Nitrogen Monoxide (NO) and Glucose

UNEXPECTED LINKS BETWEEN ENERGY METABOLISM AND NO-MEDIATED IRON MOBILIZATION FROM CELLS

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Nitrogen monoxide (NO) affects cellular iron metabolism due to its high affinity for this metal ion. Indeed, NO has been shown to increase the mRNA binding activity of the iron-regulatory protein 1, which is a major regulator of iron homeostasis. Recently, we have shown that NO generators increase $^{59}$Fe efflux from cells pre-labeled with $^{59}$Fe-transferrin (Wardrop, S. L., Watts, R. N., and Richardson, D. R. (2000) Biochemistry 39, 2748–2758). The mechanism involved in this process remains unknown, and in this investigation we demonstrate that it is potentiated upon adding d-glucose (d-Glc) to the reincubation medium. In d-Glc-free or d-Glc-containing media, 5.6 and 16.5% of cellular $^{59}$Fe was released, respectively, in the presence of S-nitrosoglutathione. This difference in $^{59}$Fe release was observed with a variety of NO generators and cell types and was not due to a change in cell viability. Kinetic studies showed that d-Glc had no effect on the rate of NO production by NO generators. Moreover, only the metabolizable monosaccharides d-Glc and d-mannose could stimulate NO-mediated $^{59}$Fe mobilization, whereas other sugars not easily metabolized by fibroblasts had no effect. Hence, metabolism of the monosaccharides was essential to increase NO-mediated $^{59}$Fe release. Incubation of cells with the citric acid cycle intermediates, citrate and pyruvate, did not enhance NO-mediated $^{59}$Fe release. Significantly, preincubation with the GSH-depleting agents, L-buthionine-[S,R]-sulfoximine or diethyl maleate, prevented NO-mediated $^{59}$Fe mobilization. This effect was reversed by incubating cells with N-acetyl-l-cysteine that reconstitutes GSH. These results indicate that GSH levels are essential for NO-mediated $^{59}$Fe efflux. Hence, d-Glc metabolism via the hexose monophosphate shunt resulting in the generation of GSH may be essential for NO-mediated $^{59}$Fe release. These results have important implications for intracellular signaling by NO and also NO-mediated cytotoxicity of activated macrophages that is due, in part, to iron release from tumor target cells.

Virtually every field of physiology has been influenced by

nitrogen monoxide (NO), a small, relatively unstable, potentially toxic, diatomic free radical gas, that is produced in a diverse variety of mammalian cells (for reviews see Refs. 1–3). NO has a physiological role as a short lived messenger molecule and has two principal functions in cells, servoregulation and cytotoxicity. Considering servoregulation, NO is produced in small amounts under physiological conditions and mediates vasorelaxation, regulates blood pressure, controls the adhesion and aggregation of platelets and neutrophils, and is involved in neurotransmission. Most of these actions are mediated through the binding of NO to iron in the heme prosthetic group of soluble guanylate cyclase (1, 3). Indeed, the high affinity of NO for iron and other metal ions is a well known branch of coordination chemistry (2).

The importance of iron in mediating the functions of NO is also apparent when examining its cytotoxic effects (3). The cytotoxic functions of NO are observed when it is produced in much larger amounts by macrophages, hepatocytes, and other cells following their exposure to cytokines or microbial products. Interestingly, NO produced via such high output systems inhibits the proliferation of intracellular pathogens and tumor cells. These effects can be explained by the reactivity of NO with iron in the Fe-S centers of important macromolecules, including aconitase and complexes I and II of the electron transport chain (4–6). The high affinity of NO for iron probably results in the removal of iron from [Fe-S] centers and the formation of dinitrosyl iron species within [Fe-S] proteins (for review see Ref. 7). NO has been shown to form complexes with a variety of important iron-containing proteins such as ferritin (8), ribonucleotide reductase (9), heme-containing proteins (10–12), and ferrochelatase (13). In fact, ferritin has been suggested to act as a store of NO (8), and NO-mediated iron release from ferritin has been shown in vitro (14).

Interestingly, co-cultivation of tumor cells with activated macrophages results in the inhibition of target cell DNA synthesis and a concomitant loss of a large fraction (64% per 24 h) of intracellular iron (15). It was speculated that the loss of iron may be due to the NO-mediated release of iron from enzymes such as mitochondrial aconitase (4, 16). In contrast to the work of Hibbs and others (4, 17, 18), it has been suggested that NO targets loosely bound pools of non-heme iron, rather than mitochondrial Fe-S clusters (19). Nonetheless, the relationship between NO and iron is clearly demonstrated by the identifi-

1 The abbreviations used are: NO, nitrogen monoxide; BSO, L-buthionine-[S,R]-sulfoximine; DEM, diethyl maleate; D-Glc, d-glucose; D-Man, d-mannose; D-Fuc, d-fructose; DFO, desferrioxamine; GSNO, S-nitroso glutathione; HMPS, hexose monophosphate shunt; IRP1, iron-regulatory protein 1; NAC, N-acetyl-L-cysteine; PIH, pyridoxal isonicotinoyl hydrazine; SNAP, S-nitroso-N-acetylcysteamine; SperNO, spermine-NONOate; Tf, transferrin; 311, 2-hydroxy-1-naphthaldehyde isonicotinoyl hydrazone.

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cation of Fe-nitrosyl complexes in activated macrophages and their tumor cell targets (17, 18, 20, 21). In these investigations, electron paramagnetic resonance spectroscopy detected signals typical of Fe-dithiol dinitrosyl (Fe(RS)2(ONO)) complexes and heme-nitrosyl complexes (17–23).

The important relationship between NO and cellular iron metabolism is underlined by the fact that NO can also increase the RNA binding of iron-regulatory protein 1 (IRP1), which plays an important role in regulating intracellular iron homeostasis (for reviews see Refs. 3 and 24). The effect of NO on IRP1 RNA binding activity occurs via two possible mechanisms, a direct effect on the [4Fe-4S] cluster and iron mobilization from cells (25–29). In terms of the ability of NO to increase iron release, we have shown that the NO produced by S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosoglutathione (GSNO), and spermine NONOate (SperNO) can mobilize 59Fe from prelabeled cells as effectively or more effectively than the clinically used iron chelator desferrioxamine (DFO) (29). However, the mechanism of NO-mediated iron efflux remains unknown (29). It has been hypothesized that NO could be released from cells as a complex composed of NO, iron, and thiol-containing ligands such as cysteine or reduced GSH (23, 30, 31).

To investigate the mechanism of NO-mediated iron release, we examined the energy dependence of this process. Surprisingly, we found that the metabolizable monosaccharides, D-glucose (D-Glc) and D-mannose (D-Man), can potentiate NO-mediated iron efflux from a variety of cell types. In contrast, monosaccharides that cannot be transported into cells or that are poorly metabolized (e.g. L-glucose and D-2-deoxyglucose) have no significant influence. The effect of D-Glc on potentiating NO-mediated 59Fe release is not due to any change in cellular viability or an effect of D-Glc on NO production by the NO generators. However, these data indicate that the levels of GSH are crucial in terms of NO-mediated iron release. These results suggest that the metabolism of D-Glc by the hexose monophosphate shunt (HMPs) and the maintenance of GSH levels are essential for NO-mediated iron mobilization. Thus, this study demonstrates that there is an important link between energy and iron metabolism and the interaction of NO.

**EXPERIMENTAL PROCEDURES**

**Cell Treatments and Reagents**

The NO generator SNAP was synthesized by established techniques (32) from the precursor compound N-acetylpenicillamine (Sigma), L-Buthionine-[SR]-sulfoximine (BSO), cytchalasin B, dipiridyl, N-arabinose, D-allose, D-glucose (D-Glc) and D-mannose (D-Man), can potentiate NO-mediated iron efflux from a variety of cell types. In contrast, monosaccharides that cannot be transported into cells or that are poorly metabolized (e.g. L-glucose and D-2-deoxyglucose) have no significant influence. The effect of D-Glc on potentiating NO-mediated 59Fe release is not due to any change in cellular viability or an effect of D-Glc on NO production by the NO generators. However, these data indicate that the levels of GSH are crucial in terms of NO-mediated iron release. These results suggest that the metabolism of D-Glc by the hexose monophosphate shunt (HMPs) and the maintenance of GSH levels are essential for NO-mediated iron mobilization. Thus, this study demonstrates that there is an important link between energy and iron metabolism and the interaction of NO.

**Cell Culture**

The mouse LMTK fibroblast cell line was obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). The human SK-N-MC neuroepithelioma cell line and mouse J774 macrophage lines were obtained from the American Type Culture Collection (Manassas, VA). The human HepG2 hepatoma cell line was kindly provided by Dr. Greg Anderson (Queensland Institute of Medical Research, Brisbane, Australia).

The LMTK and SK-N-MC cell lines were grown in Eagle’s modified minimum essential medium containing 10% fetal calf serum (CSL Ltd., Melbourne, Australia), 1% (v/v) nonessential amino acids (Life Technologies, Inc.), 1 mm sodium pyruvate (Life Technologies, Inc.), 2 mm L-glutamine, 100 μg/ml streptomycin (Life Technologies, Inc.), 100 units/ml penicillin (Life Technologies, Inc.), and 0.28 μg/ml fungizone (Bristol-Myers Squibb Co.). The J774 and HepG2 cell lines were maintained in RPMI 1640 containing D-Glc with the same additions as described above for minimally serum-containing medium. Cells were grown in an incubator (Forma Scientific) at 37 °C in a humidified atmosphere of 5% CO2, 95% air, and subcultured as described previously (35). Cellular growth and viability were monitored using phase-contrast microscopy, cell adhesion to the culture substrate, and trypan blue staining.

In experiments examining the effects of monosaccharides, disaccharides, or pyruvate on 59Fe release from cells, RPMI medium free of D-Glc and pyruvate was used, and the sugars and pyruvate were added at the concentration required.

**Nirite Determination**

The accumulation of nitrite in cell culture supernatants is commonly used as a relative measure of NO production (25, 29, 34). Nitrite was assayed using the Griess reagent that gives a characteristic spectral peak at 550 nm (36).

**ATP Assay**

Cellular ATP levels were measured as described by the Sigma ATP assay kit (catalog number 366) with some modifications. In brief, two 75-cm2 culture flasks containing confluent monolayers of LMTK fibroblasts were used for all measurements. After the appropriate incubation with the metabolic inhibitors (cyanoide, azide, or rotenone) and/or NO, the cells were removed by incubation in 1 ml EDTA/Ca-Mg-free phosphate-buffered saline, collected, and then counted using a Neubauer counting chamber. After this, the cells were homogenized in 0.3 ml of phosphate-buffered saline and 0.3 ml of 12% trichloroacetic acid by vigorous pipetting. This solution was then placed on ice for 5 min, and the supernatant was separated from the pellet by centrifugation at 3000 rpm/10 min/4 °C. The assay was continued as described by the kit.

**Glutathione Assay**

GSH was measured using the GSH assay kit from Calbiochem. The only modifications to the protocol were that 10-fold more cells (2.4 × 107 cells/assay) were used, and the cells were disrupted using two rounds of freeze-thawing before homogenization. These modifications markedly improved the sensitivity of the technique.

**Protein Preparation and Labeling**

Apotransferrin was labeled with 59Fe (Dupont NEN) or 56Fe to produce Fe2+-transferrin (TF) using standard procedures (35). In all studies, fully saturated diferric TF was used. Unbound 59Fe or 56Fe was removed by exhaustive vacuum dialysis against 0.15 M NaCl adjusted to pH 7.4 with 1.4% NaHCO3 (35).

**Efflux Assay of 59Fe From Cells**

**Efflux Assay, General Protocol—Standard techniques were used to examine the effect of NO and other agents on the efflux of 59Fe from prelabeled cells (29, 33). Briefly, cells were labeled with 59Fe-Tf (0.75 μM) for 2 h at 37 °C in D-Glc-containing RPMI. After this incubation, the cell culture dishes were placed on a tray of ice, the medium aspirated, and the cell monolayer washed four times with ice-cold balanced salt solution. The cells were subsequently preincubated for 30 min at 37 °C in the presence or absence of the appropriate sugar, and then a final efflux incubation (2 h at 37 °C) was performed in the presence or absence of the sugar and/or test reagents. After this incubation, the overlying supernatant (efflux medium) was transferred to γ-counting tubes. The cells were removed from the Petri dishes after adding 1 ml of balanced salt solution and by using a plastic spatula to attach them. Radioactivity was measured in both the cell pellet and supernatant using a γ-scintillation counter (LKB Wallace 1282 Compugamma, Finland). In some experiments the efflux medium was passed through a 5-kDa molecular mass exclusion filter (Vivaspin 500, Sartorius AG, Germany) to examine the molecular weight of the 59Fe released.**

**Iron Efflux Assay, Effect of Metabolic Inhibitors—Established procedures were used to examine the effect of metabolic inhibitors on 59Fe mobilization (37, 38). Briefly, cells were labeled for 3 h at 37 °C with 59Fe-Tf (0.75 μM) and washed as described above. The cells were then preincubated for 30 min at 37 °C with rotenone (50 μM), cyanide (5 mM), or azide (30 mM). This medium was subsequently removed, and the cells were incubated for 3 h at 37 °C with medium containing these agents. After this, the overlying supernatant and cells were collected in sepa-
monosaccharides (11 mM) and GSNO (0.5 mM). After this, the overlying
NO generator, GSNO.

Control medium had no effect on 59Fe mobilization (Fig. 1).

Reincubation of prelabeled cells with GSNO in the absence of
D-Glc was added with GSNO (Fig. 1). This experiment was
performed either in the presence or absence of D-Glc.

Iron Efflux Assay, Effect of Agents That Deplete Glutathione—We examined the effect on 59Fe efflux of two agents that deplete
GSH, namely BSO and DEM (39, 40). The cells were incubated for 20 h at
37 °C with BSO (0.01 mM), washed, and then labeled with
59Fe-Tf (0.75 μM) for 2 h at 37 °C. The cells were then washed 4 times and reincubated for 2 h at 37 °C in the presence or absence of GSH, D-glucose, or D-fructose (11 mM). The medium was then removed, and the cells
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rate tubes as described above. Experiments with metabolic inhibitors
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performed either in the presence or absence of D-Glc.
tors (azide, cyanide, and rotenone) were examined on 59Fe mobility. As a relevant control to ensure that the observed 59Fe release was not due to leakage or cell death, iron was also released from control cells incubated at 4 °C, SNAP or PIH did not increase 59Fe release over that seen with GSNO added alone (Fig. 3C). However, as observed with strong iron chelators (54), the greatest increase in 59Fe release from cells was seen after the shortest labeling period with 59Fe-Tf (i.e. 15 min; Fig. 3C). Hence, in the present studies, NO appeared to act like an iron chelator in terms of its ability to bind and release intracellular iron. Furthermore, since a much greater proportion of iron taken up from Tf is in a transit form within cells after shorter rather than longer labeling times (54), it can be suggested that NO is binding iron from a labile pool rather than that stored in ferritin.

Effect of Temperature and Metabolic Inhibitors on NO-mediated 59Fe Efflux—To characterize further NO-mediated iron efllux, the effect of temperature was investigated. As found previously for a number of iron chelators (38), NO-mediated 59Fe release was temperature-dependent. After a 3-h label at 37 °C with 59Fe-Tf (0.75 μM) followed by washing and a 3-h reincubation at 37 °C with SNAP (0.5 mM) or the iron chelator pyridoxal isonicotinoyl hydrazone (PIH; 0.5 mM), these agents increased 59Fe release from 4 ± 1% in control cells to 24 ± 2 and 42 ± 2% (three determinations), respectively. In contrast, at 4 °C, SNAP or PIH did not increase 59Fe release over that observed for control cells, which released 6% of cellular 59Fe (data not shown).

To determine whether NO-mediated 59Fe efflux was dependent on metabolic energy, the effects of three metabolic inhibitors (azide, cyanide, and rotenone) were examined on 59Fe mobilization (Fig. 4, A and B). As a relevant control to ensure ATP depletion, the efflux of the chelator PIH was also examined after incubation with the inhibitors. Previous studies have shown that depletion of intracellular ATP levels using rotenone, cyanide, azide, and other inhibitors results in a marked decrease in PIH-59Fe efflux which occurs by an energy-depend-
Energy Metabolism and NO-mediated Iron Mobilization

Collectively, these results suggested that like Fe efflux the absence or presence of D-glucose and the metabolic inhibitors cyanide, azide, or rotenone, were harvested and assayed for ATP levels as described under “Experimental Procedures.” Results are means ± S.D. of 3 determinations of a typical experiment of 3 performed.

FIG. 5. D-Glc increases 59Fe release from prelabeled cells in the presence of the NO generator GSNO but not strong iron chelators. LMTK fibroblasts were labeled as described in Fig. 1, washed, and then preincubated for 30 min at 37 °C in medium with and without D-Glc (11 mM). After this, the medium was removed and the cells then reincubated for 2 h at 37 °C in the presence and absence of D-Glc (11 mM) and GSNO (0.5 mM), PIH (50 μM), or dipyridyl (0.5 mM). After this, the overlying medium (efflux) and cells were collected in separate tubes. Results are means ± S.D. of 3 determinations of a typical experiment of 3 performed.

had no effect on the ability of DFO (0.5 mM) to increase 59Fe release from labeled LMTK- or SK-N-MC neuroblastoma cells (data not shown). Together, these results suggested that the effect of D-Glc on increasing 59Fe mobilization is relatively specific for NO and was not an effect observed with iron chelators in general.

The Effect of D-Glucose on the Rate of Nitrite Production by NO-generating Agents—It was possible that the stimulatory effect of D-Glc on NO-mediated 59Fe mobilization from cells was due to the ability of this agent to increase NO release from the NO generators as a function of time. To ensure that this was not the case, the NO generators were incubated with D-Glc in the presence and absence of cells, and the production of nitrite was determined as a function of time (Fig. 6). Nitrite was assayed as it is the direct result of NO oxidation and has been widely used to assay NO production by NO generators (55, 56). In each case, the addition of D-Glc to the NO generators had no effect on nitrite production (Fig. 6). Nitrite production by GSNO and SNAP increased linearly as a function of time, whereas nitrite generated by SperNO increased up to 15 min and then plateaued in the absence of cells or slightly decreased in the presence of cells (Fig. 6). The latter slight decrease in nitrite levels after incubation of cells with SperNO was reproducible over 3 separate experiments. Previous studies have shown that the mechanism of NO release from SperNO is specific for NO and was not an effect observed with iron chelators in general.

The Effect of Pyruvate, Citrate, and Reduced Glutathione on NO-mediated Iron Mobilization—D-Glc is metabolized in cells by two main pathways, via the glycolysis/citric acid cycle or by the hexose monophosphate shunt (HMPS). As the D-Glc-mediated increase in NO-mediated iron mobilization from cells could be due to the metabolism of this monosaccharide by either pathway, the effect on iron mobilization of intermediates from both was examined. To assess if the citric acid cycle may be involved, cells were incubated with pyruvate or citrate (1 or 11 mM) (Fig. 7). Both of these latter substrates are metabolized by the tricarboxylic acid cycle and can be efficiently transported into cells and metabolized (58–62). In contrast to D-Glc that markedly increased 59Fe release, pyruvate or citrate had no effect (Fig. 7).

rotenone at inhibiting NO-mediated 59Fe release (compare Fig. 4, A and B), even though D-Glc prevented the inhibitory effect of rotenone on PIH-mediated iron mobilization (Fig. 4B) and helped to reconstitute cellular ATP levels (Fig. 4C). These data suggest that the mechanism of action of rotenone at inhibiting NO-mediated 59Fe release is different from that of cyanide and azide. Collectively, these results suggested that like 59Fe efflux mediated by PIH (37, 38), metabolic energy was necessary for NO-mediated 59Fe mobilization from cells.

Effect of D-Glucose on the Mobilization of Iron Mediated by Strong Iron Chelators—Considering the observation that NO appeared to act like an iron chelator to increase 59Fe release (see Ref. 29 and data above), experiments were performed to assess whether the increase in 59Fe efflux in the presence of D-Glc could also be observed with other strong iron chelators (Fig. 5). Interestingly, D-Glc only stimulated 59Fe release in the presence of NO, the addition of D-Glc having no effect on 59Fe release mediated by PIH or dipyridyl (Fig. 5). Similarly, D-Glc

FIG. 4. Metabolic inhibitors prevent NO-mediated 59Fe mobilization from labeled cells in the absence of D-glucose. LMTK fibroblasts were labeled for 3 h at 37 °C with 59Fe-transferrin (0.75 μM), washed 4 times, and then preincubated for 30 min at 37 °C with cyanide (5 mM), rotenone (50 μM), or azide (30 mM) in the absence (A) or presence (B) of D-glucose (11 mM). After this preincubation, the NO generator GSNO (0.5 mM) or the iron chelator PIH (50 μM) was added in the absence (control) or presence of either cyanide (5 mM), rotenone (50 μM), or azide (30 mM), and the cells were reincubated for 3 h at 37 °C. After this, the overlying medium (efflux) and cells were collected in separate tubes. C, to examine ATP levels, cells incubated as described above in the absence or presence of D-glucose and the metabolic inhibitors cyanide, azide, or rotenone, were harvested and assayed for ATP levels as described under “Experimental Procedures.” Results are means ± S.D. (3 determinations) in a typical experiment of 4 performed.

D-Glc is metabolized in cells and can be efficiently transported into cells and metabolized (58–62). In contrast to D-Glc that markedly increased 59Fe release, pyruvate or citrate had no effect (Fig. 7).

considering the observation that NO release from SperNO is different from the other NO generators examined (57).
When D-Glc is metabolized by the HMPS, there is an increase in the level of NADPH and subsequently the ratio of GSH over GSSG increases (41). Considering this, the change in intracellular redox state may have a critical effect on intracellular iron metabolism. We hypothesized that the metabolism of D-Glc by the HMPS results in an elevation of GSH levels that may affect the redox state of the cell and/or the access of NO to chelatable 59Fe pools. Indeed, previous studies have shown that NO depletes intracellular GSH and then activates the HMPS (63). Similarly, in the present experiments, incubation with NO decreased cellular GSH levels in the absence of D-Glc, whereas when D-Glc was added to the NO generator it markedly prevented GSH depletion (Fig. 8). Statistical analysis of the results from 3 experiments demonstrated that NO caused a significant decrease \((p < 0.0001)\) in GSH levels in the absence of D-Glc. Hence, these data suggest that D-Glc acts as a metabolic substrate to largely prevent GSH depletion in the presence of NO.

Considering that the level of GSH could be a critical factor in NO-mediated iron mobilization, experiments were designed to manipulate the levels of intracellular GSH and assess its effect on 59Fe mobilization. Cells were depleted of GSH using BSO which is a highly selective and potent inhibitor of the enzyme \(\gamma\)-glutamylcysteine synthetase that is involved in GSH synthesis (39) (Fig. 9, A–C). Preincubation of cells with BSO (0.01 mM) alone had no appreciable effect on 59Fe release compared with control cells incubated with medium alone, whereas BSO markedly inhibited NO-mediated 59Fe efflux (Fig. 9A). In the same experiment, to reconstitute cellular GSH levels after exposure to BSO, cells were then incubated with NAC (1 mM) for 20 h, and the effect on NO-mediated 59Fe release was examined. Treatment of BSO-treated cells with NAC slightly increased 59Fe release compared with the control, whereas BSO markedly inhibited NO-mediated 59Fe efflux (Fig. 9A). Measurement of cellular GSH concentrations demonstrated that incubation with BSO reduced GSH levels to less than 40% that seen with the untreated control (Fig. 9C). Statistical analysis of the

**Fig. 6.** D-Glucose does not affect nitrite production by the NO generators, SNAP, GSNO, or SperNO in the presence or absence of cells. The NO generator (0.5 mM) was incubated in the presence or absence of LMTK2 fibroblasts for 5–180 min at 37 °C, and the production of nitrite was measured spectrophotometrically using the Greiss reagent. Results are means ± S.D. (3 determinations) in a typical experiment of 5 performed.

**Fig. 7.** Citrate and pyruvate do not increase NO-mediated 59Fe efflux from cells. LMTK2 fibroblasts were labeled with 56Fe-transferrin (0.75 mM) for 2 h at 37 °C, washed 4 times, and then preincubated for 30 min at 37 °C in the presence or absence of D-Glc (11 mM). The preincubation media were removed, and the cells were then reincubated for 2 h at 37 °C with media containing either D-Glc (11 mM) and/or GSNO (0.5 mM). This medium was then removed, the cells washed, and cellular GSH measured as described under “Experimental Procedures.” Results are mean ± S.D. of 3 determinations of a typical experiment of 3 performed.

**Fig. 8.** NO depletes intracellular reduced GSH, an effect that can be rescued by D-glucose. LMTK2 fibroblasts were labeled with 56Fe-transferrin (0.75 mM) for 2 h at 37 °C, washed 4 times, and then preincubated for 30 min at 37 °C in the presence or absence of D-Glc (11 mM).
results from 4 experiments showed that BSO significantly ($p < 0.00001$) reduced GSH levels compared with the control, and treatment of BSO-treated cells with NAC significantly ($p < 0.00001$) increased GSH.

To confirm the results with BSO, experiments were performed by incubating cells for 1 h at 37 °C with the oxidizing agent DEM (1 mM) which is effective at decreasing cellular GSH levels (40, 64, 65). Treatment with DEM resulted in very similar results to those reported with BSO above, significantly ($p < 0.0001$) decreasing GSH levels and inhibiting NO-mediated $^{59}$Fe release in 3 separate experiments (data not shown). Collectively, the results with BSO and DEM indicated that GSH is essential for NO-mediated $^{59}$Fe mobilization from cells.

In good agreement with the results performed with chelators in the presence or absence of D-Glc (see Fig. 5), a 20-h incubation with BSO markedly decreased $^{59}$Fe release after exposure to GSNO, viz. from 18.2 ± 0.5% to 4.6 ± 1.6% (3 determinations) (Fig. 10). In contrast, BSO had no effect on $^{59}$Fe mobilization mediated by DFO (0.5 mM), dipyridyl (0.5 mM), PHH (50 μM), or 311 (50 μM); (Fig. 10). These results indicate that the involvement of GSH in $^{59}$Fe release was an effect specific for NO rather than iron chelators in general.

**DISCUSSION**

This is the first study to demonstrate that NO-mediated iron mobilization from cells can be markedly influenced by the metabolism of D-Glc. The results are important because they demonstrate a unique relationship between glucose and iron metabolism. This link has previously been speculated to exist based on the finding that IRP1 acts as an RNA-binding protein based on the finding that IRP1 acts as an RNA-binding protein in the absence of iron and also has aconitase activity when iron is present (24).

Our studies demonstrate that the effect of D-Glc at stimulating NO-mediated iron mobilization is due to its ability to be transported into the cell and then subsequently metabolized. Mono- and disaccharides that are not transported though cell membranes or that are poorly metabolized have no effect (Fig. 1). Moreover, the effect of D-Glc at increasing NO-mediated iron release was not due to this monosaccharide acting as an iron chelator, since D-Glc had no effect on iron mobilization from control cells (Fig. 1 and Fig. 3, A and B). In addition, it is of interest that D-tagatose and D-Fuc can chelate iron (50) but did not increase NO-mediated iron release, in contrast to D-Glc and D-Man (Fig. 1). Again, these results suggest that the metabolism of the monosaccharide is important. The effect of D-Glc at stimulating NO-mediated $^{59}$Fe release appears to be due, at least in part, to the presence of reduced GSH within the cell (Figs. 8 and 9). Previous studies have also shown that in the absence of D-Glc there is a decrease in reduced GSH (41). This latter effect is due to depletion of NADPH derived from the HMPS that is necessary for the conversion of oxidized glutathione (GSSG) to its reduced counterpart (41).

We have clearly demonstrated that incubation of cells with NO decreased cellular GSH levels in the absence of D-Glc (Fig. 8). In contrast, when D-Glc was added with the NO generator it markedly prevented the depletion of GSH (Fig. 8). Our results are strongly supported by previous studies, which showed that NO rapidly caused depletion of intracellular GSH due to the formation of intracellular S-nitrosoglutathione which then results in activation of the HMPS (63). In the presence of NO and the absence of D-Glc, the ability of the HMPS to reconstitute cellular GSH levels via the production of NADPH does not appear efficient (Fig. 8). Our results add significantly to the

![Fig. 9. The GSH synthesis inhibitor BSO inhibits NO-mediated $^{59}$Fe mobilization, an effect that can be reversed by incubation of cells with NAC that reconstitutes GSH levels.](image)

![Fig. 10. The GSH synthesis inhibitor BSO prevents NO-mediated $^{59}$Fe mobilization from labeled cells but has no effect on $^{59}$Fe release mediated by strong iron chelators.](image)
observations of Clancy and associates (63) and have implications for both intracellular signaling via the NO–Fe interaction, intermediary metabolism, and NO-mediated cytotoxicity. For instance, the fact that NO markedly influences intracellular iron metabolism may be due, in part, to changes in GSH levels. This in turn could have major effects on pathways that rely on iron-containing enzymes that are vital for energy production (e.g. mitochondrial aconitase; Ref. 4) and DNA synthesis (e.g. ribonucleotide reductase; Ref. 9). Indeed, it is well known that exposure of tumor cells to NO results in the inhibition of energy production and DNA synthesis due to the action of NO on these and other iron-containing proteins (4, 9, 16). This may be important for understanding the large loss of cellular iron observed from tumor target cells (64% after 24 h) after exposure to activated macrophages producing NO (15). For instance, the increase in HMPS activity mediated by NO stimulates GSH synthesis in tumor cells, which then supplies GSH which is involved in iron release.

The process whereby GSH can facilitate NO-mediated 59Fe mobilization could be due to a number of possible mechanisms. For example, GSH may alter cellular redox state by reducing the levels of oxidants such as hydrogen peroxide (41) which may change the intracellular distribution of iron or lead to an increase in the proportion of Fe(II) that may be preferentially bound by NO. However, this hypothesis does not appear compelling, because in contrast to NO-mediated iron efflux, iron mobilization by the Fe(II) chelator dipiridyl or other chelators was not affected after incubation with either d-Glc or BSO (Figs. 5 and 10). Alternatively, the increase in GSH levels after incubation with d-Glc could result in GSH acting as a ligand that together with NO would complete the coordination shell of iron. Such a “mixed iron complex” with both NO and GSH ligands bound to iron may provide an appropriate lipophilic balance to allow diffusion through the cell membrane or transport by an appropriate carrier. Regarding this, it is of considerable interest that electron paramagnetic resonance spectroscopy can detect signals typical of Fe-dithiol dinitrosyl complexes (Fe(RS)2(NO)2) in cells exposed to NO (17–23). It is probable that the effect of NO on stimulating NO-mediated iron mobilization from cells is not just due to its effect on GSH metabolism. Indeed, our experiments have shown that like the iron chelator PIH, NO-mediated 59Fe release is both temperature- and energy-dependent, suggesting a membrane transport mechanism could be involved. Whereas the process that may be responsible for NO-mediated iron efflux has yet to be identified, it is of interest that an iron export molecule known as ferroportin 1 has recently been cloned (66). Whether this transporter can export iron bound to NO or PIH remains a question that requires further investigation.

In summary, this study has demonstrated that NO-mediated iron mobilization can be potentiated by d-Glc due to the transport and metabolism of this monosaccharide. This effect was observed with a range of NO generators (GSNO, SperNO, and SNAP) and cell types. Significantly, the increase in NO-mediated iron release is dependent on the presence of reduced GSH within the cell. Our results clearly demonstrate that there is a relationship between d-Glc and iron metabolism. This may have important implications for intracellular signaling via NO and also NO-mediated cytotoxicity of activated macrophages that is due, in part, to iron release from tumor target cells.

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