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Effect of S-nitrosoglutathione on renal mitochondrial function: a new mechanism for reversible regulation of manganese superoxide dismutase activity?

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Abstract

Mitochondria are at the heart of all cellular processes as they provide the majority of the energy needed for various metabolic processes. Nitric oxide has been shown to have numerous roles in the regulation of mitochondrial function. Mitochondria have enormous pools of glutathione (GSH $\approx$ 5–10 mM). Nitric oxide can react with glutathione to generate a physiological molecule, S-nitrosoglutathione (GSNO). The impact GSNO has on mitochondrial function has been intensively studied in recent years, and several mitochondrial electron transport chain complex proteins have been shown to be targeted by GSNO. In this study we investigated the effect of GSNO on mitochondrial function using normal rat proximal tubular kidney cells (NRK cells). GSNO treatment of NRK cells led to mitochondrial membrane depolarization and significant reduction in activities of mitochondrial complex IV and manganese superoxide dismutase enzyme (MnSOD). MnSOD is a critical endogenous antioxidant enzyme that scavenges excess superoxide radicals in the mitochondria. The decrease in MnSOD activity was not associated with a reduction in its protein levels and treatment of NRK cell lysate with dithiothreitol (a strong sulfhydryl-group-reducing agent) restored MnSOD activity to control values. GSNO is known to cause both S-nitrosylation and S-glutathionylation, which involve the addition of NO and GS groups, respectively, to protein sulfhydryl (SH) groups of cysteine residues. Endogenous GSH is an essential mediator in S-glutathionylation of cellular proteins, and the current studies revealed that GSH is required for MnSOD inactivation after GSNO or diamide treatment in rat kidney cells as well as in isolated kidneys. Further studies showed that GSNO led to glutathionylation of MnSOD; however, glutathionylated recombinant MnSOD was not inactivated. This suggests that a more complex pathway, possibly involving the participation of multiple proteins, leads to MnSOD inactivation after GSNO treatment. The major highlight of these studies is the fact that dithiothreitol can restore MnSOD activity after GSNO treatment. To our knowledge, this is the first study showing that MnSOD activity can be reversibly regulated in vivo, through a mechanism involving thiol residues.

Keywords

Nitrosoglutathione; S-glutathionylation; MnSOD; Mitochondria; Respiration; Diamide; Free radicals

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Nitric oxide is known to have an impact on mitochondrial function in numerous ways, including inhibition of mitochondrial respiration [1,2]. Many postulate that the biological effects of nitric oxide are mediated through posttranslational modification (S-nitrosylation) of critical thiol residues within mitochondrial proteins [3,4]. There have been numerous reports focusing on identifying protein targets of S-nitrosylation using supraphysiologic concentrations of the NO donor and nitrosylating agent *S*-nitrosoglutathione (GSNO) [5–7]. Many of these studies not only used high concentrations of GSNO but also simply treated pure proteins with GSNO. One purpose of this paper was to determine the effect that GSNO has on mitochondrial function of rat renal proximal tubule cells. Our laboratory has previously demonstrated that inactivation of manganese superoxide dismutase (MnSOD) during both human and rat chronic renal allograft nephropathy involves increased nitric oxide, superoxide, and peroxynitrite formation [8–10]. Consequently, the experiments in this study evaluated MnSOD activity as one endpoint of mitochondrial injury after GSNO treatment.

GSNO has been shown to be detectable at a concentration of 0.34 nmol/mg protein in liver mitochondria [11] and 6–8 μ M in rat cerebellum [12] and thus is considered a physiologically relevant nitrosylating agent [13]. GSNO can also react with mitochondrial protein thiols producing a mixed disulfide via S-glutathionylation [14,15]. Glutathione (GSH), the most abundant thiol in mammalian cells (0.5–10 mM), is often implicated in S-glutathionylation. The precise mechanisms leading to protein S-nitrosylation or S-glutathionylation remain unclear, but clearly GSNO can carry out both reactions (nicely reviewed in [16,17]). High concentrations of GSNO (0.5–5 mM) have been used to treat isolated mitochondrial fractions [5,7], recombinant proteins [18,19], or intact cells [20,6].

Dithiothreitol (DTT) is a widely used thiol group reducing agent. It effectively reduces the accessible intramolecular and intermolecular disulfide bonds in the proteins [21]. It is commonly used to reduce the posttranslational modifications induced on the thiol group of proteins and restore the original protein thiol group to its native reduced state.

Diamide (diazenedicarboxylic acid bis(*N,N*-dimethylamide)), first described by Kosower and associates, is widely used as a catalyst to promote protein–glutathione mixed disulfides (PSSGs; a process known as S-glutathionylation) [22]. It rapidly penetrates the cells and preferentially oxidizes low-molecular-weight thiols such as GSH compared to other low-molecular-weight thiols and protein thiols [22,23]. Diamide has been used extensively to study the effect of S-glutathionylation on protein and/or cell function [24–26].

Our results provide novel evidence that MnSOD is inactivated by GSNO and diamide in rat kidney cells as well as isolated kidneys. Dithiothreitol is able to reverse the MnSOD inactivation in both the cells and the rat kidneys, implicating a role for thiol groups in the observed inactivation. The immunoprecipitation studies revealed a modest increase in GSH adduct on the MnSOD in NRK cells upon GSNO treatment, demonstrating that MnSOD is a target for S-glutathionylation. But glutathionylation of recombinant human MnSOD did not alter its activity in vitro, suggesting that S-glutathionylation in itself is not the major mechanism responsible for the loss of MnSOD activity we observed upon GSNO or diamide

treatment in NRK cells and the rat kidney. In summary, these results show that GSNO leads to MnSOD inactivation in renal cells in a GSH- and thiol-dependent mechanism that probably involves the interaction of multiple proteins.

Materials and methods

Materials

Dulbecco's modified Eagle medium (DMEM), nuclear counter-stain DAPI, goat anti-rabbit IgG Alexa-594 antibody, *N*-(biotinoyl)-*N'*-(iodoacetyl)ethylenediamine (that is, biotin-iodoacetamide, BIAM), and *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl iodoacetamide (BODIPY-IAM) were purchased from Invitrogen (Carlsbad, CA, USA). *S*-nitrosoglutathione was obtained from EMD4 Biosciences (Gibbstown, NJ, USA). Diamide, L-buthionine sulfoximine (BSO), DTT, NADH, cytochrome *c* (II), bovine serum albumin, 3-nitrotyrosine, and *N*-ethylmaleimide (NEM) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Coomassie Plus protein assay reagent, Super Signal West Pico chemiluminescent substrate, NeutrAvidin Plus Ultralink resin, and protein A/G beads were purchased from Pierce (Thermo-Scientific) (Rockford, IL, USA). Amicon Ultra centrifugal filters (10,000 K MWCO) were obtained from Millipore. 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide (JC-1) was purchased from Molecular Probes (USA). Rabbit polyclonal anti-MnSOD antibody for Western blot, rabbit polyclonal anti-nitrotyrosine antibody, and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibodies were obtained from Milli-pore (Billerica, MA, USA). Rabbit polyclonal anti-MnSOD antibody used for MnSOD immunoprecipitation was purchased from Enzo Lifesciences. Mouse monoclonal anti-GSH antibody was obtained from Virogen (USA). Rabbit polyclonal β -actin antibody was from Abcam. Precision Plus Kaleidoscope molecular weight marker for Western blot was purchased from Bio-Rad (Hercules, CA, USA).

Renal cell model

Normal rat kidney proximal tubular cells (NRK-52E; American Type Culture Collection No. CRL-157, USA) were maintained in a humidified incubator gassed with 5% CO₂, 95% air at 37 °C in DMEM containing 5% fetal calf serum. Cells were grown to 60% confluency and then divided into four treatment groups: (1) untreated, (2) GSNO treated (100 μ M, 24 h), (3) diamide treated (200 μ M, 24 h), (4) BSO pretreated (0.5 mM, 24 h) and then GSNO or diamide treated. Untreated cells and cells during respective treatments remained in serum-free DMEM at 37 °C. After treatment, cells were trypsinized in 0.25% trypsin/EDTA and lysed by sonication. After lysis, the supernatant was assayed for protein concentration by Bradford assay using Coomassie Plus protein assay reagent. In some experiments, lysates were deglutathionylated/denitrosylated by treatment with DTT (0.5 mM for 30 min at room temperature), followed by ultrafiltration using Amicon Ultra centrifugal filters to remove excess DTT.

In vivo rat studies

Animals were treated according to strict University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee guidelines. Male inbred Fisher 344 rats

(Charles Rivers Laboratories, Wilmington, MA, USA) weighing 250–300 g were used in this study. Ethrane (enflurane) was used to anesthetize the rats, followed by shaving and prepping with betadine. A 2-ml bolus of 0.9% (w/v) NaCl was administered intravenously, and an incision was made 1 cm superior to the symphysis pubis to the tip of the xiphoid process. Clamps were placed on the aorta and vena cava proximal and distal to both kidney vessels. A small catheter was inserted into the aorta, and a vent was created in the vena cava close to the junction of renal vessels. Both kidneys were perfused with normal saline, the right kidney was quickly removed, and the left kidney was perfused further with saline containing diamide (0.5 mM for 5 min) to initiate protein S-glutathionylation. Renal extracts were made from frozen tissue by homogenizing (0.1 g/ml) using a Polytron homogenizer in buffer containing 50 mM potassium phosphate, pH 7.4, and 1 mM phenylmethanesulfonyl fluoride (PMSF). Solubilized extracts were sonicated and centrifuged at 10,000 rpm (5 min, 4 °C) to remove tissue debris. Protein concentrations were determined by the Bradford assay using Coomassie Plus protein assay reagent.

Mitochondrial membrane potential assessment

The relative mitochondrial membrane potential was determined using a lipophilic cationic probe, JC-1. Cells were incubated in the dark with JC-1 (7.5 μ M for 30 min). Fluorescence was observed in cells grown on coverslips in six-well dishes, using a Nikon Eclipse 800 microscope with a 60 $\times$ water immersion objective equipped with a dual filter for fluorescein and rhodamine. Green staining is indicative of the monomeric form of JC-1 (i.e., lower membrane potential) and the red staining corresponds to the aggregate form (i.e., higher membrane potential). For quantification, cells were grown on specialized coverslips and measured for fluorescence emission at 529 nm (monomer, Green) and 590 nm (J-aggregates, red) in a Hitachi spectrofluorimeter equipped with a coverslip holder using an excitation wavelength of 488 nm. Relative membrane potential was calculated using the ratio of J-aggregate/monomer (590 nm/530 nm).

Measurement of mitochondrial respiratory complex activity

NRK cell mitochondria were isolated by centrifugation on a sucrose density gradient, and the activity of mitochondrial complexes was assayed spectrophotometrically at 30 °C, as previously described [27]. Complex I (NADH:ubiquinone oxidoreductase) activity was measured by the oxidation of NADH at 340 nm. Complex IV (cytochrome *c* oxidase) activity was assessed by following the oxidation of reduced cytochrome *c* at 550 nm. The changes in absorbance in blank samples (containing no mitochondria) were recorded for all assays.

Western blot analysis

MnSOD Western analysis was performed using the polyclonal anti-MnSOD antibody (1:1000), and anti-GAPDH (1:1000) or anti- β -actin (1:1000) was used as a loading control. To evaluate the overall cellular protein S-glutathionylation status, Western blot analysis was performed according to the protocol adopted from Hill et al. [28]. Cells were lysed in phosphate buffer containing 25 mM NEM (NEM alkylates the available free protein thiols and prevents any further thiolation reactions during sample processing). The samples were

run under nonreducing conditions for the anti-GSH Western blot. Membranes were blocked in 5% milk for 1 h; however, for the PSSG adducts Western analysis, 2.5 mM NEM was added to the 5% milk during the blocking step to protect the GSH adducts from reduction by thiol-containing proteins in the milk and thereby maximized the PSSG signal on anti-GSH Western blots [28]. Membranes probed with anti-GSH primary antibody were incubated in TBS/Tween (0.1%) at 4 °C overnight as opposed to milk in the case of anti-MnSOD and anti-GAPDH antibodies. The use of TBS/Tween (0.1%) instead of milk for dilution of anti-GSH antibody was according to the Hill et al. protocol [28], to maximize the PSSG signal on Western blot. Probed membranes were washed three times in TBS/Tween (0.1%) the following day and immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

MnSOD activity

Enzymatic activity of MnSOD was determined in renal cell or tissue extracts by the cytochrome *c* reduction method in the presence of 1 mM KCN to inhibit Cu,ZnSOD activity, as previously described [29].

Recombinant MnSOD experiments

Recombinant human MnSOD (rMnSOD) expressed in an *Escherichia coli* system was prepared as previously described [10]. Briefly, 15 μ M (0.36 mg/ml) rMnSOD was incubated with varying concentrations of GSNO (1, 10, 30, 100, 300, 1000 μ M) for 1 h at room temperature in 50 mM potassium phosphate buffer (pH 7.4).

Nitrotyrosine immunocytochemistry

NRK cells were washed with cold phosphate-buffered saline (PBS), fixed for 15 min with 4% formalin, washed with PBS, and permeabilized with 0.1% Triton X-100/0.1% sodium citrate for 2 min on ice. Cells were then washed with PBS and blocked with 3% bovine serum albumin in PBS for 1 h, followed by overnight incubation at 4 °C with the rabbit polyclonal anti-nitrotyrosine antibody (1:200). The following day, the cells were washed with PBS–Tween (0.1%) and then PBS and incubated with the goat anti-rabbit IgG Alexa-594 antibody (1:1000) for 30 min in the dark at room temperature (RT). Cells were rinsed with PBS–Tween (0.1%), and nuclear counterstaining was initiated using DAPI (1:100) for 10 min at RT. Subsequently, cells were washed and coverslipped with Prolong Gold antifade reagent with DAPI. Nitrotyrosine staining was evaluated with a Nikon Eclipse 800 microscope (40 $\times$ oil). All images were captured with equal exposure times. NRK cells treated with peroxynitrite (0.8 mM) in PBS for 5 min at RT served as positive controls. The negative controls were NRK cells treated with peroxynitrite but the nitrotyrosine antibody was preincubated with excess 3-nitrotyrosine (10 mM) before being added to permeabilized cells.

Immunoprecipitation of MnSOD

NRK cells were lysed by incubation in 50 mM phosphate buffer containing 1% Triton, 1 mM PMSF, and 25 mM NEM, for 30 min at 4 °C, followed by centrifugation at 14,000 g for 10 min. A final concentration of 2 mg/ml solubilized protein was precleared with 25 μ l

protein A/G beads followed by overnight incubation with 15 µg anti-MnSOD antibody at 4 °C. On the next day, the immune complexes were precipitated by 25 µl protein A/G beads (3.5 h at 4 °C). The beads were washed and resuspended in 45 µl nonreducing sample loading buffer, boiled for 5 min at 95 °C, and followed by SDS–PAGE. Anti-GSH Western blotting was then performed as described above to detect glutathionylated MnSOD. The same blot was stripped and reprobed with anti-MnSOD antibody to analyze the amount of MnSOD immunoprecipitated.

BODIPY-IAM labeling

A snapshot of thiol status was obtained by BODIPY-IAM labeling of NRK cells according to the method described earlier by Hill et al. [30]. Briefly, untreated or GSNO-treated cells were incubated with freshly prepared BODIPY-IAM (50 µM; 30 min at 37 °C). Cells were lysed in phosphate buffer containing 1 mM DTT to get rid of excess BODIPY-IAM, followed by SDS-PAGE. The gel was then imaged for BODIPY fluorescence using a FluorChem 8900 imager from Alpha Innotech.

BIAM labeling

This was performed as previously described [31] with some modifications. Briefly, untreated and GSNO-treated cells were incubated with freshly prepared BIAM (50 µM; 30 min at 37 °C). Cells were lysed in buffer containing 20 mM NEM (NEM alkylates free thiols and thus terminates any further reaction of BIAM). BIAM-labeled proteins were immunoprecipitated by overnight incubation with 50 µl neutravidin resin at 4 °C. The following day, neutravidin beads were washed and resuspended in 45 µl reducing sample loading buffer and boiled for 5 min at 95 °C, followed by SDS–PAGE. Western blot for MnSOD was then performed as described above.

GSH measurement

GSH content in the NRK cells was determined using a modification of the Elman procedure [32].

Cell cytotoxicity assessment

NRK cytotoxicity was determined using the LDH-Cytotoxicity Assay Kit II (Biovision Research Products, USA). This kit uses a water-soluble tetrazolium salt reagent to react with NADH produced by lactate from lactate dehydrogenase (LDH), to give an intense yellow color directly correlating with the amount of LDH in the medium. Absorbance was measured at 450 nm using a SpectraMax 190 microplate reader using SpectraMax software (Molecular Devices, USA).

Statistical analysis

Results are presented as means±standard error of the mean (SEM). Means were obtained from at least three independent experiments. One-way analysis of variance was used to compare the mean values among the untreated and the treated groups, followed by Tukey's test to compare differences in means between two groups at the 95% level of confidence

using the Origin 6.0 statistical software. Differences with a *P* value less than 0.05 was considered statistically significant.

Results

The first sets of experiments were done to determine the optimal concentration of GSNO to be used in our normal rat kidney proximal tubular (NRK) cells. As mentioned earlier, previous experiments have used recombinant proteins or isolated mitochondria, rather than intact cells, and relatively high concentrations of GSNO (0.5–5 mM) [5,6,18,19]; the goal of this study was to evaluate the effect of GSNO at a concentration that did not result in excessive cell death.

NRK cells were treated with relatively low concentrations of GSNO (100 or 200 μ M) for 24 h. Our data showed that NRK cells treated with GSNO (100 μ M) for 24 h showed a small but significant increase in cell death (GSNO treated 5.69773 ± 0.64337 vs untreated 1.43333 ± 1.33333 , percentage cell cytotoxicity), when assayed using the LDH cytotoxicity kit. The cells treated with 200 μ M GSNO had obvious signs of toxicity (detached and shrunken cells). Therefore, for the remainder of experiments, cells were treated with GSNO (100 μ M) to determine the effect on mitochondrial function (membrane potential, mitochondrial respiratory chain activity, and MnSOD activity).

GSNO treatment induced mitochondrial membrane depolarization

One way to assess mitochondrial function is to measure the relative mitochondrial membrane potential, generated by oxidative phosphorylation, using the cationic probe JC-1. NRK cells incubated with GSNO (100 μ M; 24 h) exhibited significantly lower (depolarized/green) relative mitochondrial membrane potential compared to untreated cells, which show predominantly red staining (Fig. 1A). For quantitative purposes, the experiments were repeated using fluorescence spectrophotometry, which also showed a decrease in relative membrane potential, which is assessed by the intensity of JC-1 aggregates/monomers (590 nm/530 nm) (GSNO-treated cells 1.25367 ± 0.26811 vs untreated cells 5.673 ± 1.19137 ; Fig. 1B).

GSNO induced inactivation of mitochondrial complex IV activity

The activities of mitochondrial electron transport chain complexes were measured to analyze whether GSNO affects NRK cell mitochondrial respiratory function. Subunits within complex I have previously been shown to be modified by GSNO [5,6], but interestingly under conditions tested here complex I activity was unchanged after GSNO treatment of NRK cells, compared to untreated cells (Fig. 2A); complexes II and III were also not altered after GSNO treatment (data not shown). However, GSNO treatment led to a significant decrease in complex IV activity in NRK cells compared to the untreated cells (Fig. 2B). Complex IV, also known as cytochrome oxidase, is the last electron acceptor complex in the mitochondrial membrane electron transport chain.

GSNO treatment led to inactivation of MnSOD

Our laboratory has previously shown that MnSOD is inactivated via a peroxynitrite-mediated mechanism [10]. Because GSNO is considered a NO donor and given the effects on mitochondrial function shown above, we sought to determine whether GSNO treatment of NRK cells reduces MnSOD activity. As shown in Fig. 3B, GSNO treatment of NRK cells led to a significant decrease in the activity of MnSOD. There was no change in the protein levels of MnSOD after GSNO treatment (Fig. 3A). This verifies that the observed decrease in activity is not due to a reduction in MnSOD protein after GSNO treatment. Therefore, this suggests the possibility of a posttranslational modification of MnSOD playing a role in the GSNO-mediated inactivation. Because GSNO is a known nitrosylating and glutathionylating agent, one method of assessing this type of thiol modification is to treat samples with the reductant DTT (which is known to cleave the nitroso or glutathione moiety attached to protein thiol groups) and reassess activity. Therefore, NRK cell lysates were treated with DTT (0.5 mM; 30 min at room temperature), filtered, and assayed again. Interestingly, DTT treatment restored the MnSOD activity to untreated values, suggesting that the reversible regulation of MnSOD activity involves thiol residues (Fig. 3A). Interestingly, DTT treatment also restored complex IV activity to near the untreated cell values (Fig. 2).

GSNO treatment did not increase nitrotyrosine levels

Earlier studies in our laboratory [8–10,33–36], and others [35,37–43], have shown that MnSOD can be inactivated by nitration of its tyrosine residues. Nitrotyrosine is a footprint of peroxynitrite generation; therefore, nitrotyrosine immunocytochemistry of NRK cells was evaluated to determine whether GSNO increased peroxynitrite generation. No nitrotyrosine staining was evident in NRK cells incubated with GSNO for 24 h (Figs. 4A and 4B). However, as a positive control, NRK cells treated with peroxynitrite (0.8 mM) did show increased nitrotyrosine staining, which was effectively blocked when the nitrotyrosine antibody was preabsorbed with excess 3-nitrotyrosine (Figs. 4C and 4D).

Is MnSOD glutathionylated?

Immunoprecipitation of MnSOD was carried out to study its glutathionylation status after GSNO treatment. Here, we provide novel evidence that MnSOD is a target for S-glutathionylation. As shown in Fig. 5A, anti-GSH Western blot of immunoprecipitated MnSOD from NRK cells demonstrated a modestly increased glutathionylated MnSOD in GSNO-treated cells compared to untreated cells. In addition, treatment of recombinant human MnSOD with GSNO also leads to robust glutathionylation of the enzyme in vitro (Fig. 5B). However, much to our surprise, GSNO treatment of rMnSOD did not alter enzymatic activity (data not shown). As stated in the above results, thiol residues are involved in its observed inactivation because DTT restores its activity. Therefore, these results suggest that GSNO-mediated inactivation of MnSOD seems to occur via an indirect mechanism in vivo, rather than via direct glutathionylation of MnSOD.

GSH is required for GSNO-mediated MnSOD inactivation

S-glutathionylation involves adding a GSH moiety to the thiol or sulfhydryl (SH) group of an available cysteine residue within a protein. Therefore, GSH levels were depleted in NRK

cells using BSO, which irreversibly inhibits the enzyme γ -glutamylcysteine synthetase leading to GSH depletion. BSO (0.5 mM; 24 h) treatment led to an approximately 90% reduction in NRK cell GSH levels compared to control untreated cells. Additionally, diamide, a known strong thiol-oxidizing agent, which has been shown to induce protein S-glutathionylation, was also used [44,45].

Western blot analysis of NRK cell lysates showed that diamide and GSNO significantly increased the overall cellular protein glutathionylation compared to untreated cells (Fig. 6A). Pretreatment with BSO significantly decreased the levels of glutathionylated proteins in GSNO-treated cells, implying that GSH is an essential mediator for GSNO-induced glutathionylation of NRK cellular proteins.

Because diamide increased and BSO decreased the level of glutathionylation (Fig. 6A) in GSNO-treated NRK cells, we sought to determine whether these agents altered MnSOD activity. Pretreatment with BSO (0.5 mM; 24 h before GSNO treatment) completely blocked the GSNO-mediated inactivation of MnSOD (Fig. 6B). The protein levels of MnSOD in NRK cells remain unchanged after GSNO and/or BSO treatment (Fig. 6D) and there was no cell toxicity associated with BSO treatment alone (data not shown). These results suggest that GSH is necessary for the GSNO-mediated MnSOD inactivation. Treatment of NRK cells with diamide (200 μ M; 24 h) reduced MnSOD activity significantly compared to untreated cells and DTT treatment partially reversed the diamide-mediated MnSOD inactivation (Fig. 6C). In addition, BSO pretreatment (0.5 mM; 24 h before diamide treatment) also reduced the extent of MnSOD inactivation in NRK cells treated with diamide (Fig. 6C). In summary, the above results with both GSNO and diamide indicate a unique cellular GSH-dependent mechanism, which is responsible for reversible regulation of MnSOD activity in vivo, and this involves thiol residues.

Further characterization of NRK cell thiol alterations mediated by GSNO

Because DTT reversed the GSNO-mediated MnSOD inactivation, additional experiments were designed to determine the status of the reactive thiol proteome in NRK cells and also in particular the MnSOD thiol residues after GSNO treatment. The fluorophore-BODIPY-labeled iodoacetamide (IAM, a known thiol alkylating agent) was used to evaluate the reactive thiol proteome of NRK cells. IAM serves to bind to the free reactive thiols on proteins. As shown in Fig. 7A, untreated cells showed a comparatively higher amount of BODIPY fluorescence intensity compared to GSNO-treated cells, indicating that untreated cells had higher levels of free reactive thiols compared to GSNO-treated cells. Densitometry quantification of the BODIPY fluorescence showed that the amount of free thiols labeled by BODIPY-IAM was decreased by approximately 30% in GSNO-treated cells compared to the untreated cells. Therefore, GSNO treatment leads to a decrease in the availability of free reactive thiols in the NRK cells. When NRK cells were first treated with BODIPY-IAM followed by MnSOD immunoprecipitation, no distinct BODIPY signal for MnSOD was apparent, although MnSOD was efficiently immunoprecipitated as confirmed by MnSOD Western blot (data not shown).

Another approach using BIAM labeling of thiols was also applied to study the thiol status within MnSOD. The cells were treated with BIAM and the biotin-tagged proteins were then

immunoprecipitated with neutravidin beads, followed by MnSOD Western blot to detect MnSOD thiol status (Fig. 7B). GSNO appeared to reduce available thiols within MnSOD, consistent with glutathionylation of MnSOD as shown in Fig. 5A. Densitometry quantification of the MnSOD Western blot after neutravidin immunoprecipitation showed a 30% decline in MnSOD free thiols in GSNO-treated cells compared to the untreated cells.

MnSOD activity was decreased in rat kidneys treated with diamide

Systemic injection of diamide is toxic; therefore rat kidneys were isolated and perfused in vivo with saline containing diamide (0.5 mM; 5 min) to determine whether MnSOD activity could be altered in vivo after diamide treatment. As shown in Fig. 8, diamide perfusion resulted in a significant decrease in MnSOD activity compared to control unperfused kidneys. Interestingly, DTT treatment of the diamide-perfused renal homogenates restored the MnSOD activity to control values, suggesting that even in intact kidneys, MnSOD activity appears to be reversibly regulated by a novel thiol-dependent modification.

Discussion

The goal of this study was to determine whether GSNO treatment of intact NRK cells alters mitochondrial function. We demonstrated that GSNO treatment (100 μ M; 24 h) resulted in mitochondrial membrane depolarization, impaired complex IV activity, and MnSOD inactivation. Treatment with GSNO (100 μ M) for only 4 h also resulted in mitochondrial membrane depolarization, but did not alter the activities of electron transport chain complexes or MnSOD (data not shown), suggesting that mitochondrial depolarization preceded MnSOD inactivation. Interestingly, DTT treatment of renal cell lysates reversed the inactivation of complex IV and MnSOD, consistent with the hypothesis that GSNO was inducing thiol modifications that altered the activity of these proteins.

The precise mechanism leading to GSNO-induced complex IV inactivation is unknown, but complex IV has been shown to be a target for S-nitrosylation-induced inactivation [46], which could partially explain the GSNO and DTT response observed in our studies. It was indeed interesting that DTT had a partial effect on complex IV activity compared to complete restoration of MnSOD activity. One of the reasons could be the dose of DTT used (0.5 mM), as many studies use higher millimolar concentrations of DTT to reverse nitrosylation and/or glutathionylation.

GSNO has been shown to lead to S-nitrosylation [5,6,47] and S-glutathionylation [16,48] of several mitochondrial proteins; however, our new data provide the first evidence that MnSOD is altered by S-glutathionylation. One possible explanation for this oversight is that many of these studies utilized isolated mitochondrial membranes, which would not contain MnSOD, because MnSOD is located within the mitochondrial matrix. Subunits within complexes I and IV have previously been shown to be inactivated via S-glutathionylation [14,49,50]. In addition, subunits within complex I have been shown to be nitrosylated [5,7], glutathionylated [44], and tyrosine nitrated [51]. Our results showed inactivation of mitochondrial complex IV after 24 h GSNO treatment, whereas the activities of mitochondrial complexes I, II, or III were not significantly altered.

Because there was no increase in nitrotyrosine staining (footprint of peroxynitrite generation) in GSNO-treated NRK cells, it appears that peroxynitrite is not involved in the observed inactivation of MnSOD. Rather, it seems that GSNO is inducing MnSOD inactivation via a novel mechanism. This mechanism involves a key role of thiol residues because DTT treatment restored MnSOD activity. Immunoprecipitation studies show that GSNO treatment led to a modest increase in glutathionylated MnSOD (Fig. 5A), suggesting that MnSOD is a target for S-glutathionylation. GSNO also leads to glutathionylation of rMnSOD in vitro (Fig. 5B); however, glutathionylation does not alter the activity of the rMnSOD. In addition, previous reports from our laboratory using rMnSOD treated with authentic NO showed no change in enzymatic activity [10]. This suggests that glutathionylation is probably not the direct mechanism responsible for the loss of MnSOD activity after GSNO treatment of renal cells.

The iron superoxide dismutase enzyme isolated from the psychrophilic eubacterium *Pseudoalteromonas haloplanktis* (PhSOD) has been shown to be a target for S-glutathionylation after GSNO treatment in vitro [52]. However, S-glutathionylation of PhSOD did not affect its activity, but interestingly S-glutathionylation protected the enzyme from peroxynitrite-mediated inactivation. In addition, human Cu,ZnSOD has also been shown to be glutathionylated in vivo, which does not alter enzyme activity, but does alter the stability of the enzyme [53]. Therefore, other forms of recombinant SOD show results similar to those reported here in that the enzyme can be modified; however, activity was not altered. Nevertheless, the fact that GSNO treatment of intact renal cells does lead to reversible inactivation of MnSOD is a novel finding.

Our lab has previously shown that MnSOD is a target of peroxynitrite-mediated inactivation during renal transplantation and ischemic damage [8–10]. Interestingly, DTT treatment of lysates from our rat model of renal ischemia/reperfusion had *no* effect on MnSOD activity, suggesting that GSNO treatment of renal cells leads to inactivation of MnSOD via a novel thiol-dependent mechanism. The cellular mechanism regarding the intermediate steps involved in GSNO-induced MnSOD glutathionylation and inactivation in NRK cells remains unknown.

Because we know that MnSOD activity is altered by a mechanism affecting its thiol residues, we further sought to determine the role of cellular GSH in the observed loss of MnSOD activity upon GSNO treatment. Interestingly, we found that BSO pretreatment (which depletes GSH) of NRK cells before incubation with GSNO/diamide prevents MnSOD inactivation. We postulate that the GSNO-mediated MnSOD inactivation in vivo could proceed through an indirect multistep mechanism that is GSH dependent and involves thiol residues of MnSOD and/or some MnSOD-binding protein. Recently, it has been shown that MnSOD can be inactivated via acetylation of key lysine groups [54,55], but this modification is known to be neither induced by GSNO/GSH nor reversed by DTT or BSO, thereby eliminating it from consideration here.

We also used diamide (a known potent protein-glutathionylating agent and a strong thiol-oxidizing agent) as a tool to study its effect on MnSOD activity. Consistent with the effect of GSNO on MnSOD activity in NRK cells, diamide treatment of NRK cells (Fig. 6C) and

perfusion of rat kidneys with diamide (Fig. 8) led to a decrease in MnSOD activity, which was recovered upon DTT treatment. It is also possible that the effect of diamide on MnSOD could be due to thiol oxidation (or protein–protein thiol–disulfide interaction) of MnSOD instead of S-glutathionylation. But, it is known that diamide preferentially oxidizes only low-molecular-weight thiols (primarily GSH) compared to protein thiols [22].

The above results prompted us to explore the overall protein thiol status upon GSNO treatment of NRK cells. The BODIPY-IAM labeling experiment showed that GSNO treatment of NRK cells decreased the available protein free thiol groups compared to the untreated cells (Fig. 7A). Using BODIPY-IAM we were unable to detect a clear MnSOD signal after MnSOD immunoprecipitation. This could be related to the fact that there are only two cysteine residues (C140, C196) in each MnSOD monomer, and C140 is considered to be “buried” within the molecule [56], leaving a single cysteine residue available for BODIPY binding. Alternatively, this signal could have been blocked after immunoprecipitation with the MnSOD antibody. Therefore, to determine the thiol status in MnSOD we utilized the BIAM thiol-labeling technique, which did reveal a modest reduction in the availability of free thiol groups of MnSOD upon GSNO treatment compared to untreated cells (Fig. 7B). In summary, these results suggest that direct modification of MnSOD cysteine residues is probably not involved in the observed GSNO/diamide-mediated inactivation in renal cells; however, the finding that GSNO does lead to a thiol-dependent inactivation of MnSOD is exciting and will require further studies to clearly dissect the pathways involved in inactivation of MnSOD.

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Fig. 1.

GSNO treatment leads to mitochondrial membrane depolarization in NRK cells. Relative mitochondrial membrane potential of NRK cells was measured using JC-1 (7.5 μ M; 30 min). (A) Representative fluorescence microscopic images of untreated (UNTX) and GSNO-treated (100 μ M; 24 h) NRK cells. Similar results were seen in three separate experiments using different cell cultures. (B) Quantitative assessment of relative mitochondrial membrane potential using mean fluorescence intensity derived from the JC-1 aggregate/monomer ratio (590 nm/530 nm). Values are expressed as the mean $\pm$ SEM ($n=4$). * $P<0.05$, compared to untreated cells.

Fig. 2.

GSNO treatment induced inactivation of complex IV, but not complex I in NRK cells. Mitochondrial respiratory complex activities were measured using mitochondria isolated from untreated (UNTX) and GSNO-treated NRK cells (100 μ M; 24 h). (A) Complex I activity (oxidation of NADH) and (B) complex IV (oxidation of cytochrome *c*) activity. Corresponding untreated and GSNO-treated cell lysates were also treated with DTT in vitro (0.5 mM; 30 min) followed by similar mitochondrial respiratory activity measurement. Values are expressed as the mean $\pm$ SEM ($n=3$). * $P<0.05$, compared to untreated cells.

Fig. 3.

MnSOD activity was significantly decreased in NRK cells treated with GSNO (100 μ M; 24 h) without a change in its protein levels. Corresponding untreated and GSNO-treated NRK cell lysates were also treated with DTT in vitro (0.5 mM; 30 min). (A) Expression of MnSOD in NRK cell lysates after exposure to GSNO $\pm$ DTT assessed by Western blot analysis. GAPDH was used as a loading control. Similar findings were seen in three separate experiments. (B) MnSOD activity (U/mg protein) in NRK cell lysates was measured using the cytochrome *c* reduction method. Values are expressed as the mean $\pm$ SEM ($n=4$).

* $P<0.05$, compared to untreated cells.

Nitrotyrosine Immunocytochemistry

Fig. 4.

GSNO treatment of NRK cells did not increase tyrosine nitration. Nitrotyrosine formation in NRK cells was assessed as described under Materials and methods. Briefly, NRK cells were incubated with a nitrotyrosine polyclonal antibody (1:200), followed by the secondary antibody conjugated with Alexa-594 (1:1000). Cells positive for nitrotyrosine fluoresce red, and nuclear counterstaining (blue fluorescence) was evaluated using DAPI (1:100). Fluorescence microscopic images of (A) untreated (Untx) cells, (B) cells exposed to GSNO (100 μ M; 24 h), (C) cells treated with peroxynitrite (ONOO⁻, 800 μ M; positive control), and (D) cells treated with peroxynitrite, but anti-nitrotyrosine antibody preincubated with excess nitrotyrosine (negative control, ONOO⁻+Block) are shown. All results shown are representative of three experiments using different cell cultures.

Fig. 5.

GSNO induced glutathionylation of MnSOD in NRK cells and rMnSOD. (A) Representative image of immunoprecipitation of MnSOD followed by Western blot with anti-GSH antibody. The same blot was stripped and reprobed with anti-MnSOD antibody. (B) Western blot showing glutathionylation of rMnSOD (5 µg) after treatment with various concentrations of GSNO (1–1000 µM). (C) The Coomassie gel image of the rMnSOD Western blot showing equal loading of protein.

Fig. 6. Effect of GSNO, diamide, and BSO on overall protein glutathionylation and MnSOD activity in NRK cells. (A) Western blot depicting the overall glutathionylation of NRK cell proteins in the following treatment groups—untreated (U), diamide treated (D; 200 μ M; 24 h), BSO pretreatment (0.5 mM; 24 h) followed by diamide (B+D), GSNO treated (G; 100 μ M; 24 h), and BSO pretreatment (0.5 mM; 24 h) followed by GSNO (B+G). GAPDH was used as a loading control. (B) MnSOD activity (U/mg protein) in NRK cell lysates was measured using the cytochrome *c* reduction method. For depletion of GSH, NRK cells were preincubated with BSO (0.5 mM; 24 h) followed by treatment with GSNO (GSNO+BSO). (C) MnSOD activity (U/mg protein) was also measured in the following groups—untreated (UNTX) cells, diamide-treated cells (200 μ M; 24 h), diamide-treated cells followed by DTT (0.5 mM; 30 min) treatment of the cell lysate (diamide+DTT), and BSO-pretreated cells (0.5 mM, 24 h) followed by diamide treatment (diamide+BSO). Values are expressed as the mean $\pm$ SEM ($n=3$). * $P<0.05$, compared to untreated cells. # $P<0.05$, compared to diamide treated cells. (D) MnSOD expression in NRK cell lysates was assessed by Western blot. Actin was used as a loading control; similar findings were observed in three separate experiments.

Fig. 7.

Assessment of the overall protein thiol status and MnSOD thiol status in NRK cells using BODIPY fluorescence and BIAM labeling. (A) Representative BODIPY fluorescence image of the untreated (Untx) and GSNO-treated cells (GSNO) of three separate experiments. The graph below the image shows the densitometric quantification of the data ($n=4$). (B) Representative Western blot of MnSOD after labeling the NRK cell proteins with BIAM followed by precipitation with neutravidin beads. The graph shows the densitometric quantification of the BIAM data ($n=3$).

Fig. 8.

Diamide treatment in vivo decreases renal MnSOD activity. Rat left kidneys were perfused with diamide in saline (0.5 mM; 5 min) and right kidneys served as control with only saline perfusion. MnSOD activity (U/mg protein) was measured using the cytochrome *c* reduction method in rat kidney lysates. Values are expressed as the mean $\pm$ SEM ($n=3$). * $P<0.05$, compared to control right kidneys.