**Cytochrome c-mediated formation of S-nitrosothiol in cells**

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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 formation 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]), 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 depletion of cytochrome \( c \) 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 to either NO-donor or NO-producing macrophages. Finally, antimycin A, an inhibitor of mitochondrial electron transport that enhances the level of ferric cytochrome \( c \), increased S-nitrosothiol formation in murine macrophages stimulated with LPS (lipopolysaccharide). 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. Taken together, these data provide evidence that cytochrome \( c \) may be an important cellular mediator of protein S-nitrosation.

MATERIALS AND METHODS

Materials

NO 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 trichloroacetic acid.
precipitation step (catalogue 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 (diethylene triamine penta-acetic acid; 100 μM) and EDTA (100 μM).

**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 2 h before the start of experiments to ensure complete anaerobiosis.

**MLR (multilinear regression analysis)**

Absorption spectra were recorded between 450 and 700 nm every 10 s in a 1-cm-pathlength 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 [17].

**Cell culture and treatments**

RAW 264.7 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium: Invitrogen) supplemented with streptomycin (200 μg/ml), penicillin (200 units/ml) and 10 % (v/v) FBS (fetal bovine serum; Invitrogen). Murine embryonic cells lacking cytochrome c were a gift from Dr M. Celeste Simon (University of Pennsylvania, Philadelphia, PA, U.S.A.) and were maintained in DMEM with 4.5 g glucose/ml, 20 % FBS, 25 mM Hepes, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), 0.1 mM MEM non-essential amino acids, 50 μM 2-mercaptoethanol (Cell and Molecular Technologies), 500–1000 units/ml mouse leukaemia inhibitory factor (Chemicon ESGRO), 2 mM sodium pyruvate and 50 μg/ml uridine as described previously [18]. Cells were grown on gelatin-covered six-well plates. Control mouse embryonic cells were cultured under similar conditions, but in the absence of additional pyruvate, uridine and Hepes. Medium without 2-mercaptopethanol and leukaemia inhibitory factor was used upon the exposure of mouse embryonic cells to LPS-stimulated RAW 264.7 macrophages.

**S-nitrosation in cell lysates**

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

**S-nitrosothiol determination**

The S-nitrosothiol levels were determined using the tri-iodide-dependent oxygen-based chemiluminescence method using a Sievers model 280 NO analyser as described previously [19,20]. Briefly, the reaction solution was made fresh daily from potassium iodide (28 mg) and I₂ (18 mg) in acetic acid (3.75 ml) and double-distilled water (1.25 ml). This solution was added into the reaction vessel together with an anti-foaming agent and maintained at 30°C. Samples were pre-treated with 10 % (v/v) sulfanilamide (100 mM in 2 M 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 the Griess assay [21]. Briefly, 200 μl of the sample was mixed with 10 μl of sulfanilamide (30 mM in 2 M HCl), followed by 10 μl of N-(1-naphthyl)ethylenediamine dihydrochloride (30 mM in 0.1 M HCl). The attenuation was measured at 540 nm and compared with a 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 lystate (450 μg) was incubated with antibody-coated beads for 3 h at 4°C, followed by centrifugation. The resulting supernatants were incubated under anaerobic conditions with Proli/NO (1m31-(hydroxyl-NNO-azoxy)-L-proline (ProliNONOate)) 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 by SDS/PAGE (4–20 % 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 means ± S.E.M. unless otherwise indicated. Statistical analysis of data was carried out using 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]. In the present study, we have examined the efficiency of cytochrome c-mediated S-nitrosation under fully aerobic conditions. The addition of Proli/NO (100 μM) to GSH under aerobic conditions leads to the formation of approximately 20 μM GSNO (Figure 1A). In the presence of cytochrome c, the levels of GSNO formed in this system increased approximately 2 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 1(B) 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, either 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 (results not shown). This is in agreement with our previous studies under anaerobic conditions [17].
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 a 10 kDa filter, and GSNO was quantified in the low-molecular-mass fraction with tri-iodide-chemiluminescence. Values are means ± S.E.M. (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 and 700 nm with a rate of 2.5 s per cycle and subject to MLR. Continuous line: ferric cytochrome c; dotted line: ferric nitrosyl cytochrome c; dashed line: ferrous cytochrome c.

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: HSA (human serum albumin), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) 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 (results 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, each bar represents total S-nitrosothiol and is divided into low-molecular-mass (grey) and high-molecular-mass (white) as determined by passage through a 10 kDa cut-off 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 into 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 \{N-[4-[(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (sperrmine NONOate)\} [half-life: 2.3 s and 230 min at room temperature (25°C)] respectively] (Figures 2B and 2C). 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 with 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-life 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
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. Results were normalized to protein concentration and are means ± S.E.M. (n = 3).

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 cell 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 (Figure 4A). When deoxygenated lysates were treated with Proli/NO, the levels of S-nitrosothiol were decreased by 40% in cytochrome c immunodepleted samples compared with the control (Figure 4B).

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 blot analysis (Figure 5A, inset). Figure 5(A) shows that cells lacking cytochrome c protein produced over 3 times lower levels of S-nitrosothiols compared with 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 S-nitrosothiol formation.
The presence of ferric cytochrome c results in an almost stoichiometric conversion of NO into GSNO. This is the most efficient mechanism of S-nitrosation described so far. 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 caeruloplasmin 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 probably due to kinetic competition between GSH/cytochrome c and oxygen for NO. As the reaction between NO and oxygen is a second-order reaction in NO [26,27], this competition will increasingly favour reaction with oxygen as NO concentrations increase. Although 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 with cytosol and matrix. Hu et al. [28] have utilized redox-sensitive YFP (yellow fluorescent protein) to probe the intermembrane space in yeast and calculated the GSH/GSSG ratio of 250:1, based on the assumption that GSH concentration in this compartment does not differ from cytosol [29]. In comparison, the 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 (green fluorescent protein) in the mitochondrial intermembrane space, as compared with 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 a vast majority of the 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 reoxidation 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 whether 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 the 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 shown recently, 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 signalling 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 signalling paradigm. In the present study, 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 of cellular proteins. The functional consequences of this pathway remain to be uncovered.

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**REFERENCES**

281 Wang, X., Bryan, N. S., MacArthur, P. H., Rodriguez, J., Gladwin, M. T. and Feelisch, M.

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