combined effect of these proteins on NO metabolism and its cytotoxicity were unknown. Our studies show that when exposed to NO, VP cells were more resistant than their respective WT cells (Fig. 7A, panel i). However, although there was no significant difference in cytotoxicity between the VP Vector* and the VP MRP1 + GST P1-1 (23), the VP MRP1 + GST P1-1 was 15% more resistant than their respective WT cells (Fig. 7B, panels i and ii). The discordance may relate to the metabolic differences between rat and human cells and the cell types studied as GSTs demonstrate tissue-specific distributions and functions (27). Consistent with this idea, it is worth discussing that MCF7 cells naturally express GST P1-1 rather than GST A1-1 or M1-1 (31). Thus, the metabolic machinery necessary for the interaction between GST P1-1 and MRP1 and the handling of DNICs could be present in these cells, whereas that for GST A1-1 or M1-1 may not exist.

Central to our hypothesis is the concept of DNICs as a common currency for storage and transport of NO (Fig. 8). In fact, DNICs are a natural storage form of NO that possesses a longer half-life than NO alone (15). In addition, DNICs associate with GST to stabilize NO for many hours (15), which markedly exceeds the half-life of “free NO” (2 ms–2 s) (33). Further, DNICs permeate cells to donate iron and trans-nitrosylate molecular targets, demonstrating their bioavailability (1, 34).

Previous studies in vitro showed that DNICs inactivate glutathione reductase and that GSTs can prevent this and act to protect cells (27). Our work corroborates and extends these findings and demonstrates that GST P1-1 acts as an NO store that regulates DNIC release via MRP1 (Fig. 8). In this way, MRP1 could act to efflux DNICs once the binding capacity of GST P1-1 for DNICs is exceeded, preventing DNIC cytotoxicity.

**REFERENCES**


**FIGURE 8. Schematic illustrating the respective roles of GST P1-1 and MRP1 in NO storage and transport.** NO can diffuse through the membrane or be actively transported into cells by protein disulfide isomerase (PDI) (37). Due to the ability of NO to act as a ligand, it can bind iron transported into cells and released from transferrin. GSH completes the NO-Fe complex to form a DNIC. DNICs can be bound by GST P1-1 or effluxed out of cells via MRP1.
Iron, GST P1-1, and Dinitrosyl Iron Complexes