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S-Nitrosation of monocarboxylate transporter 1: Inhibition of pyruvate-fueled respiration and proliferation of breast cancer cells

Anne R. Diers^{1,3,†}, Katarzyna A. Broniowska^{1,3,†}, Ching-Fang Chang^{1,3}, R. Blake Hill², and Neil Hogg^{1,3}

¹Department of Biophysics, Medical College of Wisconsin

²Department of Biochemistry, Medical College of Wisconsin

³Redox Biology Program, Medical College of Wisconsin

Summary

Energy substrates metabolized through mitochondria (e.g., pyruvate, glutamine) are required for biosynthesis of macromolecules in proliferating cells. Since several mitochondrial proteins are known to be targets of S-nitrosation, we determined whether bioenergetics are modulated by S-nitrosation and defined the subsequent effects on proliferation. The nitrosating agent S-nitroso-L-cysteine (L-CysNO) was used to initiate intracellular S-nitrosation, and treatment decreased mitochondrial function and inhibited proliferation of MCF7 mammary adenocarcinoma cells. Surprisingly, the D isomer of CysNO (D-CysNO) which is not transported into cells also caused mitochondrial dysfunction and limited proliferation. Both L- and D-CysNO also inhibited cellular pyruvate uptake and caused S-nitrosation of thiol groups on monocarboxylate transporter 1, a proton-linked pyruvate transporter. These data demonstrate the importance of mitochondrial metabolism in proliferative responses in breast cancer and highlight a novel role for inhibition of metabolic substrate uptake through S-nitrosation of exofacial protein thiols in cellular responses to nitrosative stress.

Keywords

redox; thiol; extracellular flux analysis; nitric oxide; nitrosylation

Introduction

An altered metabolic phenotype is an emerging hallmark of cancer [1]. Otto Warburg was the first to make the observation that, compared to their normal counterparts, cancer cells

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To whom correspondence should be addressed: Anne R. Diers, Department of Biophysics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA, Phone: +1 (414) 955-3605, Fax: +1 (414) 456-6512, annerdiers@gmail.com.

[†]Authors contributed equally to this manuscript

This manuscript is dedicated to the fond memory of Victor Fung, Ph.D., a former Program Officer at NCI and former Scientific Review Officer of the Cancer Etiology study section of CSR, NIH, for his wisdom, compassion, integrity, his love of sciences and the arts, his incredible culinary skills, and above all, his contributions to the career development of so many investigators during his own distinguished career.

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preferentially metabolize glucose even in the presence of adequate oxygen (termed the Warburg effect) [2]. Although upregulation of glycolysis is certainly important for the initiation and maintenance of malignancy, we and others have demonstrated that metabolism of energy substrates through mitochondria (e.g., pyruvate, glutamine) is required to support the rapid proliferation of multiple cancer cell types [3–5]. In this context, attention has shifted away from the traditional role of mitochondria as the energy transduction organelle towards a central role for mitochondria in generating building blocks essential for protein, lipid, and DNA synthesis [6]. For example, production of the citric acid cycle intermediate citrate is required for lipid biosynthesis, and blocking its production at the level of ATP citrate lyase attenuates growth of lung, prostate, ovarian, and hematopoietic cancers both *in vitro* and *in vivo* [7]. Thus, targeting cancer metabolism is now thought to be a viable therapeutic strategy [8]. The metabolic phenotype of a cancer cell is driven by concerted alterations in the expression/activity of metabolic substrate transporters, enzymes required to metabolize these substrates as well as the cellular systems used to export resultant byproducts (e.g., lactate excretion as the result of glucose metabolism). The monocarboxylate transporter (MCT) family has garnered much attention for their dual role in exporting lactate from highly glycolytic tumors and importing pyruvate to support mitochondrial function in more oxidative tumors. In fact, both lactate- and pyruvate-dependent mechanisms account for the therapeutic effects of MCT inhibitors in preclinical cancer models [3;9].

Several metabolic enzymes are known targets of S-nitrosation, a nitric oxide (NO)-dependent protein cysteine thiol modification. In all cases, modification of critical cysteine residues inhibits their activity. Mitochondrial Complex I [10] and several glycolytic enzymes, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [11] and aldolase [12], have been implicated as sites of NO-dependent modulation of cellular energy transduction through this modification. The development of low-molecular weight S-nitrosothiols and other cysteine modifying agents as therapeutics (termed ‘redox therapeutics’) to target these pathways has captured significant interest in recent years for the prevention and treatment of cancer, neurodegeneration, cardiovascular disease, and diabetes (reviewed in [13]). The therapeutic efficacy of S-nitrosothiols specifically has been best established in the cardiovascular system, as their ability to reversibly S-nitrosate metabolic enzymes limits ischemia-reperfusion injury [14]. Despite these exciting findings, S-nitrosothiol-based approaches have not been extended to other pathological conditions, such as cancer, in which targeting metabolism may be beneficial.

We have used delivery of the low-molecular weight S-nitrosothiol S-nitroso-cysteine (CysNO) to specifically probe the effects of cellular S-nitrosation in breast cancer and define its potential as a redox-based therapeutic agent. The L-isomer of CysNO (L-CysNO) is taken up into cells via amino acid transporter system L (L-AT) and can transfer the nitroso group to proteins, eliciting S-nitrosothiol-dependent signals [15;16]. In contrast, the D isomer (D-CysNO) can participate in the same chemistry, but is not a good substrate for L-AT [17]; thus, it can be used as a control for S-nitrosothiol uptake-independent processes (shown schematically in Fig. 1a). This approach has also been extremely useful to delineate S-nitrosothiol-dependent pathways and isolate them from the direct effects of NO [11;18].

Here, we define the effects of CysNO isomers on cellular bioenergetics in human mammary adenocarcinoma cells (MCF7). We show that the L isomer of CysNO impairs multiple aspects of mitochondrial function and causes depletion of adenine nucleotide pools. In contrast, the D isomer of CysNO only inhibits the reserve respiratory capacity. Further studies demonstrate that both L-CysNO and D-CysNO inhibit pyruvate uptake into cells, and permeabilization of the plasma membrane alleviates D-CysNO-dependent mitochondrial dysfunction, indicating plasma membrane transport is critical point of regulation. Our

studies link changes in mitochondrial function to attenuated proliferation of breast cancer cells and identify that the pyruvate transporter monocarboxylate transporter 1 (MCT1) is an important target for S-nitrosation by both L- and D-CysNO. These data demonstrate the importance of mitochondrial metabolism in proliferative responses in breast cancer and highlight metabolic substrate transporters as targets of S-nitrosation and potentially other redox therapeutics.

Experimental Procedures

Materials

All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. N-biotinylaminoethyl methanethiosulfonate (bt-MTSEA) was purchased from Toronto Research Chemicals Inc. (North York, Ontario). CysNO was synthesized by incubating cysteine with acidified nitrite as previously described [15].

Cell culture

MCF7 were a generous gift from Dr. B. Kalyanaraman and were maintained in MEMα media (5.56 mM glucose, 1 mM pyruvate, 2 mM glutamine; Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum, 200 U/mL penicillin, 200 µg/mL streptomycin, and non-essential amino acids (10 µM). Cultures were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. Cells were used between passages 4 and 30 after thawing. For all extracellular flux measurements, cells were seeded at 40,000 cells/well in specialized Seahorse Bioscience microplates 24 h prior to assays. For all other experiments, cells at ~80% confluence in 6-well cluster plates were used. For exposure to S-nitroso-cysteine isomers, cells were rinsed with PBS and treated in XF running media (Dulbecco's phosphate buffered saline with Ca⁺⁺ and Mg⁺⁺ supplemented with 5.56 mM glucose, 1 mM sodium pyruvate, 100 µM diethylene triamine pentaacetic acid (DTPA)) for the indicated time.

S-Nitrosothiol measurements

S-Nitrosothiols were detected using triiodide-dependent ozone-based chemiluminescence as previously described [19–21]. Samples were pretreated with 10% (vol/vol) sulfanilamide (100 mM in 2 N HCl) to remove nitrite, and mercury chloride (5 mM) was used to verify the presence of S-nitrosothiols. A standard curve was generated using S-nitroso-glutathione.

Adenine nucleotide extraction and HPLC analysis

Perchloric acid precipitation was used to extract adenine nucleotides as described previously [22]. Solvent A (75 µL; 0.1 M potassium phosphate, 4 mM tetrabutylammonium bisulfate, pH 6.0, and water 64:36) was added to supernatants, and they were filtered and used immediately for HPLC analysis. Protein pellets were resuspended, and protein concentration was determined by the method of Bradford. HPLC analysis of adenine nucleotides, based on a previously described method [23], was performed on Kromasil C-18 column using solvent A and solvent B (0.1 M potassium phosphate, 4 mM tetrabutylammonium bisulfate, pH 6.0, and methanol 64:36) with a flow rate of 1 mL/min. ATP and ADP peaks were measured for each sample and compared to the standards.

Extracellular flux technology

To measure mitochondrial function in intact MCF7 cells, a Seahorse Bioscience XF24 Extracellular Flux Analyzer was used [24]. The mitochondrial function assay employed sequential injection of oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and antimycin A to define a number of mitochondrial parameters such as basal

oxygen consumption rate (OCR), ATP-linked OCR, proton leak, maximal respiratory capacity, reserve respiratory capacity, and non-mitochondrial oxygen consumption [25]. Optimal concentrations of oligomycin and FCCP which elicit maximal responses were determined empirically (Fig. S1). All assays were conducted using a seeding density of 40,000 cells/well in XF running media, and the AKOS algorithm was used to calculate OCR [26]. For experiments using permeabilized cells, XF Plasma Membrane Permeabilizer (XF PMP, 5 nM) was added to a standard Mitochondrial Assay Solution (MAS; 70 mM sucrose, 220 mM mannitol, 10 mM KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES, 1 mM EGTA, and 0.2% (w/v) fatty acid-free BSA, pH 7.2 at 37°C). Permeabilized cells were then provided pyruvate (10 mM) and malate (0.5 mM) as substrates and 4 mM ADP to initiate State 3 respiration immediately prior to beginning the XF assay. A shortened measurement sequence was used (0.5 min mix, 0.5 min wait, 2 min measure) with no 'equilibration' step to minimize the assay length per manufacturer's protocol.

Glucose and pyruvate consumption assays

For analysis of glucose consumption and pyruvate consumption, cells were incubated with indicated compounds in 0.5 mL assay media without FBS supplemented with 5.56 mM glucose or 1 mM sodium pyruvate, respectively. Media levels of glucose and pyruvate were assayed using commercially available kits (BioVision; Mountain View, CA). Consumption of these substrates from the media was calculated and normalized to the total cell number.

Western blotting for MCT1 expression in breast cancer cell lines

Equal amounts of protein from MCF7, T-47D, COH-BR1, MDA-MB468, MDA-MB231, MDA-MB436, MDA-MB453, and SKBR3 human breast cancer cell lysates were resolved using SDS-PAGE, transferred to nitrocellulose membranes, and probed using antibodies specific for MCT1 (Millipore #AB3538P), MCT4 (Santa Cruz #sc-50329), or tubulin (Cell Signaling #2125) with chemiluminescent detection.

MTSEA-bt extracellular thiol labeling and immunoprecipitation

Reduced, solvent-exposed thiols on the cell surface were labeled with biotin-MTSEA (10 mM) for 15 min protected from light, resolved using non-reducing SDS-PAGE, and visualized with Streptavidin conjugate to horseradish peroxidase (HRP) [27]. Pretreatment with DTT (1 mM, 10 min) prior to labeling was used as a control for total cell-surface thiols. Biotinylated proteins were immunoprecipitated with streptavidin beads and probed using antibodies specific for MCT1 and GAPDH (Cell Signaling #2118).

Biotin-switch detection of S-nitrosated proteins

After treatment, cells were subjected to biotin-switch assay using copper and ascorbate as reducing agents with minor modifications [28;29]. Briefly, cells were harvested in lysis buffer containing 50 mM N-ethylmaleimide to block free thiols. S-nitrosated thiols were reduced using copper (II) sulfate (1 μ M) and ascorbate (1 mM) and labeled with biotin-HPDP (1 mM). Biotinylated proteins were immunoprecipitated and probed using antibodies specific for MCT1. Membranes were stained with Amido Black to visualize total protein as previously described [30].

Trypan Blue exclusion assays

After indicated treatment, cells were trypsin-harvested and diluted 1:1 with 0.4% Trypan Blue. The Countess Automated Cell Counter (Invitrogen) was then used to count the total number of cells and score the percentage of cells which excluded Trypan Blue.

Colony formation assay

Colony formation was measured after indicated treatments. Briefly, cells were plated in 6-well cluster plates at low density (100–200 cells per well), and clones were allowed to grow for 7 days in complete culture medium. Cells were then fixed with 70% ethanol and stained with Coomassie blue for analysis of colony formation as previously described [31].

Statistical analysis

Results are reported as means $\pm$ standard error of the mean (s.e.m.) for $n = 3$ biological replicates. Statistical analysis was performed using Origin 8.6 software, and significance was evaluated by two-tailed Student's *t*-test or one-way ANOVA with Tukey post-hoc testing. The alpha value was set at 0.05 for all analyses.

Results

S-Nitrosothiols after exposure to L- and D-CysNO

We first validated the cellular uptake pathways for S-nitroso-cysteine isomers in MCF7 cells by examining the levels of S-nitrosothiols after administration of L- or D-CysNO. As expected, the intracellular nitrosating agent L-CysNO caused a significant, concentration-dependent increase S-nitrosothiols in the low nmol/mg protein range (Fig. 1b,c). In contrast, treatment with the less permeable D isomer caused 70% lower cellular S-nitrosothiol levels (compared to L-CysNO at 100 μ M).

Effect of S-nitroso-cysteine isomers on adenine nucleotide pools

As a general indicator of metabolic pathway activity, we examined the effect of exposure to S-nitroso-cysteine isomers on ATP and ADP levels. MCF7 cells were exposed to increasing concentrations of L- or D-CysNO (5–100 μ M) for 1 h, and ATP and ADP were quantified using HPLC analysis. Consistent with our previous findings in endothelial cells [18], treatment with L-CysNO resulted in a concentration-dependent decrease in ATP and concomitant increase in ADP while D-CysNO had no effect on these parameters (Fig. 2).

Regulation of mitochondrial function by S-nitroso-cysteine isomers

We next sought to explore the effects of L- and D-CysNO on mitochondrial function. To this end, we used extracellular flux analysis to non-invasively monitor basal oxygen consumption rate (OCR) in intact MCF7 cells. Injection of mitochondrial electron transport chain toxins (oligomycin, FCCP, and Antimycin A) was used to probe the effects of S-nitroso-cysteine on multiple mitochondrial function parameters as described previously [30] and shown schematically in Figure S2a [25;30].

Mitochondrial function was assessed in MCF7 cells exposed to increasing concentrations of L- or D-CysNO (5–100 μ M) for 1 h. Representative time-resolved OCR traces are shown for 100 μ M treatments (Fig. S2b). Basal OCR and ATP-linked OCR were inhibited by treatment with L-CysNO (Fig. 3a,b), and D-CysNO affected only ATP-linked OCR at the highest concentration tested (100 μ M). There was little effect of either L- or D-CysNO on proton leak or oxygen consumption independent of Complex IV (Fig. S3a,b). The reserve capacity was the most sensitive to inhibition by S-nitroso-cysteine, and both L- and D-CysNO significantly impaired this mitochondrial function parameter (Fig. 3c).

Effect of S-nitroso-cysteine isomers on metabolic substrate uptake by cells

We previously reported that pyruvate is necessary to support the reserve capacity of MCF7 cells [3], and we confirmed this finding under the conditions used for S-nitroso-cysteine exposure (Fig. S4). Since we observed that both L- and D-CysNO significantly impaired the

reserve capacity, we next examined the effect of administration of S-nitroso-cysteine isomers on uptake of the metabolic substrates glucose and pyruvate. There was no significant effect of L- or D-CysNO on glucose uptake by MCF7 cells (Fig. S5). However, treatment with either isomer of S-nitroso-cysteine decreased cellular pyruvate consumption (Fig. 4a). Interestingly, when pyruvate consumption is plotted against reserve capacity under conditions of S-nitroso-cysteine exposure, there is a direct relationship between the extent of inhibition of pyruvate consumption and the loss of the reserve capacity ($y=0.92x+27.43$, $R^2=0.9826$; Fig. 4b). The ratio of pyruvate uptake to oxygen consumption is approximately 3, as is expected if pyruvate is metabolized solely through mitochondrial oxidative phosphorylation. Additional studies using the mitochondrial pyruvate carrier inhibitor α -cyano-4-hydroxycinnamic acid (CHC) [32] demonstrate it also significantly limits pyruvate consumption by cells and diminishes the reserve capacity (Fig. S6), further supporting the critical role of pyruvate in fueling this aspect of mitochondrial function.

S-Nitroso-cysteine-dependent changes in mitochondrial function in cells provided different metabolic substrates

To determine if the S-nitroso-cysteine-dependent changes in mitochondrial function required pyruvate, we assessed their effects on MCF7 cells provided only glucose as a metabolic substrate in the assay media or glucose and pyruvate. Consistent with our previous study [3], cells provided only glucose or glucose and pyruvate have similar basal respiration (basal OCR, mean $\pm$ s.e.m., $n=3$; for glucose only = 22.7 ± 2.3 pmol/min/ μ g protein; for glucose and pyruvate = 22.8 ± 3.7 pmol/min/ μ g protein); however, glucose alone is not sufficient to support the reserve capacity of MCF7 (Fig. 5b). When one overlays the effects of isomers of S-nitroso-cysteine, it is clear that L-CysNO significantly impairs basal OCR in the presence and absence of pyruvate (Fig. 5a) while the D isomer has no effect. In contrast, both L- and D-CysNO decrease the reserve capacity only in the presence of pyruvate (Fig. 5b).

Identification of the plasma membrane as a site for S-nitroso-cysteine action

As D-CysNO is a much poorer substrate for L-AT, its ability to affect intracellular targets is limited; however, our data indicate it has a significant effect on mitochondrial function in the whole-cell context. This suggests that the point of regulation is on the cell surface and does not require S-nitrosation of an intracellular protein target. To examine this possibility in more detail, MCF7 cells were treated with L- or D-CysNO (100 μ M) for 1 h, washed extensively, and then the plasma membrane was permeabilized using XF PMP, a recombinant perfringolysin O derivative selective for plasma membrane disruption without effects on mitochondrial membranes [33], for analysis of mitochondrial function. L- and D-CysNO-dependent inhibition of the reserve capacity remains after the compounds are removed in unpermeabilized cells (Fig. S7), providing an experimental window in which to examine the effect of plasma membrane permeabilization. Time-resolved OCR traces of the permeabilization protocol are shown in Figure S8. L-CysNO significantly inhibited State 3 respiration while D-CysNO-treated cells were similar to controls (Fig. 6a); however, there was no difference in oligomycin-dependent State 4 respiration in any treatment group (Fig. 6b). This suggests the site of action for L-CysNO-dependent effects on mitochondrial function is at the mitochondrion while D-CysNO clearly acts in an extra-mitochondrial location, potentially through modification of a cell-surface protein target. Together, these data are consistent with the idea that the plasma membrane is an important point of control for mitochondrial respiration after exposure to D-CysNO, likely through regulation of cellular uptake of mitochondrial substrates such as pyruvate.

Effect of S-nitroso-cysteine isomers on colony formation in breast cancer cells

To link the changes in metabolic substrate uptake and mitochondrial function to a biological endpoint, we examined the effect of S-nitroso-cysteine isomers on cancer cell proliferation

and cell death. MCF7 cells were treated with L- or D-CysNO for 4 h, and then Trypan Blue exclusion was assessed as a measure of cytotoxicity. There was no significant decrease in the number of cells which exclude Trypan Blue (scored as viable) after exposure to L- or D-CysNO (Fig. 7a). In contrast, when these cells were seeded at low density and allowed to form colonies over 1 week, there was a significant decrease in colony formation after exposure to either isomer of S-nitroso-cysteine (Fig. 7b).

Examination of exofacial thiol modification after exposure to S-nitroso-cysteine isomers

Our data indicate that both L- and D-CysNO significantly alter cellular pyruvate consumption and mitochondrial function, thus we hypothesized the protein target(s) of S-nitrosation which regulates these effects may not be an intracellular protein thiol, but a solvent-exposed, exofacial thiol on the cell surface. We examined the expression of MCT1 and MCT4 as potential plasma membrane transporters which may mediate the observed effects because of their critical role in monocarboxylate influx/efflux [34]. The expression profile of MCF7 cells was compared to a panel of other human breast cancer cells lines. MCT1 is the predominant MCT isoform in MCF7 as well as COH-BR1 cells (Fig. 8a) while MCT4 is more highly expressed in MB231 cells. Since MCT1 is the isoform better adapted for pyruvate influx as opposed to lactate efflux [35;36], we first sought to determine if it has solvent-exposed thiols which could be targets for S-nitrosation. A cell-impermeable, biotin-tagged thiol label (MTSEA-bt) was used to label exofacial thiols (scheme shown in Fig. S9a), and a number of proteins were labeled under basal conditions (Fig. 8b). In addition, treatment with DTT for 10 min prior to MTSEA-bt labeling resulted in an increase in the number and intensity of protein bands indicating that some cell surface cysteines are reversibly oxidized under basal conditions. MTSEA-bt-tagged proteins were then immunoprecipitated using streptavidin beads, and proteins of interest were identified using Western blotting techniques. MCT1 was present at a similar level in the immunoprecipitated fraction with and without DTT (Fig. 8c; densitometry analysis, mean $\pm$ s.e.m, n=3; 0.50 ± 0.06 for control and 0.29 ± 0.06 for DTT treated) while the intracellular thiol-containing protein GAPDH was absent in both cases. These findings demonstrate that MCT1 has a solvent-exposed, exofacial thiol(s) in the reduced state under basal conditions.

To probe for S-nitrosation of MCT1, the biotin-switch assay was performed on samples prepared from MCF7 cells treated with L- or D-CysNO (100 μ M, 1 h). After reduction with copper and ascorbate (Cu/Asc) and labeling with HPDP-bt (shown schematically in Fig. S9b), MCT1 was detected in immunoprecipitated fractions (Fig. 9a). This suggests that MCT1, or a protein covalently bonded to MCT1, is, in fact, S-nitrosated by both L- and D-CysNO. Moreover, when Cu/Asc was omitted during labeling with HPDP-bt, no protein signal was observed (Fig. 9 and S9c), further supporting this conclusion [37]. The fact that D-CysNO exposure results in less modification of MCT than L-CysNO also suggests this S-nitrosation may have some chiral specificity.

Discussion

The use of compounds which modify protein cysteine residues and control specific cellular responses has emerged as a unique strategy in the development of therapeutic agents. Electrophilic compounds from endogenous (e.g., nitroalkenes, cyclopentenone prostaglandins) and dietary (e.g., curcumin, sulforaphane) sources mediate their biological responses through the post-translational modification of thiols. These compounds have also shown promise as therapeutic agents in preclinical and clinical models of inflammatory conditions, cardiovascular disease, and cancer among others [13]. S-Nitrosating agents such as mitochondrial-targeted S-nitroso-N-acetylpenicillamine (termed 'MitoSNO1') have garnered particular attention for their ability to mitigate ischemia-reperfusion injury by S-nitrosating metabolic enzymes to promote 'gradual reawakening' of metabolic pathways

[38;39]. It is now clear that nearly all aspects of cellular function can be modulated through the post-translational modification of specific protein targets [40], and thus, agents which modify thiol residues have potential use in many pathological contexts.

Here, we have examined the effect of isomers of S-nitroso-cysteine, agents which initiate S-nitrosation of protein thiols, on mitochondrial function and proliferative responses in breast cancer cells. Despite the extensive literature examining the effects of S-nitrosating compounds on metabolic enzyme function, particularly in the area of cardiovascular disease, far fewer studies have examined their potential therapeutic effects in cancer. We used isomers of S-nitroso-cysteine to identify changes in mitochondrial function which can be attributed to S-nitrosation of intracellular protein targets versus extracellular targets. Consistent with our previous findings in endothelial cells [18], L-CysNO significantly depressed mitochondrial function (Fig. 3). We found a concentration-dependent inhibition of basal OCR, ATP-linked OCR, and the reserve capacity. The loss of mitochondrial function was also mirrored by depletion of ATP pools and the accumulation of ADP (Fig. 2). On the other hand, with the exception of the reserve capacity, D-CysNO did not affect these parameters. We and others have largely attributed the effects of S-nitrosating compounds to the modification of glycolytic enzymes (e.g., GAPDH) [11] and components of the mitochondrial electron transport chain (e.g., Complex I) [10]. Recent studies also indicate that the newly identified mitochondrial pyruvate carrier may be redox-sensitive, expanding the role of redox control in mitochondrial function [33]. The particularly surprising aspect of our studies on breast cancer cells, however, was that both isomers of S-nitroso-cysteine impaired the reserve capacity (Fig. 3).

Our previous studies suggest that pyruvate is required to fuel mitochondrial function and, specifically, support the reserve capacity and proliferative responses of breast cancer cells [3]. We have confirmed this finding in the current experimental system (Fig. 5 and S4). The role of pyruvate in supporting proliferative responses is also consistent with the emerging concept that metabolism of energy substrates through the mitochondrial citric acid cycle is required to produce biosynthetic intermediates in rapidly proliferating cancer cells [6;41]. Here, we examined the effect of L- and D-CysNO on pyruvate consumption by cells and found that both S-nitroso-cysteine isomers were capable of inhibiting pyruvate uptake (Fig. 4), but had no effect on glucose uptake (Fig. S5). Moreover, there was a direct correlation between the inhibition of pyruvate consumption and loss of the reserve capacity, and plasma membrane permeabilization studies indicate that the site of action for D-CysNO is not mitochondrial but at the plasma membrane (Fig. 6). Together, these data imply that the protein target(s) involved must not be within the cells, as D-CysNO is not readily transported into cells [17]. However, this is not consistent with any known targets of S-nitrosation [10–12].

A logical explanation for our findings is that the modified protein(s) is present on the cell surface with cysteine residues exposed to the extracellular space. Consequently, we examined the potential role for modification of MCTs themselves. Studies in the 1990s by Poole and Halestrap first identified MCTs in red blood cells using 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) to label and purify the transporters [42]. It was later determined that DIDS also inhibits MCT activity [43;44], and the chemical properties of the isothiocyanate moiety of DIDS make it reactive with both the thiols on cysteine residues [45;46] and primary amines on lysine residues [47]. Our data demonstrate that MCT1 is S-nitrosated after exposure to both isomers of S-nitroso-cysteine (Fig. 9). Very few proteins have been shown to be modified on exofacial thiols [48;49]; thus, MCT1 is a member of a unique proteome of redox-sensitive cell surface proteins.

MCT1 and MCT4 are the isoforms which are most ubiquitously expressed in human tissues and have been shown to be up-regulated in many cancer types [50–52]. Increased expression of these proteins also correlates with higher grade tumors and poor prognosis. Recent studies have also demonstrated that inhibition of MCTs decreased tumor burden in preclinical models of colon cancer [9]. While the structures of MCT family members are not yet available, general topology maps and homology models have been created. The proteins are thought to have twelve transmembrane domains which compose a pore through which monocarboxylate molecules can be transported [53]. While it is yet unknown how these cysteine residues interact within the protein structure as a whole, our data clearly indicated that one or more cysteine residues are solvent-exposed and are in the reduced state under basal conditions (Fig. 8). This is further supported by examination of the rat MCT1 homology model proposed by Wilson et al [54]. Cys106, corresponding to Cys106 of the human homolog, appears to be present on the extracellular face of the transporter. Cys393 (corresponding to human Cys399) seems to be in the lumen of the channel while Cys336 and Cys350 (corresponding to human Cys343 and Cys357), for example, are clearly buried in the lipid bilayer. Interestingly, MCT4 has eleven cysteine residues which are also in similar positions compared to MCT1; thus, it is possible that it is also a target for thiol modification, representing the possibility for redox control of lactate efflux pathways. Alternatively, since MCT1 and MCT4 both require an ancillary protein for transmembrane transport (e.g., basigin) [54], the possibility exists that S-nitrosation of this binding partner may also alter MCT function as has been reported for organomercurial inhibition of these transporters [55]. If a tightly-bound ancillary protein is S-nitrosated, and subsequently biotinylated in the switch assay, MCT1 would likely be co-immunoprecipitated with streptavidin, even if it were itself unlabeled. Consequently, the switch assay cannot *per se* distinguish between MCT1 and covalently-bound partners [56]. Importantly, because S-nitrosation is a nitric oxide-dependent protein modification, it is possible that under (patho)physiologic conditions of elevated nitric oxide production, S-nitrosation is an endogenous control mechanism for MCT activity *in vivo*.

We and others have shown the metabolism of mitochondrial substrates is required for proliferative responses in cancer cells [3–5]. Our data demonstrate that isomers of S-nitroso-cysteine can inhibit mitochondrial function, at least in part, by limiting the uptake of the mitochondrial substrate pyruvate into cells. It is likely that this is also the reason we observed similar effects on colony formation after exposure to either isomer of S-nitroso-cysteine (Fig. 7), demonstrating important functional consequences for redox control of MCT activity; however, we recognize that S-nitroso-cysteine has many protein targets, and thus modification of additional targets may be required for the observed effects.

Taken together, these data demonstrate a novel target for S-nitrosation, MCT1, and highlight the biological effects of modification of MCTs. Moreover, since metabolic pathways are now thought of as relevant targets for the treatment of multiple malignancies, our findings provide increased rationale for the development of therapies which target mitochondrial metabolism, in particular, at the level of metabolic substrate uptake. Finally, compounds which are pleiotropic in nature such as redox therapeutics may be effective therapeutic agents since they have the potential to target multiple aspects of metabolism simultaneously.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

Asc	ascorbate
HPDP-bt	N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide
MTSEA-bt	N-biotinylaminoethyl methanethiosulfonate
Cu	copper
cGMP	cyclic guanosine monophosphate
D-CysNO	S-nitroso-D-cysteine
DIDS	4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid
DTT	dithiothreitol
DTPA	diethylene triamine pentaacetic acid
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HCl	hydrochloric acid
HRP	horseradish peroxidase
L-AT	amino acid transporter system L
L-CysNO	S-nitroso-L-cysteine
MCT	monocarboxylate transporter
MitoSNO1	S-nitroso-N-acetylpenicillamine
NO	nitric oxide
OCR	oxygen consumption rate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sGC	soluble guanylyl cyclase

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Highlights

Exofacial thiols are targets of S-nitrosation

S-nitrosation of MCT1 limits pyruvate uptake

Pyruvate fuels mitochondrial respiration and proliferation

Figure 1.

Effect of S-nitroso-cysteine isomers on S-nitrosothiol levels. Panel (a) shows a scheme for protein S-nitrosation by L- and D-CysNO. MCF7 cells were treated with L- or D-CysNO (50 or 100 μ M) for 1 h, and then cell lysates were prepared for tri-iodide-based chemiluminescent detection of S-nitrosothiols. Representative chemiluminescence traces are shown (b). Injection volume was 40 μ L for each sample except 100 μ M L-CysNO which was 20 μ L. Traces were quantified and normalized to total protein (c), and values represent means $\pm$ s.e.m., $n = 3$ (1-way ANOVA, $\alpha = 0.05$, * $p < 0.05$ compared to control, # $p < 0.05$ compared to D-CysNO at same concentration). Under these conditions, the level of S-nitrosothiols in control samples was below the limit of detection.

Figure 2.

Effect of S-nitroso-cysteine isomers on adenine nucleotide levels. MCF7 cells were treated with increasing concentrations of L- (◆) or D-CysNO (□; 5–100 μM) for 1 h, and then cell lysates were prepared for HPLC analysis of adenine nucleotide levels. ATP (a) and ADP (b) levels were normalized to total protein, and values represent means $\pm$ s.e.m., $n = 3$ (two-tailed Student's t -test, $\alpha = 0.05$, * $p < 0.05$ compared to control, # $p < 0.05$ compared to D-CysNO at same concentration).

Figure 3.

Effect of S-nitroso-cysteine isomers on mitochondrial function. MCF7 cells were seeded in specialized microplates and allowed to attach and grow for 24 h prior to assessment of mitochondrial function. After baseline was established, increasing concentrations of L- (◆) or D-CysNO (□; 5–100 μM) were injected into assay wells as shown in Fig. S2, and basal oxygen consumption rate (OCR) was monitored for 1 h prior to assessment of mitochondrial function parameters using sequential injection of oligomycin (1 μg/mL), FCCP (0.3 μM), and Antimycin A (10 μM). Basal OCR (a), ATP-linked OCR (b), and reserve capacity OCR (c) are shown. OCR were normalized to total protein/well at the end of the assay, and values

represent means $\pm$ s.e.m., $n = 3-4$ (two-tailed Student's t-test, $\alpha = 0.05$, * $p < 0.05$ compared to control, # $p < 0.05$ compared to D-CysNO at same concentration).

Figure 4.

Effect of S-nitroso-cysteine isomers on cellular pyruvate uptake. MCF7 cells were treated with L- or D-CysNO (50 or 100 μM) for 1 h in the presence of FCCP (0.3 μM) to elicit maximal respiration, and then media levels of pyruvate were assayed. Consumption of pyruvate by cells was calculated and normalized to total cell number/well (a). Values represent means ± s.e.m., n = 3–4 (1-way ANOVA, α = 0.05, * p < 0.05 compared to control, # p < 0.05 compared to D-CysNO at same concentration). Pyruvate consumption was plotted against reserve capacity OCR, and linear regression analysis was performed (b).

Figure 5. Effect of S-nitroso-cysteine isomers in the presence of different metabolic substrates. MCF7 cells were seeded in specialized microplates and allowed to attached and grow for 24 h prior to assessment of mitochondrial function. Cells were assayed in media containing glucose only (5.56 mM) or glucose + pyruvate (1 mM). Cells were treated with vehicle (open bars), L-CysNO (100 μ M; closed bars), or D-CysNO (100 μ M; hashed bars) for 1 h prior to assessment of mitochondrial function. Basal OCR (a) and reserve capacity OCR (b) are shown. OCR were normalized to total protein/well at the end of the assay, and values represent means $\pm$ s.e.m., $n = 3-4$ (1-way ANOVA, $3 = 0.05$, * $p < 0.05$ compared to control, # $p < 0.05$ compared to D-CysNO at in same assay media).

Figure 6.

Changes in mitochondrial function in permeabilized cells after treatment with S-nitroso-cysteine isomers. MCF7 cells were seeded in specialized microplates and allowed to attach and grow for 24 h. Cells were then treated with L- or D-CysNO (100 μ M) for 1 h, washed, and treatment media was replaced with MAS containing XF PMP (5 nM), pyruvate (10 mM), malate (0.5 mM), and ADP (4 mM) to initiate State 3 respiration. State 3, oligomycin-dependent State 4 (State 4o), and FCCP-stimulated uncoupled State 3 respiration were assessed using sequential injection of oligomycin (1 μ g/mL), FCCP (two 3 μ M injections), and Antimycin A (10 μ M). State 3 (a) and State 4o (b) are shown. Values represent means $\pm$ s.e.m., $n = 5-7$ (1-way ANOVA, $\alpha = 0.05$, * $p < 0.05$ compared to control, # $p < 0.05$ compared to D-CysNO).

Figure 7.

Effect of S-nitroso-cysteine isomers on Trypan Blue exclusion and colony formation. MCF7 cells were treated with L- or D-CysNO (50 or 100 μM) for 4 h, and the percentage of cells which exclude Trypan Blue was counted at the end of treatment (a). Cells were then seeded at low density (100–200 cells/well), and colony formation was measured after 7 days (b). Values represent means ± s.e.m., n = 3 (1-way ANOVA, α = 0.05, * p < 0.05 compared to control).

Figure 8.

Identification of exofacial thiol-containing proteins using MTSEA-biotin (MTSEA-bt). Expression of MCT1 and MCT4 was examined in a panel of breast cancer cell lines after SDS-PAGE and Western blot analysis (a). Reduced exofacial thiols were labeled with MTSEA-bt (1 mM) in MCF7 cells with or without pre-treatment with DTT (1 mM, 10 min). Proteins were resolved using non-reducing SDS-PAGE, transferred to nitrocellulose membranes, and probed using streptavidin conjugated to HRP (b). Biotinylated proteins were then immunoprecipitated with avidin beads, and MCT1 and GAPDH were detected using Western blot analysis (c). Representative images are shown, n = 3.

Figure 9.

Biotin-switch identification of S-nitrosated MCT1. MCF7 cells were treated with L- or D-CysNO (100 μ M) for 1 h, and then the biotin-switch assay was performed on cell lysates with and without copper (Cu) and ascorbate (Asc) reduction. HPDP-biotin (HPDP-bt) labeled proteins were immunoprecipitated with avidin beads and detected using Western blot analysis and anti-MCT1 antibodies (a). Representative blots are shown. The relative amount of MCT1 which was immunoprecipitated was quantified using densitometry analysis (b). Values represent means $\pm$ s.e.m., $n = 3$ (1-way ANOVA, $\alpha = 0.05$, * $p < 0.05$ compared to control, # $p < 0.05$ compared to D-CysNO).