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Cytochrome c-mediated formation of S-nitrosothiol in cells

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Abstract

S-Nitrosothiols are products of nitric oxide metabolism that have been implicated in a plethora of signaling processes. However, mechanisms of S-nitrosothiol formation in biological systems are uncertain and no efficient protein-mediated process has been identified. Recently, we observed that ferric cytochrome c can promote S-nitrosoglutathione formation from nitric oxide and glutathione by acting as an electron acceptor under anaerobic conditions. In the current study we show that this mechanism also is robust under oxygenated conditions, that cytochrome c can promote protein S-nitrosation via a transnitrosation reaction, and that cell lysate depleted of cytochrome c exhibits lower capacity to synthesize S-nitrosothiols. Importantly, we also demonstrate that this mechanism is functional in living cells. Lower S-nitrosothiol synthesis activity, from donor and nitric oxide synthase-generated nitric oxide, was found in cytochrome c deficient mouse embryonic cells as compared to wild-type controls. In total, these data point to cytochrome c as a biological mediator of protein S-nitrosation in cells. This is the most efficient and concerted mechanism of S-nitrosothiol formation reported to date.

Keywords

S-nitrosation; nitrosylation; cytochrome c; glutathione; S-nitrosoglutathione; S-nitrosothiols

INTRODUCTION

The S-nitrosation of cellular proteins by nitric oxide (NO)-dependent processes has been widely recognized as an important post-translational modification involved in cellular signal transduction [1–3]. However, mechanisms of S-nitrosation in biological systems are poorly understood. Although NO can be easily oxidized to nitrogen dioxide and dinitrogen trioxide (both implicated in mechanisms of S-nitrosation [4–6]) and at high levels of NO and oxygen, the third order kinetics of this reaction limit or even preclude its involvement under biologically relevant conditions [7]. It has been suggested that the reaction between NO and oxygen is enhanced in hydrophobic environments due to local concentration effects [8;9],

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AUTHOR CONTRIBUTION

K.A.B designed and performed experiments with cell lysates and cells, analyzed the data and co-wrote the paper, A.K. designed and performed experiments GSNO and purified proteins, analyzed the data and co-wrote the paper, S.B. and D. K-S. provided helpful discussion and edited the paper, N.H. designed the experiments and co-wrote the paper.

CONFLICTS OF INTEREST

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but there is little evidence that this effect is important in vivo. The reaction of NO with thiol radical has been reported by some [4;6], but not others [10], to form S-nitrosothiols, but again the relevance of this process in any meaningful biological system has not been established. There has been significant interest in the role of metal ions and metalloproteins in S-nitrosothiol formation [11]; peroxidases and hemoglobin [12;13], as well as dinitrosyl iron complexes [14;15] have all been invoked as intermediates or promoters of nitrosation. Gow et al [16] proposed that electron acceptors could facilitate S-nitrosation by oxidizing an intermediate thionitroxyl radical, formed from the addition of NO to a thiol, suggesting that single electron acceptors may facilitate S-nitrosothiol formation. We have recently observed that ferric cytochrome c, under anaerobic conditions, can efficiently promote glutathione S-nitrosation by acting as an electron acceptor [17]. The mechanism appears to involve the initial weak binding of glutathione to cytochrome c, followed by reaction with NO to generate ferrous cytochrome c and S-nitrosoglutathione (GSNO). This mechanism would become catalytic if cytochrome c is subsequently re-oxidized to the ferric form. This reaction is highly efficient with over 50% of NO converted to GSNO.

In this study we have further examined the role of cytochrome c in facilitating S-nitrosothiol formation in purified protein samples and in cellular systems. We show here that cytochrome c facilitates S-nitrosation in both the absence and presence of oxygen. Additionally, cytochrome c can promote the S-nitrosation of purified proteins in the presence of glutathione and can also increase S-nitrosation in cell lysate. Immuno-depletion of cytochrome c from lysate results in a decrease in S-nitrosothiol formation. In addition, embryonic stem cells that lack cytochrome c have significantly lower S-nitrosothiol generating capacity than wild-type controls, when they are exposed either to NO-donor or NO-producing macrophages. Finally, antimycin A, an inhibitor of mitochondrial electron transport that would enhance the level of ferric cytochrome c, increased S-nitrosothiol formation in murine macrophages stimulated with LPS. Similarly, treatment with NO in the presence of antimycin A led to elevated S-nitrosation in wild-type, but not in cytochrome c deficient cells. In total, these data provide evidence that cytochrome c may be an important cellular mediator of protein S-nitrosation.

METHODS

Materials

Nitric oxide donors were purchased from Cayman Chemicals; all other materials were obtained from Sigma-Aldrich unless otherwise noted. All experiments were carried out using cytochrome c that was purified without TCA precipitation step (catalog number C7752). Purified proteins were used as supplied, without further treatment or refining and prepared in phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μ M) and EDTA (100 μ M).

Anaerobic experiments

Anaerobic experiments were performed using a Coy anaerobic chamber under an atmosphere of 95% nitrogen and 5% hydrogen. Buffers were equilibrated overnight inside the chamber and other solutions were stirred within the chamber for two hours prior to experiments to ensure complete anaerobiosis.

Multilinear Regression Analysis (MLR)

Absorption spectra were recorded between 450–700 nm ranges at every 10 s in a 1 cm pathway cuvette with an Agilent 8453 UV-visible Spectrophotometer. Deconvolution of spectra into individual species was accomplished with MLR, using a set of pure spectra of all components as a basis. The pure components used were those shown in reference [17].

Cell culture and treatments

RAW 264.7 cells were cultured in DMEM (Invitrogen) supplemented with streptomycin (200 µg/ml), penicillin (200 U/ml), and 10% FBS (Invitrogen). Murine embryonic cells lacking cytochrome c were a kind gift from Dr. M. Celeste Simon at the University of Pennsylvania and were maintained in DMEM with 4.5 g glucose/ml, 20% FBS (Invitrogen), 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), 0.1 mM MEM nonessential amino acids, 50 µM β-mercaptoethanol (Cell & Molecular Technologies), 500–1000 units/ml mouse leukemia inhibitory factor (Chemicon ESGRO), 2 mM sodium pyruvate, and 50 µg/ml uridine as previously described [18]. Cells were grown on gelatin-covered 6-well plates. Control mouse embryonic cells were cultured under similar conditions but in the absence, of additional pyruvate, uridine and HEPES. Medium without β-mercaptoethanol and leukemia inhibitory factor was used upon exposure of mouse embryonic cells to LPS-stimulated RAW 264.7 macrophages.

S-Nitrosation in cell lysates

Cells were lysed in lysis buffer (Tris 20 mM, pH 8.0, NaCl 137 mM, DTPA 1 mM, glycerol 10 %, Triton X-100 1 %, protease inhibitor cocktail 1 %) and incubated with an NO donor under anaerobic or aerobic conditions for 30 min in the presence or absence of ferric cytochrome c for 30 minutes. To prevent any subsequent S-nitrosation reactions free thiols were blocked with 10 mM NEM. For anaerobic experiments the lysates were incubated under anaerobic conditions for 2 h prior to the treatment.

S-Nitrosothiol determination

The S-nitrosothiol levels in purified proteins and cell lysates were determined using the triiodide-dependent ozone-based chemiluminescence method using a Sievers Model 280 NO analyzer as described previously [19;20]. Briefly, the reaction solution was made fresh daily from potassium iodide (28 mg) and I₂ (18 mg) in glacial acetic acid (3.75 ml) and double-distilled H₂O (1.25 ml). This solution was added into the reaction vessel together with antifoaming agent and maintained at 30°C. Samples were pretreated with 10% (vol/vol) of sulfanilamide (100 mM in 2 N HCl) to remove nitrite. Mercuric chloride (5 mM for 10 min) was used to verify the presence of S-nitrosothiols. A standard curve was generated based on the detector response to GSNO.

Nitrite measurements

The nitrite level in the medium was measured by Griess assay [21]. Briefly, 200 µl of sample was mixed with 10 µl of sulfanilamide (30 mM in 2 N HCl), followed by 10 µl of N-1-naphthylethylenediamine dihydrochloride (30 mM in 0.1 N HCl). The absorbance was measured at 540 nm and compared to standard curve generated using sodium nitrite.

Cytochrome c immunodepletion

Protein A/G beads were coated with anti-cytochrome c antibody (BD Biosciences) or isotype matched control, IgG, (Sigma) for 2 h at 4°C and then beads were washed to remove unbound antibody. Cell lysate (450 µg) was incubated with antibody-coated beads for 3 h at 4°C followed by centrifugation. Resulting supernatants were incubated under anaerobic conditions with Proli/NO in the presence or absence of cytochrome c.

Determination of cytochrome c levels

The protein levels of cytochrome c and β-actin were probed using Western blot analysis after reducing SDS-PAGE. Briefly, the cells were harvested in lysis buffer and cellular proteins were separated on a 4–20 % SDS-PAGE gel. The levels of cytochrome c and β-

actin were detected using specific antibodies (MitoSciences and Sigma, respectively) and visualized with enhanced chemiluminescence.

Statistics

All data are reported as the mean $\pm$ standard error of means unless otherwise indicated. Statistical analysis of data was made with a Student's t-test. Changes were considered statistically significant when $p < 0.05$.

RESULTS

Cytochrome c-mediated S-nitrosation of GSH under aerobic conditions

In our previous study we demonstrated that cytochrome c could efficiently promote the S-nitrosation of GSH by NO under anaerobic conditions and also in the presence of 1% oxygen [17]. Here, we have examined the efficiency of cytochrome c-mediated S-nitrosation under fully aerobic conditions. The addition of Proli/NO (100 μ M) to GSH under aerobic conditions leads to formation of about 20 μ M S-nitrosoglutathione (GSNO) (Figure 1A). In the presence of cytochrome c, the levels of GSNO formed in this system increase about two times (Figure 1A), indicating that even in the presence of high concentrations of both NO and oxygen, cytochrome c-mediated GSNO formation is still competitive with NO oxidation. Figure 1B shows kinetic traces of the reaction between glutathione, ferric cytochrome c and NO. As one can observe, ferric cytochrome c rapidly transforms into a ferric nitrosyl form, which decays over time, with concomitant generation of ferrous cytochrome c. Under the same conditions both GSH or Proli/NO alone reduces ferric cytochrome c much more slowly and to a lesser extent, than when GSH and Proli/NO were both present (data not shown). This is in agreement with our previous studies under anaerobic conditions [17].

S-Nitrosation of purified proteins by cytochrome c

To determine if cytochrome c could directly or indirectly facilitate the S-nitrosation of proteins, we incubated several purified proteins: human serum albumin (HSA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and aldolase, with cytochrome c and Proli/NO in the presence and absence of GSH under anaerobic conditions. Without GSH negligible levels of S-nitrosated proteins could be detected in the presence or absence of cytochrome c (data not shown). When GSH was introduced to the system, robust cytochrome c-dependent GSNO formation, and significant protein S-nitrosation were observed (Figure 2A). In this figure the bar represents total S-nitrosothiol and is divided into low molecular weight (gray) and high molecular weight (white) as determined by passage through a 10 KDa cutoff filter.). These data suggest that protein S-nitrosation occurs via the intermediacy of GSNO as a result of transnitrosation between GSNO and the protein thiol. The efficiency of protein S-nitrosation is therefore likely to depend on the equilibrium position of the transnitrosation reaction with GSNO. When comparing the conversion of protein thiol to S-nitrosothiol, we found that HSA and aldolase were more sensitive targets to S-nitrosation than GAPDH (0.42, 0.37, and 0.26 mol:mol respectively).

We further investigated the efficiency of S-nitrosation of HSA under anaerobic and aerobic conditions, using Proli/NO and Sper/NO (half-life: 2.3 s and 230 minutes at room temperature, respectively) (Figure 2B and C). The longer half-life of Sper/NO will create a sustained steady-state level of NO rather than an initial burst that would be generated with Proli/NO. In all cases increased S-nitrosothiol formation was detected in the presence of GSH and cytochrome c, compared to mixtures deficient in either or both of these compounds. Under aerobic conditions cytochrome c also promoted HSA S-nitrosation, although to a smaller extent than observed anaerobically. A similar pattern of protein S-

nitrosation and GSNO formation was observed with both NO donors, although in general Sper/NO generated lower levels of S-nitrosothiols, as expected by its longer half-time of decomposition.

Cytochrome c-dependent S-nitrosation in cell lysates

To examine whether cytochrome c can facilitate protein S-nitrosation in a model biological system, cell lysate obtained from RAW 264.7 cells was incubated with NO donors in the presence or absence of cytochrome c. Under anaerobic conditions NO, generated from Proli/NO, led to a dose-dependent increase in S-nitrosothiol levels (Fig. 3A inset). When the lysate was supplemented with ferric cytochrome c (100 μ M), the S-nitrosation was greatly enhanced achieving levels of 3.8 nmol/mg protein (Fig 3A). Figure 3B shows the treatment of cell lysate with Sper/NO with and without ferric cytochrome c under aerobic conditions. Similarly, in the presence of cytochrome c, the S-nitrosation was more efficient. Sper/NO instead of Proli/NO was used under aerobic conditions due to its longer half-life to provide steady-state level of NO. Although absolute levels of S-nitrosothiols were higher under anaerobic than under aerobic conditions, ferric cytochrome c enhanced S-nitrosation in both cases.

Effect of endogenous cytochrome c on S-nitrosothiol formation

To study the involvement of endogenous cytochrome c in the S-nitrosation process, cytochrome c was immunodepleted from the lysate. Cell lysate was equally divided and incubated with either IgG-coated protein A/G beads or anti-cytochrome c antibody. The absence of cytochrome c in the immunodepleted lysate was confirmed by Western blotting (Fig 4A). When deoxygenated lysates were treated with Proli/NO, the levels of S-nitrosothiols were decreased by 40% in cytochrome c immunodepleted samples compared to control (Fig. 4B). To ascertain if the immunodepleted lysate was still capable of supporting S-nitrosation, exogenous ferric cytochrome c was added to these samples. S-nitrosation was increased equally in both control and cytochrome c-depleted samples. This indicates that sample manipulation did not affect the ability of the lysates to support S-nitrosothiol formation.

S-Nitrosation in cytochrome c deficient mouse embryonic cells

To further explore the role of cytochrome c in facilitating the S-nitrosothiol production, murine cells obtained from cytochrome c^{-/-} embryos or from wild type littermate embryos, were treated with Sper/NO to determine whether endogenous cytochrome c plays a role in S-nitrosation in live cells. The cytochrome c-deficient status of these cells was confirmed by Western analysis (Fig 5A, inset). Figure 5A shows that cells lacking cytochrome c protein produced over 3 times lower levels of S-nitrosothiols compared to wild type counterparts after exposure to Sper/NO.

As S-nitrosothiol synthesis requires ferric cytochrome c, we hypothesized that inhibition of mitochondrial electron transport would increase cytochrome c oxidation and therefore enhance S-nitrosothiol formation. Inhibition of mitochondrial electron transport with antimycin A significantly increased the levels of S-nitrosothiols in wild-type cells but not in cytochrome c null cells. These data support the role of cytochrome c in S-nitrosothiol formation; we show that S-nitrosation is less efficient in the absence of cytochrome c and that by putatively increasing the level of ferric cytochrome c, one can facilitate the nitrosothiol formation.

S-Nitrosation from iNOS-derived NO

We next explored S-nitrosothiol formation from iNOS-derived NO in the presence and absence of antimycin A. RAW 264.7 macrophages were treated with LPS for 13 h to induce iNOS (nitrite levels in the medium: control untreated cells, $2.3 \pm 0.1 \mu\text{M}$; LPS, $72.6 \pm 0.9 \mu\text{M}$). To prevent the production of NO and formation of S-nitrosothiols during the overnight induction of iNOS, an iNOS inhibitor, L-NAME, was added. L-NAME decreased medium nitrite levels to $5.0 \pm 0.3 \mu\text{M}$ in LPS-exposed macrophages. After iNOS induction, cells that had been treated with LPS and L-NAME were subsequently washed and cultured in L-NAME-free medium in the presence and absence of antimycin A, and cellular S-nitrosothiol formation and nitrite accumulation in the medium were monitored. Figure 5B shows that antimycin A did not affect nitrite accumulation during 4 h of incubation. Nevertheless, the inhibition of complex III led to an almost 2-fold increase of S-nitrosation in RAW 264.7 cells.

Finally, to directly probe the involvement of endogenous cytochrome c in S-nitrosation from iNOS-derived NO, wild type and cytochrome c null cells were exposed to LPS-stimulated RAW 264.7 cell. In this experiment, RAW 264.7 macrophages, cultured on transwell inserts, were stimulated with LPS in the presence of L-NAME for 12 h. Next, the cells were washed and inserts were transferred onto plates containing mouse embryonic cells. Cells were co-cultured together for 4 h and S-nitrosothiol levels were measured in wild type and cytochrome c mouse embryonic cells. We detected S-nitrosothiols only in wild type, but not cytochrome c null, mouse embryonic cells, which were exposed to LPS-stimulated macrophages (Fig. 6). Although the levels of S-nitrosothiols were close to the detection limit of the technique, peaks that were amenable to integration were observed in the wild-type cells but not in the cytochrome c null cells. Inset of Figure 6 shows the raw traces obtained after injection of equal amounts of the wild type and cytochrome c null samples ($\sim 350 \mu\text{g}$ each) into the NO analyzer.

DISCUSSION

In our previous study we observed that ferric cytochrome c could act as an electron acceptor for the formation of GSNO from GSH and NO [17]. In this work we extend previous findings and show that this mechanism is unlikely to facilitate direct protein S-nitrosation, but is able to stimulate protein S-nitrosation through the intermediacy of GSNO. In addition, we demonstrate that immunodepletion of cytochrome c from RAW 264.7 cell lysate results in a decrease in S-nitrosothiol formation after exposure to NO. Finally, we report decreased S-nitrosothiol formation in cytochrome c deficient cells. We previously proposed a mechanism that involved the weak binding of GSH to cytochrome c, followed by reaction of this complex with NO to form GSNO and reduced cytochrome c [17]. In many ways this mechanism is similar to that proposed by Gow et al [16] which invoked the one-electron oxidation of an intermediate thionitroxyl radical formed from the addition of NO to thiol by oxygen or NADP^+ . While we have found no evidence that either oxygen or NADP^+ [5] can act as one-electron acceptors in this reaction, ferric cytochrome c is able to act in this role. The specificity for cytochrome c may come from the fact that it has a weak binding site for GSH that facilitates this reaction [22]. However, other heme proteins, particularly hemoglobin, have been shown to support S-nitrosothiol formation, although with very low efficiency [12;13;23;24].

The presence of ferric cytochrome c results in an almost stoichiometric conversion of NO to form GSNO. This is the most efficient mechanism of S-nitrosothiol formation yet described. Previous studies have identified transition metals and metalloproteins as mediators of S-nitrosation through their ability to act as electron acceptors. In particular, the plasma protein ceruloplasmin has been shown to support NO-dependent S-nitrosation in plasma [25]. In

addition, the 'free iron pool' has been implicated in cellular S-nitrosothiol formation through the intermediate formation of dinitrosyl iron complexes [15]. Cytochrome c-dependent GSNO formation is quite strongly oxygen-dependent with greater yields under anaerobic conditions. This is likely due to kinetic competition between GSH/cytochrome c and oxygen for NO. As the reaction between NO and oxygen is second order in NO [26;27], this competition will increasingly favor reaction with oxygen as NO concentrations increase. While the reaction of NO with oxygen generates nitrosating agents that can generate S-nitrosothiols, the efficiency is low, and the major product is thiol disulfide [4;5]. Our data indicate that, even in the presence of atmospheric oxygen concentrations, the cytochrome c/GSH reaction is still operative and increases the efficiency of thiol S-nitrosation. At physiological concentration of NO and oxygen, this difference will be significantly increased.

Cytochrome c-dependent S-nitrosothiol formation strongly suggests the intermembrane space as a locus for thiol nitrosation. The glutathione redox buffer of this compartment is more oxidizing as compared to cytosol and matrix. Hu et al have utilized redox-sensitive YFP to probe the intermembrane space in yeast and calculated the GSH:GSSG ratio of 250:1 [28], based on the assumption that GSH concentration in this compartment does not differ from cytosol [29]. In comparison, cytosolic and mitochondrial matrix GSH:GSSG ratio is 3000:1 and 9000:1, respectively. In agreement with these studies, an increased oxidation of the redox-sensitive GFP in the mitochondrial intermembrane space, as compared to cytosol, was also observed in the smooth muscle cells (intermembrane space: 47.7 %, cytosol: 18.6 %) [30]. Although these reports indicate more oxidizing character of the intermembrane space, they also point out that vast majority of glutathione pool is in the reduced state.

Experiments performed with cytochrome c deficient cells strongly indicate that S-nitrosothiols are largely formed by a cytochrome c-dependent mechanism in a cellular environment. These cells are derived from mouse embryos at day 8.5 as cytochrome c deficiency is embryonically lethal [31]. It has been demonstrated that reintroduction of cytochrome c into these cells restores their ability to respire, indicating that they contain otherwise functional mitochondria [18]. Cells lacking cytochrome c generated significantly lower levels of S-nitrosothiols when exposed to NO donor. Moreover, S-nitrosation was observed only in wild type cells when mouse embryonic cells were co-cultured with LPS-stimulated RAW 264.7 cells.

As the mechanism of S-nitrosation requires ferric cytochrome c, we examined if antimycin A, an inhibitor of cytochrome c reduction from mitochondrial complex III, was able to stimulate S-nitrosation. We observed a significant increase in cytochrome c formation in the presence of antimycin A only in the wild-type cells and not in the cytochrome c null cells. Similarly, in case of LPS-stimulated RAW 264.7 macrophages, we detected enhanced S-nitrosation when cells were treated with antimycin A. This observation suggests that the redox state of the mitochondrial electron transport chain may be a variable in controlling the rate of cellular S-nitrosation. This control of S-nitrosation by the oxidation state of the mitochondrial electron transport chain has several intriguing possible consequences. It would be expected that NO, by inhibiting respiration at complex IV [32;33] would force cytochrome c into the ferrous state and so inhibit S-nitrosothiol formation. In contrast the inhibition of electron flow upstream of cytochrome c by (for example) S-nitrosation or oxidation of complexes I or III, would facilitate S-nitrosothiol formation. We have recently shown, in endothelial cells, that the effects of NO on cellular respiration are quite distinct from S-nitrosation [34] and are explainable solely by reversible binding of NO to complex IV. However, it is possible that in the presence of increased oxidative stress, the mitochondrial electron transfer proteins may become inhibited leading to the promotion of

S-nitrosation via a ferric cytochrome c-dependent process. We are currently investigating these speculations.

Although S-nitrosation has been celebrated as an important NO-dependent signaling paradigm, robust mechanisms of S-nitrosothiol formation *in vivo* have been elusive. Much emphasis has been placed on the reaction of NO with oxygen but there is a strong argument that this reaction is not fast enough to represent a feasible mechanism of S-nitrosothiol formation *in vivo*. Other mechanisms discussed above involving metal ions and metalloproteins have been examined, but the fact that no efficient, concerted mechanism of S-nitrosothiol synthesis in cells has been reported has been a major impediment to the concept of S-nitrosation as a signaling paradigm. Here we have demonstrated that the cytochrome c-dependent formation of S-nitrosothiols that we previously observed, functions in living cells and is a viable route to the S-nitrosation cellular proteins. The functional consequences of this pathway remain to be uncovered.

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ABBREVIATIONS

DTPA	diethylene triamine pentaacetic acid
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSNO	S-nitrosoglutathione
HSA	human serum albumin
MLR	Multilinear Regression Analysis
NEM	N-ethylmaleimide
Prolⁱ/NO	1-(hydroxyl-NNO-azoxy)-L-proline (Prol ⁱ NONOate)
Sper/NO	N-[4-[1-(3-Aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (Spermine NONOate)
TCA	trichloro-acetic acid
Tris	tris(hydroxymethyl)aminomethane

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Figure 1. Cytochrome c-mediated S-nitrosation of glutathione with Proli/NO under aerobic conditions

A: Glutathione (1 mM) was incubated with Proli/NO (100 μM) without or with cytochrome c (100 μM) under aerobic conditions for 30 min followed by blocking of free thiol with excess NEM. Cytochrome c was separated on 10 kDa filter, and GSNO was quantified in the low molecular weight fraction with triiodide-chemiluminescence. Values are means ± SEM, (n=6).

B: Kinetic traces of the reaction between glutathione (1 mM), cytochrome c (100 μM), and Proli/NO (100 μM) under aerobic conditions. Spectra were recorded between 450 nm and 700 nm with a rate of 2.5s/cycle, and subjected to multiple linear regression. Solid line:

ferric cytochrome c, dotted line ferric nitrosyl cytochrome c, dashed line: ferrous cytochrome c.

Figure 2. Cytochrome c-mediated S-nitrosation of purified proteins

A: HSA, GAPDH, or aldolase (15 μM) was incubated with Proli/NO (100 μM) and GSH (1 mM) in the presence or absence of cytochrome c (100 μM) under anaerobic conditions for 30 min. Then free thiol was blocked with excess NEM. Protein was separated from low molecular weight fraction on a 10 kDa filter, and S-nitrosothiol levels were quantified in both fractions by chemiluminescence. Open bars: protein S-nitrosothiol, shaded bars: GSNO. Values are means ± SEM, (n=3).

B and C: HSA (100 μM) was incubated with Proli/NO (**B**) or with Sper/NO (**C**) (100 μM) in the presence or absence of cytochrome c (100 μM), and with or without GSH (1 mM) for 30

min. Then free thiol was blocked with excess NEM. Protein was separated from low molecule weight fraction on a 10 kDa filter, and S-nitrosothiol levels were quantified in both fractions by chemiluminescence. Open bars: protein nitrosothiol, shaded bars: GSNO. Values are means $\pm$ SEM, (n=3).

Figure 3. Cytochrome c-mediated S-nitrosation in RAW 264.7 lysates

RAW 264.7 cell lysates were exposed to increasing concentrations of NO donor in the presence (closed symbols) or absence (open symbols) of cytochrome c (100 μM). **A:** Samples were exposed to various concentrations of Proli/NO under anaerobic conditions in the presence and absence of cytochrome c for 30 min. Inset: Cell lysate treated with Proli/NO. **B:** Samples were exposed to various concentrations of Sper/NO under aerobic conditions in the presence and absence of cytochrome c for 30 min. S-Nitrosothiol levels were determined by chemiluminescence. Data were normalized to protein concentration and represent mean ± SEM (n = 3).

Figure 4. Cytochrome c depletion

A: Western blot analysis of immunodepleted lysates. **B:** RAW 264.7 cell lysates were incubated with specific anti cytochrome c antibody to deplete endogenous cytochrome c or with control IgG. Lysates were then exposed to Proli/NO (100 μ M) in the presence and absence of cytochrome c (100 μ M) under anaerobic conditions for 30 min. S-Nitrosothiol levels were measured by chemiluminescence. Data were normalized to protein concentration and represent mean $\pm$ SEM (n = 3, * p < 0.005).

Figure 5. Cytochrome c-mediated S-nitrosation – the effect of antimycin A

A. Murine embryonic cells, wild type and cytochrome c null, were treated with Sper/NO (200 μM) for 1 h in HBSS in the absence (closed bars) or presence (open bars) of antimycin A (10 μM). Cells were lysed in the presence of NEM and S-nitrosothiol levels were measured by chemiluminescence. Data were normalized to protein concentration and represent mean ± SEM (n = 3, * p < 0.05, ** p < 0.01 as compared to wild type treated with Sper/NO). Inset: Western blot analysis of mouse embryonic cells. **B.** RAW 264.7 macrophages were treated with LPS (0.5 μg/ml) in the presence of L-NAME (4 mM) for 13 h, after which the medium was replaced and cells were incubated in the presence and

absence of antimycin A (10 μ M) for 4 h. S-Nitrosothiol levels (closed bars) were measured by chemiluminescence and normalized to protein concentration. The nitrite concentration in the medium (grey bars) was measured by Griess assay. Data represent mean $\pm$ SEM (n = 3, * p < 0.01).

Figure 6. S-Nitrosation in co-culture studies

Murine embryonic cells, wild type and cytochrome c null, were co-cultured with LPS-stimulated RAW 264.7 macrophages for 4 h. Murine embryonic cells were lysed and S-nitrosothiol levels were measured by chemiluminescence. Data were normalized to protein concentration and represent mean $\pm$ SEM ($n = 3$). S-Nitrosothiol levels were below detection limit in cytochrome c null cells (not detectable, N.D.). Inset: Raw chemiluminescence data showing the size of the signal from wild type and cytochrome c null cells. Arrows indicate the injection of the sample.