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Review

The nitric oxide–iron interplay in mammalian cells: Transport and storage of dinitrosyl iron complexes

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Abstract

Nitrogen monoxide (NO) is a vital effector and messenger molecule that plays roles in a variety of biological processes. Many of the functions of NO are mediated by its high affinity for iron (Fe) in the active centres of proteins. Indeed, NO possesses a rich coordination chemistry with this metal and the formation of dinitrosyl–dithiolato–Fe complexes (DNICs) is well known to occur intracellularly. In mammals, NO produced by activated macrophages acts as a cytotoxic effector against tumour cells by binding and releasing cancer cell Fe that is vital for proliferation. Glucose metabolism and the subsequent generation of glutathione (GSH) are critical for NO-mediated Fe efflux and this process occurs by active transport. Our previous studies showed that GSH is required for Fe mobilisation from tumour cells and we hypothesized it was effluxed with Fe as a dinitrosyl–diglutathionyl–Fe complex (DNDGIC). It is well known that Fe and GSH release from cells induces apoptosis, a crucial property for a cytotoxic effector like NO. Furthermore, NO-mediated Fe release is mediated from cells expressing the GSH transporter, multi-drug resistance protein 1 (MRP1). Interestingly, the glutathione-*S*-transferase (GST) enzymes act to bind DNDGICs with high affinity and some members of the GST family act as storage intermediates for these complexes. Since the GST enzymes and MRP1 form a coordinated system for removing toxic substances from cells, it is possible to hypothesize these molecules regulate NO levels by binding and transporting DNDGICs.

Keywords: Nitrogen monoxide; Nitric oxide; Transferrin; Iron; Glutathione-*S*-transferase; Multi-drug resistance protein 1

1. General introduction: nitrogen monoxide is a vital messenger molecule and cytotoxic effector

Nitrogen monoxide (NO) is a versatile effector and messenger molecule that plays roles in a wide variety of biological processes in animals and plants [1,2]. It has been established that NO exists in a variety of redox states that leads to the molecule being able to affect a variety of molecular targets [3]. For instance, the reduced form of NO is known as either nitric oxide or the NO radical ($NO^{\bullet}$) and forms complexes with iron (Fe). Indeed, the high affinity of $NO^{\bullet}$ for Fe is a well known branch of coordination chemistry [3]. Many regulatory functions of $NO^{\bullet}$ are due to its ability to bind Fe within the haem prosthetic group of guanylate cyclase [2–4].

There are also other redox-related forms of NO that have different molecular targets [3,5]. The oxidized form of NO is known as the nitrosonium ion (NO^{+}) and reacts with sulphydryl groups leading to *S*-nitrosylation [3]. This modification is thought

Abbreviations: BSO, buthionine sulphoximine; CO, carbon monoxide; D-Glc, D-glucose; DMT1, divalent metal ion transporter 1; DNDGIC, dinitrosyl–diglutathionyl–Fe complex; DNIC, dinitrosyl–dithiolato–Fe complex; EPR, electron paramagnetic resonance spectroscopy; GSH, glutathione; GSNO, *S*-nitroso-glutathione; GST, glutathione-*S*-transferase; HFE, haemochromatosis gene product; IRE, iron-responsive element; IRP, iron-regulatory protein; MRP1, multi-drug resistance protein 1; $NO^{\bullet}$, nitric oxide; NO, nitrogen monoxide; NO^{+}, nitrosonium ion; NO^{-}, nitroxyl anion; PPP, pentose phosphate pathway; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; TCA, tricarboxylic acid cycle; Tf, transferrin; TfR1, transferrin receptor 1; UTR, untranslated region

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to act similarly to phosphorylation, leading to alterations in the activity of a range of proteins [3,5–9]. Another redox-related form of NO is the reduced congener, nitroxyl anion (NO^-) [3], that in contrast to $NO^\bullet$ favours Fe(III) in haem rather than Fe(II) and has been demonstrated to be endogenously generated [10]. Recent studies have shown that the NO^- donor, Angeli's salt ($Na_2N_2O_3$), increases force development in isolated rat cardiac muscle [11]. In addition, it resulted in coronary vasculature dilation through soluble guanylate cyclase-mediated calcitonin gene-related peptide release and K(ATP) channel activation [12,13]. Hence, NO^- could play an important role in regulating vascular tone and blood pressure.

The equilibrium between the various redox-related states of NO has been suggested to play a role in mediating the many biological functions of NO. Certainly, NO plays many roles in neurotransmission, smooth muscle relaxation, blood clotting, Fe metabolism and contributes to the cytotoxicity of activated macrophages against tumour cells and intracellular parasites [2,14–18]. The production of NO in living organisms is mediated *via* the nitric oxide synthase (NOS) family of enzymes that are flavo-haem proteins [1,19,20]. There are three well characterised NOS isoforms encoded by three different genes, namely neuronal, endothelial and the inducible isoforms of the enzyme [21]. In addition, a number of reports suggest the existence of a mitochondrial NOS [22,23].

In terms of the biological reactions of NO, the most important of these are with oxygen, superoxide and metal ions [3]. The half-life of uncomplexed NO is in the order of 2 ms–2 s [24] and it reacts rapidly with superoxide to form the cytotoxin, peroxynitrite ($ONOO^-$) [25]. While $ONOO^-$ has well known cytotoxic effects leading to generation of the hydroxyl radical [25], there is also evidence that it can *S*-nitrosylate crucial sulphydryl groups of the enzyme, glyceraldehyde dehydrogenase [26]. In addition to these latter reactions, the interaction of NO with intracellular Fe-containing proteins is vital for its many effector functions and these are discussed below.

2. Nitrogen monoxide forms intracellular complexes with Fe

Vanin and colleagues have demonstrated that $NO^\bullet$ forms intracellular complexes within cells which play important roles in biological processes [27–30]. Apart from the formation of haem–NO complexes in guanylate cyclase, it is well known that the effect of NO is mediated, at least in part, due to its interaction with Fe in a number of proteins, including: (i) the rate-limiting enzyme in DNA synthesis, ribonucleotide reductase [31]; (ii) the Fe storage protein, ferritin [32]; (iii) haem-containing proteins [33–35]; (iv) the [Fe–S] cluster enzyme ferrochelatase that catalyses the final step in the haem synthesis pathway [36]; (v) other [Fe–S] cluster proteins involved in energy metabolism such as mitochondrial aconitase and those in complex I and II of the electron transport chain [37,38]; and (vi) the [Fe–S] cluster-containing molecule, iron-regulatory protein-1 (IRP1), that plays a role in regulating Fe homeostasis [39–44].

The interaction of NO with Fe-containing proteins and its subsequent effects is demonstrated by the studies of John Hibbs and co-workers. These investigators demonstrated that when tumour cells are co-cultured with activated macrophages there is a decrease in DNA synthesis with a concomitant loss of 64% of tumour cell Fe over 24 h [3,4]. The Fe released was postulated to be derived from [Fe–S]-containing proteins and enzymes [15,37,45,46] and has been hypothesized to be a complex of NO and Fe with thiol-containing ligands, for example GSH or cysteine (Cys) [27,28,47]. In fact, a wide variety of studies using electron paramagnetic resonance (EPR) spectroscopy have demonstrated that NO forms dinitrosyl–dithiolato–Fe complexes (DNICs) in activated macrophages, tumour target cells and other tissues [28,45,46,48–51]. Most commonly, these coordination complexes exist in solution as the Fe atom ligated to two NO molecules, two thiols (*e.g.*, GSH) and the remaining two positions being filled by water [30].

In the current review, we will focus on the interaction of $NO^\bullet$ with intracellular Fe in the formation of DNICs that can be transported *via* the multi-drug resistance protein 1 (MRP1) and/or stored within the glutathione-*S*-transferase group of enzymes.

3. Cellular Fe metabolism

Before describing the effects of NO on cellular Fe pools, it is important to discuss the mechanisms involved in Fe metabolism. Below, we first describe the uptake of Fe from the gut followed by its subsequent transport in the blood, uptake by cells, intracellular metabolism and storage. This series of events represents the set of metabolic processes necessary for the assimilation of Fe by a mammalian organism and is vital to describe in order to understand the effect of NO on this metal ion.

Iron is fundamental for life as it is a cofactor of enzymes essential for ATP production and DNA synthesis (for reviews see [4,52,53]). The maintenance of mammalian Fe homeostasis begins with the ability to control dietary Fe uptake from the gut *via* enterocytes (for review see [54]). The absorption of dietary Fe requires its reduction and this was thought to be achieved by duodenal cytochrome *b* (Dcyt*b*) [55] (Fig. 1). However, more recent studies using Dcyt*b* knockout mice have demonstrated it is not essential for normal Fe metabolism [56]. Once Fe(III) is reduced to Fe(II), it is transported into the cell by the divalent metal ion transporter 1 (DMT1) [57–59]. Haem can also be transported into enterocytes and recently a candidate molecule that possesses this activity (haem carrier protein 1; HCP1) has been described [60]. However, HCP1 was subsequently demonstrated to be a folate transporter [61], questioning its role in haem uptake [62]. Intriguingly, another haem transporter known as the human feline leukemia virus subgroup C receptor (FLVCR) has also been identified [63].

Enterocyte Fe trafficking (Fig. 1) involves the function of hephaestin and ferroportin-1 [64]. This latter molecule is also known as metal transporter protein 1 (MTP1; [65]) or Ireg1 [66]. Hephaestin is a transmembrane, multi-copper ferroxidase with homology to serum ceruloplasmin [4]. By way of its ferroxidase activity, hephaestin facilitates Fe export from enterocytes, perhaps in cooperation with the basolateral Fe transporter, ferroportin-1 [67] (Fig. 1). Ferroportin-1 is down-regulated by the binding of the Fe-regulatory hormone, hepcidin [68] (see below), leading to decreased cellular Fe efflux [68]. However, Fe release does not

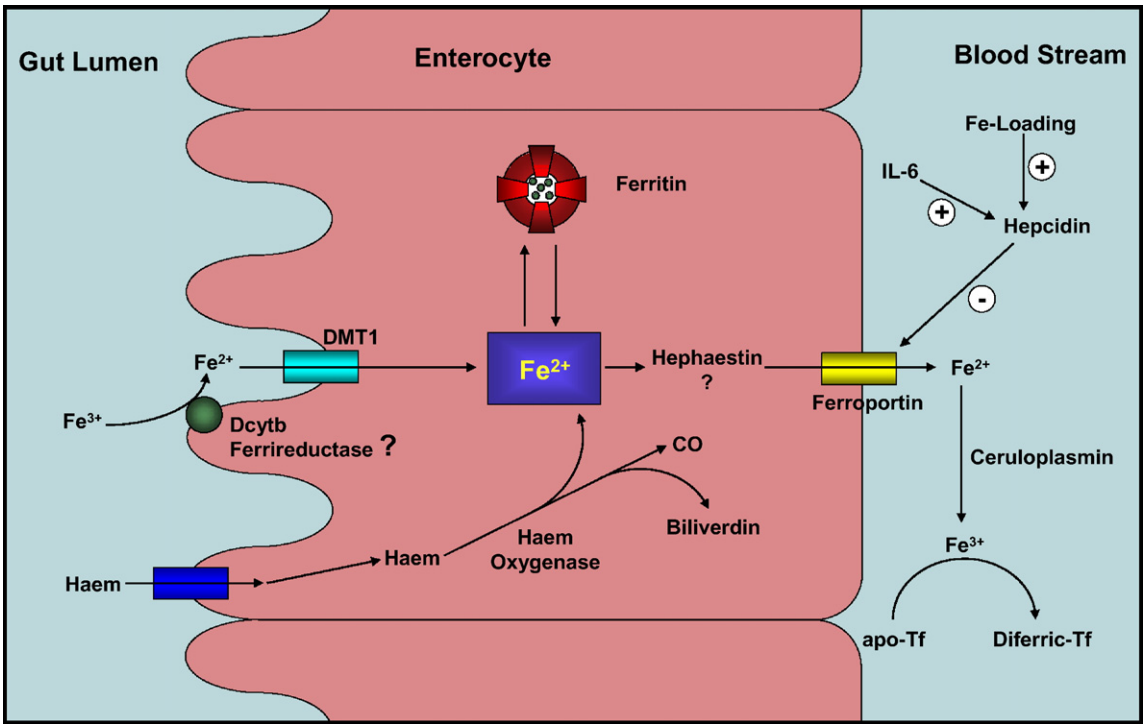

Fig. 1. Schematic illustration of the mechanism of Fe uptake by the enterocyte. Iron is internalized after inorganic Fe(III) in the diet is reduced to Fe(II) possibly by a ferrireductase known as Dcyt*b*. However, the identity of this ferrireductase remains controversial [38]. Haem may be taken up from the gut lumen *via* specific transporters *e.g.*, haem carrier protein 1 (HCP1) [60] and/or feline leukemia virus subgroup C receptor [63]. Internalized haem is then metabolised by haem oxygenase 1 to Fe(II), bilirubin and carbon monoxide (CO). The Fe(II) probably then enters a compartment known as the intracellular Fe pool or is stored in ferritin. In a sequence of events that remains unclear, the ferroxidase hephaestin may be involved in conversion of Fe(II) to Fe(III) and the subsequent release from the cell *via* ferroportin-1. The Fe efflux mediated by ferroportin-1 is thought to be as Fe(II) which may then be oxidized by the ferroxidase activity of ceruloplasmin or apo-transferrin in the serum. The Fe(III) is then subsequently bound by apo-Tf to form diferric Tf. The release of Fe from the enterocyte is regulated at least to some degree by the peptide, hepcidin, which is synthesized by the liver. High levels of storage Fe in the liver or the inflammatory cytokine, interleukin-6 (IL-6), increase hepcidin expression that decreases Fe release from the enterocyte, probably by down-regulation of ferroportin-1 (see text for details). Taken from: Richardson, D.R. (2005) Curr. Med. Chem. 12, 763–771.

only appear to be dependent upon these molecules. Indeed, ceruloplasmin also plays a role in Fe efflux [69]. Iron released is subsequently bound to the serum Fe-binding and transport protein, transferrin (Tf) (Fig. 1).

The absorption of Fe from the gut is under the control of DMT1 [57,58], hepcidin [70], hemojuvelin [71] and the hemochromatosis gene product (HFE) [72]. The expression of DMT1 at the apical surface of the enterocyte is controlled by the presence of an iron-responsive element (IRE) in the 3′-untranslated region (UTR) of its mRNA that is bound by the iron-regulatory proteins (IRPs), namely IRP1 and IRP2 [4,73]. High Fe levels result in low IRP-RNA-binding activity that prevents binding of IRPs to the IRE in the 3′-UTR of DMT1 [57,73]. This leads to reduced DMT1 mRNA stability and a decrease in its translation. The opposite occurs when Fe levels are low, leading to high DMT1 expression.

4. Iron transport, uptake and its intracellular metabolism and storage

Transferrin (Tf) binds two Fe(III) atoms and is bound by cells expressing the transferrin receptor 1 (TfR1; for reviews see [4,74]). The uptake of Fe from Tf is controlled by TfR1 expression which is modulated by intracellular Fe *via* IRP1 and IRP2 (for reviews see [4,73,74]). The interaction of Tf with the TfR1 is also regulated by HFE binding to this receptor [75].

Once Tf is bound to TfR1, the complex is internalized *via* receptor-mediated endocytosis [4,74]. After release of Fe(III) from Tf, it is reduced to the ferrous state by an endosomal ferrireductase. A candidate for this molecule is the six-transmembrane epithelial antigen of the prostate 3 (Steap3), [76]. Transfer of the so-formed Fe(II) through the endosomal membrane into the cell is then mediated by DMT1 [57–59].

After Fe release from Tf and its transport through the endosome, Fe(II) becomes part of the elusive intracellular Fe pool [77]. The nature of the labile Fe pool (LIP) remains unknown and is controversial. For instance, while Fe(II) or Fe(III) exists within cells [78], the mechanism of how Fe is transported within cells remains an unsolved question. Initially, Fe was suggested to be bound to low M_r Fe complexes [77], while other studies found no evidence of such intermediates [79,80]. While procedures exist for the measurement of an apparent "LIP" through the use of the fluorescent chelator, calcein or Phen Green SK [81,82], it is likely that this ligand like any other chelator will lead to marked alterations in the intracellular equilibrium of Fe. This will probably lead to the artificial creation of a LIP that is dependent on the Fe-binding properties of calcein or Phen Green SK. Considering this in conjunction with recent criticism of the method [83], it is

important that care be exercised when interpreting data generated from this technique. Recently, it has been suggested that there are siderophore-like molecules in human cells that can be bound by the acute phase protein, lipocalin-2 [84]. While this latter study is intriguing, no mammalian siderophore has ever been conclusively identified [85].

Like other toxic metals, it is unlikely that cellular Fe exists in its free state and is probably bound by ligands that result in specific trafficking and protect it from the aqueous environment. Already, evidence for the trafficking of copper *via* chaperones and organelle interactions has been presented [86–88] and Fe may utilize similar, but probably distinct mechanisms [89]. In fact, some evidence for the specific intracellular trafficking of Fe has been presented *via* organelle interactions and chaperone proteins [90]. Ponka and colleagues have presented evidence in support of the "kiss and run" hypothesis, that is, endosomes can directly interact with the mitochondrion to lead to highly efficient Fe transfer [91,92].

After entrance of Fe into the cell it can be used for general metabolism and become incorporated into Fe-binding sites of haem and non-haem Fe-containing molecules. Alternatively, Fe can be assimilated within the Fe storage protein, ferritin [93]. Ferritin is a hetero-polymer consisting of H- and L-subunits that can accommodate up to 4500 atoms of Fe [4,93]. The mechanism of Fe release from ferritin remains unclear, although the lysosome and proteasome are probably involved [94,95]. The regulation of ferritin expression by Fe occurs post-transcriptionally and is mediated by the IRE-IRP mechanism [73,96].

There are other Fe-binding proteins which share high homology with Tf, including lactoferrin and melanotransferrin [53]. Lactoferrin is found in secretions and its ability to bind Fe plays a role in its bacteriostatic effects [97]. Melanotransferrin is a membrane-bound Tf homologue that is highly expressed in human melanoma cells and has only 1 Fe-binding site [98]. However, melanotransferrin does not act as an Fe transporter nor does it modulate Fe homeostasis [99,100]. In fact, it plays a role in the proliferation, migration, invasion and tumourigenesis of melanoma cells [101,102].

5. Effect of nitrogen monoxide on intracellular Fe metabolism

The high affinity of NO for Fe means that it will affect the activity of many Fe-containing proteins [4,18]. One of the most important examples of NO interacting with Fe-containing molecules is its effect on IRP1 that is known to play a role in the regulation of Fe metabolism [73]. In fact, NO can activate the RNA-binding activity of IRP1 that plays a role in the homeostatic regulation of cellular Fe metabolism [39–41]. The mechanism by which NO exerts its effects on IRP1 is probably by both depleting intracellular Fe and by interacting with its [Fe–S] cluster [39,40,44,103]. In fact, it has been shown that NO increases the size of the LIP in some cell types [82,104], but decreases it in others [105]. The difference in results between these latter studies could relate to the cell types used and experimental conditions implemented in each case. The source of the Fe pool altered in endothelial cells was suggested to reside within the mitochondrion as the mitochondrial protein synthesis inhibitor, chloramphenicol, prevented the increase in the LIP induced by NO [82]. This is a clearly debatable inference, as chloramphenicol could non-specifically affect many cellular processes.

As will be described in detail below, NO can enter cells and act to some degree like a chelator by binding Fe and inducing its release [44,106,107]. The NO-mediated Fe release is physiologically-relevant, as it is observed upon the interaction of activated macrophages with tumour cells and other targets [14,15,108]. In addition, it is of interest to note that the effect of NO at inducing Fe release is quite specific for this molecule, as it is not observed with the closely related diatomic effector, carbon monoxide (CO) [107].

The relative roles of NO-mediated intracellular Fe release or direct interaction with the [Fe–S] cluster in activating IRP1-RNA-binding activity depend on the source of NO, the redox-related state of NO generated and the amount produced [44]. In contrast to IRP1, NO and $ONOO^-$ have been reported to decrease IRP2-RNA-binding activity [109,110], perhaps by a mechanism involving NO^+-mediated degradation [110]. Direct measurement of *S*-nitrosylation of critical sulphydryl groups of IRP2 has been shown [7], suggesting that this mechanism could be a physiological regulator of this molecule. More recent reports have indicated that there is also IRP-independent regulation of ferritin synthesis by NO [111]. This latter process is a post-transcriptional mechanism that is regulated by NO^+ and remains uncharacterised at present [111]. These latter studies used the NO^+ donor, sodium nitroprusside (SNP), which is a coordination complex containing Fe [5]. A number of investigations have suggested that the influence of SNP on cellular Fe metabolism is due to its ability to act as an Fe donor rather than through its NO^+ character [112,113]. However, there is evidence that the Fe in SNP does not play a role since chelators such as EDTA and DFO do not prevent its effects on ferritin synthesis [41,111]. In addition, the effect of SNP on ferritin expression is more rapid than the soluble Fe salt, ferric ammonium citrate, suggesting that SNP works by a different mechanism than Fe donation [111].

Considering that iNOS can be regulated by intracellular Fe, this could lead to an auto-regulatory-loop whereby low Fe levels induce iNOS expression [114]. This enzyme subsequently generates NO which activates IRP1-RNA-binding activity that has a number of downstream effects on mRNAs containing the IRE [114–116]. For instance, increased IRP1 binding to the 3′-IRE of *TfR1* mRNA would up-regulate TfR1 expression that theoretically leads to elevated Fe uptake from Tf [73]. The enhanced cellular Fe levels would then induce the opposite effect by reducing iNOS transcription, resulting in decreased NO levels and thus, IRP1-RNA-binding activity [114]. Upon this complex level of control, other studies using murine macrophages have shown that IRP1 gene expression can be down-regulated by NO [117] at the transcriptional level [118]. In this latter investigation it was demonstrated that signal transducer and activator of transcription proteins are involved in down-regulating IRP1 after exposure to NO *via* their interaction with the interferon-gamma-activated sequence in the IRP1 promoter [118].

It has been shown that NO can decrease Fe uptake from Tf by cells *via* interfering with intracellular Fe trafficking and inducing cellular Fe mobilisation [44,106,107]. The effect of NO at reducing total cellular Fe uptake and Fe incorporation into ferritin is also seen with CO, which like NO has a high affinity for Fe (Fig. 2A,B) [107]. Of interest, a recent study has suggested that NO down-regulates expression of the Fe transporter DMT1 [119] and potentially this could also be responsible for the decrease in Fe uptake from Tf after incubation with NO [120,121].

Recently, a signalling cascade has been discovered in neurons involving the regulation of DMT1 by NO [9]. In this pathway, stimulation of NMDA receptors activates neuronal NOS that leads to *S*-nitrosylation and activation of a G protein of the Ras superfamily known as Dexras1 [9]. This alteration has been suggested to induce Fe uptake *via* DMT1 [9], although its physiological relevance has yet to be confirmed and it is unknown if such a pathway exists in non-neuronal cells.

Since NO was reported to form a complex with Fe in lactoferrin [122], it could be expected that based upon their high homology [4], a comparable reaction could occur with Tf. Such a reaction could also potentially interfere with Fe uptake from this protein. However, studies using electron paramagnetic (EPR) spectroscopy demonstrated that the ability of NO to reduce Fe uptake from Tf [120] was not due to direct removal of Fe from the protein nor the formation of an NO–Fe complex within this molecule [120,121]. In fact, the effect of both NO and CO at reducing Tf-bound Fe uptake appeared to be mediated by its ability to inhibit cellular ATP production [107,121], which is essential for cellular Fe internalization *via* Tf endocytosis [123]. Considering that Tf binds high spin Fe(III) and that its Fe-binding site does not interact with NO [121], it is questionable whether NO can form an NO–Fe complex within lactoferrin as reported by others [122]. This is because the Fe-binding sites of these molecules are very similar [121].

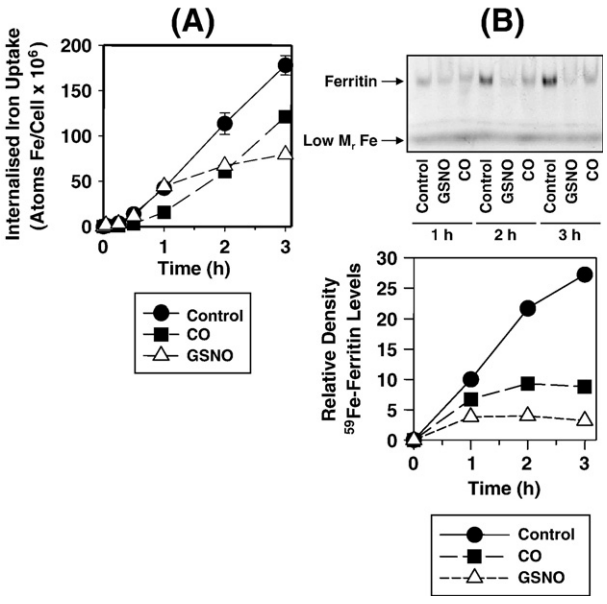

Fig. 2. Both CO gas and GSNO reduce: (A) ^{59}Fe uptake from ^{59}Fe-transferrin (^{59}Fe-Tf) and (B) ^{59}Fe incorporation into ferritin by LMTK$^-$ fibroblasts. (A) Cells were labelled with ^{59}Fe-Tf (0.75 μM) for 5–180 min at 37 °C in the presence or absence of 2% CO gas or GSNO (0.5 mM). The cells were then washed four times on ice and incubated for 30 min with Pronase (1 mg/ml) at 4 °C to separate internalized from membrane-bound ^{59}Fe. (B) The LMTK$^-$ cells were labelled with ^{59}Fe-Tf (0.75 μM) for 60–180 min at 37 °C, washed, and native-PAGE ^{59}Fe-autoradiography performed. Densitometric analysis is presented below the autoradiograph. The results in (A) are expressed as mean of duplicate determinations in a typical experiment of 5 performed, while the results in (B) are representative of 3 separate experiments. Taken from: Watts, R.N. and Richardson, D.R. (2004) Biochim. Biophys. Acta 1692, 1–15.

6. The mechanism of NO-mediated Fe release from cells

Regarding the effect of NO on cellular Fe release, initial studies using NO^+ generators (*e.g.*, SNP) or $ONOO^-$ donors (*e.g.*, SIN-1) [124] demonstrated that these agents did not result in appreciable Fe mobilisation from cells [120]. In contrast, $NO^\bullet$-releasing agents (*e.g.*, *S*-nitroso-*N*-acetylpenicillamine (SNAP), *S*-nitrosoglutathione (GSNO) or spermine-NONOate) showed high efficacy [44,106,125]. The effect of these latter agents at inducing Fe release was due to their ability to generate $NO^\bullet$, as their precursor compounds which do not bear the NO-moiety had no effect [44,106]. Hence, it was the $NO^\bullet$ redox state which was important for forming intracellular Fe complexes, probably because this form of NO is capable of generating coordination complexes [3]. While NO could markedly induce intracellular release, other similar diatomic molecules such as CO had little effect (Fig. 3A) [107]. Further, the ability of NO to mobilise cellular Fe could not be augmented by CO (Fig. 3B), despite their similar chemistry and the potential of CO to directly ligate Fe pools [3,107,126]. Like synthetic high affinity Fe chelators [127], the efficacy of NO at mobilising Fe from cells decreased as the labelling time with ^{59}Fe-Tf increased (Fig. 3B) [107]. This can be explained by the entrance of Fe into less accessible cellular pools (*e.g.*, ferritin) as the incubation time increased [127].

Recently, our laboratory has extensively examined the mechanism of NO-mediated Fe release from cells. These studies have demonstrated that it is glutathione (GSH)- and energy-dependent and reliant on the uptake and metabolism of D-glucose (D-Glc) [106,125]. In fact, only sugars that can be taken up and metabolised by cells were effective at increasing NO-mediated Fe release [106]. Fig. 4 is a schematic illustration summarising a model of NO-mediated Fe release based upon these investigations [106,125]. Glucose enters the cell by the well characterised family of glucose transporters [128] and is subsequently phosphorylated to glucose-6-phosphate (G-6-P; Fig. 4) [126]. Glucose-6-phosphate is metabolised by two major pathways, either through glycolysis and/or the tricarboxylic acid cycle (TCA) to form ATP, or through the pentose phosphate pathway (PPP; that is also known as the hexose monophosphate shunt) to form reduced NADPH (*e.g.*, for GSH synthesis) and pentose sugars (Fig. 4) [107].

Our studies demonstrated that D-Glc uptake and metabolism by the PPP was essential for NO-mediated Fe release [106]. Significantly, depletion of GSH using the specific GSH synthesis inhibitor, buthionine sulphoximine (BSO) [129], prevented NO-mediated Fe release from cells [106,107,125]. In addition, Fe

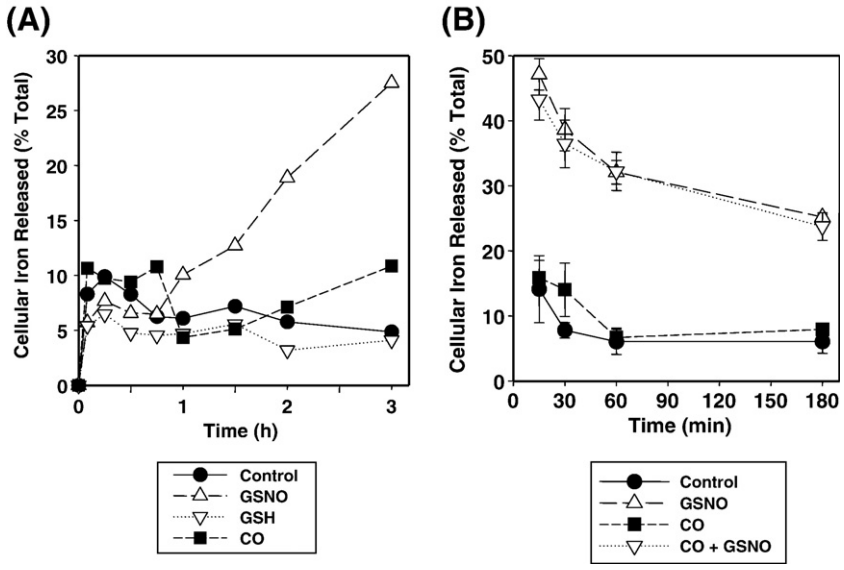

Fig. 3. (A) The NO-donor GSNO but not CO gas results in ^{59}Fe mobilisation from prelabelled LMTK$^-$ fibroblasts. (B) Iron mobilisation from LMTK$^-$ fibroblasts prelabelled for various times in the presence of GSNO, CO gas, or a combination of both. (A) Cells were prelabelled with ^{59}Fe-Tf (0.75 μM) for 180 min at 37 °C, washed, and then reincubated for up to 180 min at 37 °C with GSNO (0.5 mM), GSH (0.5 mM) or 2% CO gas. Results are means of duplicate determinations in a typical experiment from 3 performed. (B) Cells were prelabelled with ^{59}Fe-Tf (0.75 μM) for 15–180 min at 37 °C, washed and then reincubated for 180 min with GSNO (0.5 mM), 2% CO gas, or GSNO (0.5 mM) and 2% CO gas. Results are expressed as mean±SD of triplicate determinations in a typical experiment of three experiments performed. Taken from: Watts, R.N. and Richardson, D.R. (2004) Biochim. Biophys. Acta 1692, 1–15.

mobilisation after GSH-depletion could be reconstituted by incubation of cells with *N*-acetylcysteine that increased cellular GSH levels [106].

It is probable that the effect of D-Glc on stimulating NO-mediated Fe mobilisation was not just due to its effect on GSH metabolism. Indeed, our experiments showed that NO-mediated ^{59}Fe release was temperature- and energy-dependent, suggesting a membrane transport mechanism could be involved [106]. As shown in Fig. 4, NO can enter cells through diffusion and there has been some evidence that this can occur by a transport molecule such as the protein disulfide isomerase that catalyses *trans*-nitrosylation [130].

Work in our laboratory indicated that a major intracellular target of NO appeared to be the Fe storage molecule ferritin [125]. NO prevented the uptake of Fe into ferritin and also appeared to indirectly mobilise Fe from this protein [125]. An indirect mechanism of Fe release from this protein was postulated, as NO-generating agents added to cellular lysates had no direct effect on ferritin-Fe mobilisation [125]. The efflux of Fe from cells and its removal from ferritin was GSH-dependent and could be inhibited using BSO [106,125]. It was speculated that GSH may assist in the removal of the Fe from cells by either acting as a reducing agent or by filling the coordination shell of an Fe complex composed of NO and GSH ligands. This complex could be lipophilic enough to pass through the plasma membrane to exit the cell. However, our experiments using a variety of metabolic inhibitors showed that NO-mediated Fe removal from cells was an ATP-dependent event [106,125]. While NO appeared to act like a typical synthetic chelator (*e.g.*, DFO or dipyridyl) in terms of its ability to mobilise cellular Fe, the mechanism was quite different, as chelator-mediated Fe release was not dependent on cellular GSH levels [106].

7. Nitrogen monoxide mediates Fe export from cells by the GSH transporter, MRP1

As discussed above, NO-mediated Fe release from cells was a temperature- and ATP-dependent event suggesting the involvement of a transport system [125]. Considering possible transport

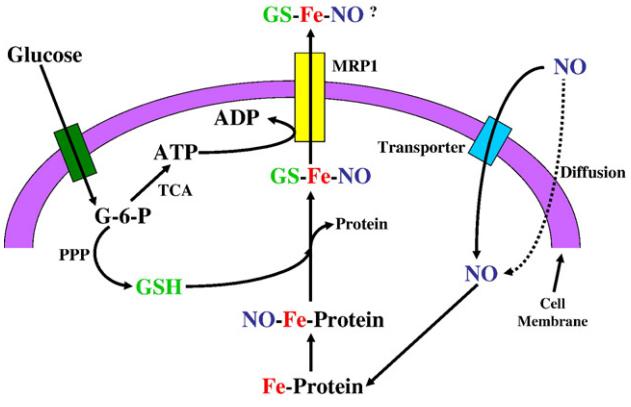

Fig. 4. Hypothetical model of D-glucose-dependent NO-mediated Fe mobilisation from cells. D-Glucose is transported into cells and is used by the tricarboxylic acid cycle (TCA) for the production of ATP and by the pentose phosphate pathway for the generation of pentose sugars and NADPH. This reductant is involved in the production of reduced glutathione (GSH). Nitrogen monoxide (NO) either diffuses or is transported into cells where it intercepts and binds Fe bound to proteins or Fe that is en route to ferritin. The high affinity of NO for Fe results in the formation of an NO–Fe complex and GSH may either be involved as a reductant to remove Fe from endogenous ligands or may complete the Fe coordination shell along with NO. This complex may then be released from the cell by an active process requiring a transporter that has recently been identified as multi-drug resistance-associated protein 1 (MRP1). Modified from Watts, R.N. and Richardson, D.R. (2002) Eur. J. Biochem. 269, 3383–3392.

molecules responsible for NO-mediated Fe release, recently our attention became focused on the multi-drug resistance-associated protein 1 (MRP1 or ABCC1) [131]. MRP1 is an ABC transporter that is expressed ubiquitously in tissues [131]. Apart from the role of MRP1 as a detoxifying mechanism for the efflux of drugs from cells, this transporter also plays physiological roles where it is involved in the export of GSH and leukotriene C4 [131–135]. Interestingly, it has also been demonstrated that MRP1 can transport GSH coordinated to heavy metals such as As and Sb [132–135], but its role in Fe transport has not been previously proposed.

We examined NO-mediated ^{59}Fe mobilisation using a well characterised cell model expressing high MRP1 levels, namely MCF7-VP cells [136]. These cells were compared to their wild-type parental counterparts (MCF7-WT) which do not express high MRP1 levels. To confirm functionality and expression of MRP1 in MCF7-VP cells, efflux of the classical MRP1 substrate, tritiated-vincristine (^{3}H-VCR) [137], was examined and found to significantly increase ($p<0.05$–0.0001) compared to MCF7-WT parental cells [138]. This suggested that MRP1 was expressed on the plasma membrane and was functional. These results were further confirmed in studies showing the higher levels of *MRP1* mRNA and protein in MCF-VP cells compared to their WT counterparts [138]. In addition, expression of several other potential GSH transporters, namely MRP2–4 or cystic fibrosis transmembrane conductance regulator (CFTR) [131] were not up-regulated in MCF7-VP cells compared to MCF7-WT. MCF7-VP cells are well known to hyper-express MRP1, but not other drug transporters such as P-glycoprotein [139,140]. Hence, these cells were an appropriate model to characterise ^{59}Fe and GSH efflux *via* MRP1 after incubation with NO.

Using the MCF7-VP and MCF7-WT cell lines, we showed [136] that MRP1 was involved in NO-mediated ^{59}Fe and GSH efflux from studies demonstrating that: (1) NO-mediated ^{59}Fe release (Fig. 5A,B) and GSH efflux (Fig. 5D,E) were significantly greater in MCF7-VP cells hyper-expressing MRP1 compared to MCF7-WT; (2) Cellular ^{59}Fe release and GSH efflux occurred by temperature- and metabolic energy-dependent mechanisms consistent with active transport; (3) The specific GSH inhibitor, BSO, that inhibits MRP1 transport activity [131] markedly prevented both NO-mediated ^{59}Fe and GSH efflux from cells; (4) Well characterised inhibitors of MRP1 such as MK571, difloxacin, verapamil and probenecid prevented NO-mediated ^{59}Fe efflux; and (5) Potent inhibitors of MRP1 transport activity such as MK571 and probenecid resulted in an intracellular build-up of

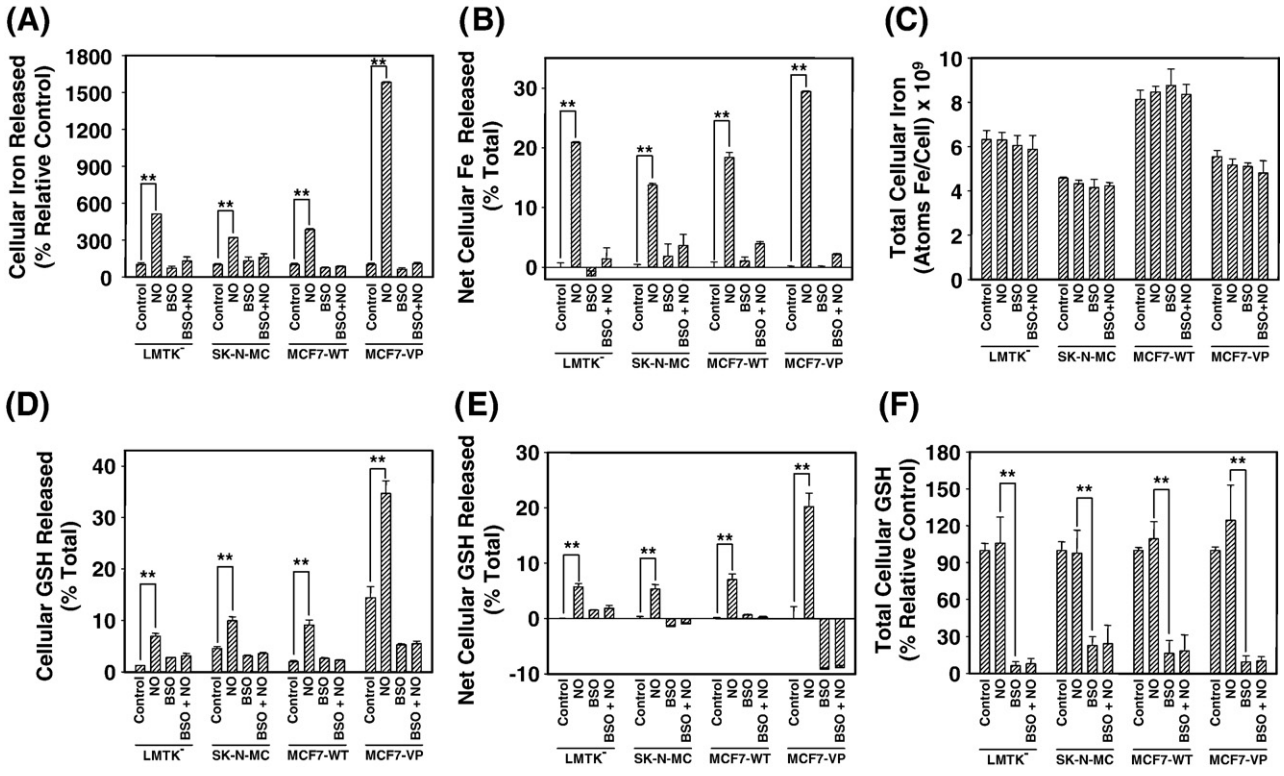

Fig. 5. Iron (Fe) and glutathione (GSH) efflux increase concurrently after incubation with the NO-generator, spermine-NONOate (SperNO), and are more marked in MRP1 hyper-expressing MCF7-VP cells than MCF7-WT. Both NO-mediated ^{59}Fe and GSH efflux were inhibited by the GSH synthesis inhibitor, buthionine sulphoximine (BSO). NO-mediated ^{59}Fe and GSH efflux was examined by preincubating cells with or without 0.1 mM BSO for 20 h at 37 °C before labelling for 3 h at 37 °C with ^{59}Fe-transferrin (0.75 μM), washing, and subsequent incubation with the NO-generator, SperNO (0.5 mM) for 3 h at 37 °C. (A) NO-mediated ^{59}Fe efflux results are expressed as a percentage of the control. (B) Results calculated as a percentage of total cell ^{59}Fe. These data are expressed as net ^{59}Fe efflux over that found for the control. (C) Total cellular ^{59}Fe (i.e., cell ^{59}Fe+efflux ^{59}Fe). (D) NO-mediated GSH efflux expressed as a percentage of total GSH. (E) GSH efflux data expressed as net GSH efflux over that found for the control. (F) Total GSH measured showing the GSH-depletion in the presence of BSO. Results are mean±SD (4 determinations) in a typical experiment of 4. **Denotes $p<0.0001$. Taken from Watts, R., Hawkins, C., Ponka, P. and Richardson, D.R. (2006) Proc. Natl. Acad. Sci. USA 103(20), 7670–7675.

EPR-detectable DNICs. Moreover, the extent of accumulation of these later species correlates with the ability of these inhibitors to prevent NO-mediated ^{59}Fe efflux from MRP1-hyper-expressing cells [136].

8. Biological relevance of NO-mediated transport *via* MRP1

The transport of NO into and out of cells is of great importance, particularly as it relates to its messenger and cytotoxic effector functions. We have demonstrated that NO can stimulate cellular ^{59}Fe and GSH release by a mechanism that is mediated by MRP1 (Fig. 5) [136]. This may be important for a number of reasons. For instance, the release of DNICs from the cell by a specific transport process could be important for intercellular responses to this messenger molecule and may impart NO with a greater half-life. In fact, as already discussed, a number of investigations have reported that DNICs are natural carriers and storage forms of NO that possess a greater half-life than NO alone [[141–145]. Considering this, it is relevant to note that DNICs have been found in animal tissues, human sera, activated macrophages and a range of different cell types [28,45,46,48–51,141].

A recent study has suggested that the dinitrosyl–diglutathionyl–Fe complex (DNDGICs) can associate with glutathione-*S*-transferase enzymes (see below) to stabilize NO for many hours ($t_{1/2}$=4.5–8 h; [143]) which markedly exceeds the $t_{1/2}$ of "free NO" which is 2 ms–2 s [24]. Furthermore, DNICs can transverse cell membranes to donate Fe to tissues [145] and can *trans*-nitrosylate acceptor targets *in vitro* and *in vivo* [144,146,147] demonstrating their bioavailability and potential role as NO-carrier molecules.

The efficient efflux of DNICs by an active transport mechanism could be crucial at sites where NO is produced in small physiological quantities as a messenger molecule [136]. For example, in blood vessels where the small quantities of DNICs released from endothelial cells could be important for regulating smooth muscle tone [34] (Fig. 6A). Indeed, the ability of cells to actively transport and traffic NO overcomes the random process of diffusion that would be inefficient and non-targeted.

Conversely to the situation when NO acts as a messenger molecule, under conditions where NO is used as a cytotoxic effector, the substantial quantities generated by inducible NOS of activated macrophages could lead to the efflux of a relatively large proportion of Fe and GSH from tumour target cells (Fig. 6B) [136]. Since Fe and GSH are critical for proliferation [4,131], their release from tumour cells in large amounts would be cytotoxic. In addition to these results, this hypothesis is strongly supported by previous studies where cytotoxic macrophages induced the release of a large proportion (64%) of intracellular Fe from tumour target cells [14], an effect mediated by NO [15]. Previous investigations have shown that increased GSH efflux from cells is a key signal that mediates apoptosis [148] and it is well known that Fe mobilisation from cells using chelators results in marked anti-tumour activity [149]. Hence, the dual action of NO resulting in both Fe and GSH mobilisation may play a vital role in the

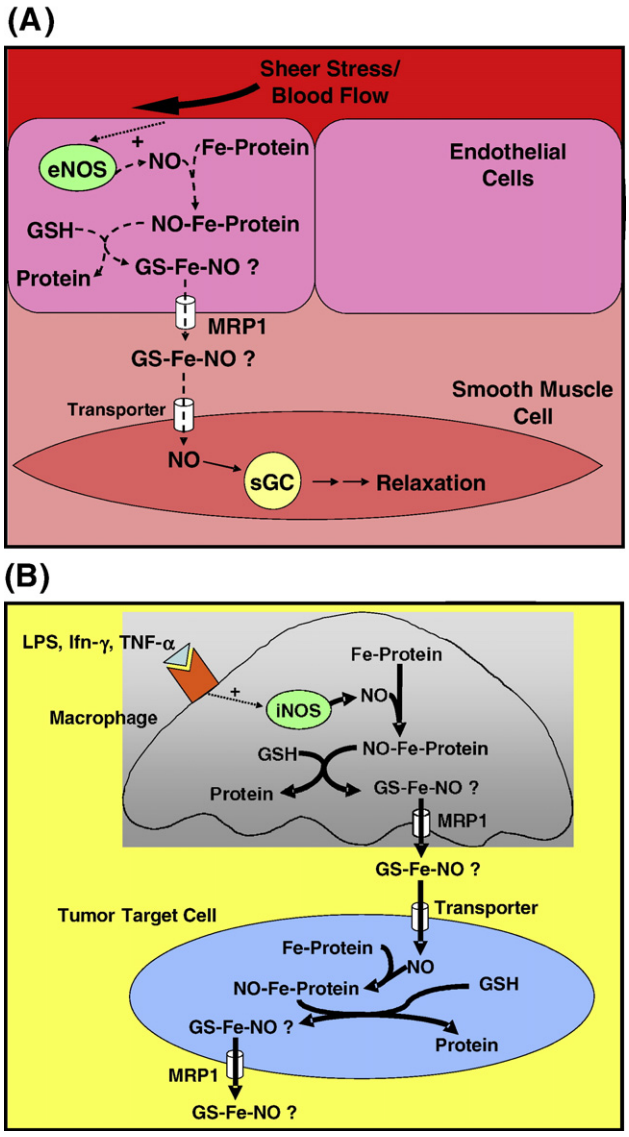

Fig. 6. Schematic illustration of hypotheses proposing the consequences of MRP1-mediated DNIC efflux from cells. (A) The efficient efflux of DNICs by an active transport mechanism could be crucial where NO is produced in small quantities as a messenger molecule *e.g.*, in blood vessels where endothelial NOS (eNOS) generates NO. The ability of cells to actively transport NO overcomes diffusion that would be inefficient. The small quantities of GS–Fe–NO complexes released from endothelial cells may be taken up by smooth muscle cells through diffusion or active transport *e.g.*, *via* protein sulfide isomerase for instance [130]. (B) Where NO is used as a cytotoxic effector, the substantial quantities generated by inducible NOS (iNOS) of activated macrophages could lead to the efflux of a large proportion of Fe and GSH from tumour cell targets that would be cytotoxic. From Watts, R., Hawkins, C., Ponka, P. and Richardson, D.R. (2006) Proc. Natl. Acad. Sci. USA 103(20), 7670–7675.

cytotoxic activity of activated macrophages against tumour cells. It was also shown that under conditions that lead to Fe and GSH efflux, proliferation of MCF7-VP cells hyper-expressing MRP1 was more sensitive to NO than their wild-type counterparts [136]. This supports the hypothesis that enhanced GSH and Fe efflux from cells hyper-expressing MRP1 leads to greater anti-proliferative activity. In addition, GSH-depletion increased the cytotoxicity of NO particularly in MRP1 hyper-expressing

MCF7-VP cells, suggesting the critical role played by GSH in the anti-proliferative activity of NO [150].

The role of GSH in NO-mediated Fe release may not only be important for its transport out of the cell *via* the GSH transporter, MRP1. In fact, we hypothesize that it is also essential for Fe release from cellular proteins targeted by NO such as those with [Fe–S] clusters *e.g.*, aconitase [37,151]. Thus, we suggest that there is an intracellular equilibrium between protein-bound DNICs and low M_r DNICs and that GSH is necessary for the conversion to the low M_r form that is transported out of the cell by MRP1 [136]. This idea is supported by studies showing that incubation of cells with BSO markedly prevented GSH and ^{59}Fe release (Fig. 5A,D) and the potent MRP1 transport inhibitors, MK571 or probenecid, resulted in DNIC accumulation [136]. Our hypothesis is also consistent with studies of Ding and colleagues that postulate that glutathione and/or cysteine are necessary for release of DNICs from [Fe–S] clusters [152,153].

Considering how Fe and GSH are transported by MRP1, five potential mechanisms have been proposed to account for interaction of the transporter with GSH and its substrates [131]. First, GSH itself can directly act as a MRP1 substrate and be transported out of the cell. Second, GSH can form a complex or conjugate with a metal or compound that is then transported by MRP1 *e.g.*, leukotriene C4 [154] or the $As(GS)_3$ complex [155]. Third, GSH can be co-transported with some MRP1 substrates *e.g.*, vincristine. Fourth, GSH can stimulate the transport of certain compounds (*e.g.*, estrone-3-sulfate) by MRP1 without itself being transported out of the cell, and fifth, GSH transport by MRP1 can be enhanced by interaction with certain compounds (*e.g.*, verapamil) that are not translocated themselves across the membrane [131,156,157].

We showed that the GSH synthesis inhibitor, BSO, markedly inhibited both NO-mediated ^{59}Fe and GSH efflux suggesting a dependence on GSH for transport [106] (Fig. 5). Considering this, it can be suggested that either a complex containing both ^{59}Fe and GSH are effluxed together or ^{59}Fe and GSH are separately transported across the membrane by MRP1. We favour a hypothesis necessitating the formation of a complex intracellularly. This is because we can detect intracellular DNICs directly by EPR and the fact that these accumulate after incubation with the MRP1 inhibitors MK571 and probenecid [136]. Relevant to the molecular mechanism of GSH and Fe transport by MRP1, it is of interest that verapamil and difloxacin markedly prevent NO-mediated ^{59}Fe efflux from MCF7-VP and -WT cells, while both of these MRP1 inhibitors stimulate GSH release [150]. Hence, at least in the presence of these inhibitors, GSH and ^{59}Fe appear to be separate transportable entities [136].

Iron efflux from cells has been described for many years [4], and recently, ferroportin-1 has been shown to be a physiologically-relevant Fe exporter [64–66]. It is probable that the role of MRP1 is quite different to that of ferroportin-1 and this is suggested by differences in the pattern of expression of these molecules. Certainly, MRP1 is ubiquitously expressed in tissues [158], while ferroportin-1 expression appears more restricted, being found at high levels in macrophages, placenta and hepatocytes [64–66]. Moreover, ferroportin-1 has not been reported to transport GSH and its function appears to be the regulation of Fe homeostasis [68]. Finally, while we have shown that MRP1 facilitates NO-mediated Fe efflux, we cannot rule out that other transporters not examined in this study could also perform this function.

9. The potential intermediary or storage role of DNICs by the glutathione-*S*-transferase enzymes

The glutathione-*S*-transferase (GST; EC 2.5.1.18) family of enzymes forms an integrated detoxification mechanism in cells along with MRP1 that eliminate toxic exogenous (*e.g.*, anti-cancer chemotherapeutics) and endogenous compounds (*e.g.*, oxidative stress by-products) as glutathione conjugates from cells [159,160]. These enzymes catalyse the nucleophilic attack of reduced GSH on non-polar compounds to form glutathione conjugates that are more amenable to elimination. Cytosolic GSTs in humans are dimeric proteins that are grouped into at least 7 classes (Alpha, Mu, Pi, Sigma, Theta, Omega and Zeta) [159,160].

Considering the coordinated role of GSTs and MRP1 in detoxification [161–163] and the role of MRP1 in transport of DNICs [150], it is of interest that GSTP1-1, Alpha, Mu and Theta bind DNDGICs with high affinity (K_d 10^{-7} to 10^{-10} M) [141,143,164–166]. This led the latter authors to suggest these enzymes are scavengers or intracellular NO carriers.

A crystal structure of the DNDGIC–GSTP1-1 complex revealed that Tyr-7 in the active site of the enzyme coordinates through its phenolate group to Fe displacing one GSH molecule from the complex [166]. Using EPR, the half-life of the DNDGIC–GSTP1-1 complex has been assessed in intact bacterial cells. This work demonstrated that the binding of DNDGIC to this protein was reversible, with the loss of the EPR signal occurring within 1 h unless Fe was supplied from an exogenous source [166]. *In vitro* in tissue homogenates, the half-life of the complex was found to be markedly longer, namely 4.5 h for the Alpha and Mu GST classes and 8 h for GSTP1-1 [143]. The generation of the DNDGIC–GSTP1-1 complex opposes the complete loss of the detoxifying activity of this enzyme, as binding in one subunit lowers the affinity for the complex in the second subunit [141]. Indeed, such negative cooperativity due to inter-subunit communication in GSTP1-1 and GSTM2-2, prevents loss of catalytic activity of the second subunit [141].

Most of the studies examining the formation of the DNDGIC–GST lysosome complexes have been performed on the isolated and purified mammalian proteins or the expression of these proteins in bacterial cells. Two recent studies have demonstrated using hepatocytes that DNDGIC binds to the alpha class of the GST enzymes that are present in these cells at high concentrations (0.3 mM) [167,168]. It was suggested that the NO spontaneously removes Fe from ferritin and transferrin to form the DNDGIC [167]. However, previous studies have demonstrated that NO does not directly remove Fe(III) from the Fe-binding site of Tf or ferritin *in vitro* [121,125]. More likely, NO may intercept Fe released from these proteins when it is in the Fe(II) state and in a more labile form *e.g.*, when ferritin is degraded within the lysosomes or after Fe(II) is released from Tf and transported out of the endosome. In these stages of its metabolism, Fe is in a more

easily bound state and can be intercepted to form a complex with NO. Considering this, a recent study has suggested that DNICs are generated from the breakdown of metalloproteins in the lysosome and that lysosomotropic agents can prevent the generation of these complexes [169].

The specific interaction of DNDGICs with GSTs raises the question of their function and whether there could be an interaction with MRP1 considering the known role of these proteins in detoxification processes [161–163]. Studies *in vitro* have shown that DNDGICs lead to the inactivation of glutathione reductase and that this only occurs when the GST-binding capacity is exceeded [167]. Hence, it has been hypothesized GSTs may act as a physiological protective mechanism against the generation of high intracellular levels of DNDGICs. An interesting finding is that 20% of the GST–DNDGIC is found associated with subcellular components, particularly the outer membrane of the nuclear envelope, suggesting a protective role against DNA damage [168]. Alternatively, or in combination with this latter function, GSTs may act as a convenient NO store for the cell and prevent the release of DNDGICs *via* MRP1.

10. Conclusions

The interaction of NO with intracellular Fe to form DNICs represents an important biologically-relevant reaction. Interestingly, NO mediates the efflux of Fe and GSH from cells by an active mechanism mediated by the GSH transporter, MRP1 [136]. The ability of the cell to actively transport and traffic NO overcomes the random process of diffusion that would be inefficient and non-targeted. This could have important biologically-relevant consequences for intra- and extra-cellular NO-signalling, NO-mediated apoptosis and the cytotoxicity of activated macrophages that is due, in part, to Fe release from tumour cell targets. Furthermore, the GST enzymes that bind DNDGICs with high affinity could act as an intermediate form of NO for regulating intracellular biological processes. Since the GST enzymes and MRP1 form an integrated system for removing a variety of toxic substances [161–163], it is possible to hypothesize that these molecules coordinately regulate NO levels by binding and then transporting DNDGICs from cells.

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