Mechanisms of the interaction of nitroxyl with mitochondria


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INTRODUCTION

NO* (nitric oxide) has been recognized to be an integral component of several physiological signalling pathways, including cGMP-mediated vasorelaxation, neurotransmission and, as recognized more recently, regulation of cytochrome c oxidase in the mitochondrial respiratory chain [1–3]. While these effects of NO are the result of a direct interaction of the molecule with haem iron, other signal transduction pathways are thought to be mediated by secondary products of NO*. Notable examples of these are RSNO (S-nitrosothiols), which can modify protein function through post-translational modification [4,5]. Initially, it was proposed that simple one-electron reactions with NO could yield either the oxidized nitrosonium cation or the reduced nitroxyl anion (NO•−), which would be capable of S-nitrosation or oxidation reactions respectively [6]. This proposal has evolved to encompass reactions of NO• with oxygen or superoxide, providing alternative routes for the formation of RSNO or ONOO− (peroxynitrite) [7]. While the mechanisms of formation and reaction of ONOO− have been studied extensively, insights into the chemistry of NO• are only now emerging. For example, it was once thought that NO• could be formed by the reduction of NO and remain as an anion in vivo. However, it has recently been shown that the reduction potential for NO is too negative for this reaction to occur, and the pKa for NO• has been re-evaluated from 4.7 to a value of 11.6, suggesting that it exists in its protonated form (HNO; nitroxyl) under most biological conditions [8]. Various studies have highlighted important biological effects of HNO, including the formation of NO•, cytotoxicity and the enhancement of ischaemic cell damage (as discussed and reviewed in [9–13]).

Although HNO has been implicated in several of the same pathways as NO*, such as vasodilation, the unique chemistry of this molecule, including its direct reaction with thiols, allows it to mediate effects distinct from those of NO*. For example, HNO has been shown to be more cytotoxic than NO• in cell culture, much like ONOO− [10]. Recently it has been suggested that HNO mediates oxidative reactions through the formation of hydroxyl radicals [14]. The effects of HNO on biological systems also depend on the prevailing metabolic status of the cell, since it has been shown to potentiate myocardial ischaemia/reperfusion injury when added during the reperfusion phase, but is protective if added prior to ischaemia [10,12,15]. Investigation of these distinct effects of HNO is important for advancing our understanding of the contrasting biological effects of RNS (reactive nitrogen species) in a wide variety of physiological and pathological contexts. Although the production of HNO in vivo is still controversial, it is important to understand in more detail the responses of biological systems to this RNS and its commonly used donor AS (Angeli’s salt) for insight it may offer into both the basic mechanisms of RNS biology and potential therapeutic applications.

The mitochondrion provides a unique arena in which to investigate the effects of HNO, since the regulation of mitochondrial function by other RNS has been studied extensively and is reasonably well understood [16]. NO• binds reversibly to the oxygen binding site of cytochrome c oxidase, the terminal member of the electron transport chain, resulting in the inhibition of respiration. The mechanisms involved are complex, with greater inhibition occurring as the oxygen concentration decreases, through a mechanism that includes regulation by the
mitochondrial inner membrane [17–19]. Other RNS, such as ONOO−, mediate effects distinct from those of NO+ in the organelle, such as the inhibition of complex II, inhibition of the ATP synthase and nitration of Mn superoxide dismutase [16,20,21]. However, the effects of HNO on mitochondrial function have not been examined previously.

Using isolated mitochondria and SMPs (submitochondrial particles), we show here that mitochondria convert HNO into NO+ in a process that is dependent on components of the mitochondrial inner membrane and the concentration of the HNO donor AS. We also demonstrate that HNO inhibits mitochondrial respiration at complex II and modifies thiols in the organelle. The effects of HNO on mitochondrial function will be compared with the NO+-dependent regulation of respiration and discussed in the context of its cytotoxic and cytoprotective properties.

EXPERIMENTAL

Male Sprague–Dawley rats (body weight 250 g; from Harlan, Indianapolis, IN, U.S.A.) were handled in accordance with the recommendations of the NIH Guide for the Care and Use of Laboratory Animals, and food and water were available ad libitum. Liver mitochondria and SMPs were prepared as described previously [19], and the protein concentration was determined using the Folin-phenol reagent using a standard curve constructed with BSA. SMPs were prepared as described previously [19] and their purity was >99%, as determined by spectrophotometric measurement of citrate synthase, a marker of mitochondrial matrix contamination. Papa (PapaNONOate) was from Alexis measurement of citrate synthase, a marker of mitochondrial matrix contamination. Papa (PapaNONOate) was from Alexis and ONOO− experiment. The molar absorption coefficients for Papa, AS were determined spectrophotometrically before beginning the experiment. The molar absorption coefficients for Papa, AS were determined spectrophotometrically before beginning the experiment.

Liver mitochondria and SMPs were prepared as described previously [19], and the NO+ concentration was measured simultaneously [22–24]. Human haemoglobin was prepared as described previously [19]. Oxygen and NO+ concentrations were determined simultaneously using Clark-type electrodes (from Inotech, Plymouth Meeting, PA, U.S.A., Harvard Apparatus, Holliston, MA, U.S.A., World Precision Instruments, Sarasota, FL, U.S.A.) in a 1 ml sealed chamber, magnetically stirred at 37 °C. Mitochondria or SMPs (0–1 mg/ml) were incubated in respiration buffer (120 mM KCl, 10 mM Hepes, 1 mM EGTA, 25 mM sucrose, 5 mM MgCl2, 5 mM KH2PO4, pH 7.4). Data were collected using a digital recording device (Dataq, Akron, OH, U.S.A.) connected to a PC. A saturated solution of NO+ was prepared, and the NO+ concentration of this solution was determined spectrophotometrically by monitoring the conversion of oxyhaemoglobin into methaemoglobin [25]. The NO electrode was calibrated by bolus additions of NO (0.1–3.0 μM) to the electrode chamber, to establish the relationship between the concentration of NO and the electrode current [25].

NO+ was also measured using a chemiluminescence detector (Antek Instruments, Houston, TX, U.S.A.) attached to a sealed reaction chamber water-jacketed to maintain a temperature of 37 °C. Helium was passed over the surface of the buffer in the reaction chamber and the gas mixture was introduced into the detector, where the reaction with ozone occurred. The resulting chemiluminescence produced a signal, which was recorded using a digital recording device (Dataq) connected to a PC. In experiments to detect mitochondrial protein modifications, chemiluminescence was measured in samples incubated in 1 mM reagent at 37 °C after no treatment, treatment with sulphhalamide (0.5 %) or treatment with sulphhalamide plus Hg2+ (5 mM) to determine NO−, SNO and other adducts.

Mitochondrial enzyme activities were also measured spectrophotometrically. Complex II activity was determined by measuring the reduction of dichloroindophenol at 600 nm, which was coupled to the oxidation of CoQ2 using succinate as a substrate. SMPs were incubated (37 °C) with AS (20 μM) for 15 min and then re-isolated by centrifugation at 12 000 g for 20 min and incubated for a further 20 min at 22 °C with either 1 or 5 mM glutathione. Cytochrome c oxidase activity was measured by monitoring the oxidation of ferrocytochrome c at 550 nm. Citrate synthase activity was measured at 412 nm by using the coupled reaction of oxaloacetate, acetyl-CoA and DTNB [5,5′-dithiobis-(2,4-nitrobenzoic acid) and complex I activity as described [26]. Total glutathione equivalents (GSH + GSSG) were measured in lysed mitochondria using the Tietze recycling assay, and reduced protein thiols were measured by adding DTNB to the mitochondria and measuring absorbance at 412 nm. All experiments were performed a minimum of three times, and data are presented as means ± S.E.M. Statistical significance was determined using Student’s t test, with P < 0.05 representing significance. Curve fitting was performed using Origin™ software (Microcal, Northampton, MA, U.S.A.).

Nitrotyrosine was detected by probing a Western blot with an antibody to 3-nitrotyrosine (Cayman Chemicals, Ann Arbor, MI, U.S.A.). Samples of mitochondria (1 mg) treated or not with AS or malonate (1 mM) were centrifuged at 13 000 g, resuspended in 5 mM Tris buffer (pH 8.0)/0.1% Triton X-100 and labelled with 100 μM BIAM [N-(biotinyl)-N′-(iodoacetyl)ethylenediamine] for a period of 15 min at room temperature in the dark before analysis by electrophoresis. Two-dimensional gel analysis was performed by rehydrating immobilized pH gradient strips (Biogel, Hercules, CA, U.S.A.) with the sample in resolubilizing buffer (5 M urea, 2 M thiourea, 2% CHAPS. 0.5% lauryl maltoside, 30 mM dithiothreitol), and subjecting these strips to isoelectric focusing using an IPG Zoomrunner (Invitrogen, Carlsbad, CA, U.S.A.). The strips were then soaked in equilibrating buffer [6 M urea, 2 M SDS, 0.375 M Tris, 20% (v/v) glycerol] and subjected to electrophoresis in a denaturing gel. The gels were either silver stained using the SilverSnap kit (Pierce, Rockford, IL, U.S.A.) or Western blotted and probed for BIAM with streptavidin conjugated to HRP (horseradish peroxidase).

RESULTS

Generation of NO from mitochondria incubated with AS

It has been suggested that mitochondria have the ability to convert HNO into NO+: by providing electron acceptors, possibly in the form of ubiquinone or cytochrome c [27]. To examine this possibility, AS (20 μM) was added to intact mitochondria (1 mg/ml) in the oxidized (non-respiring) state and the NO+ concentration was monitored polarographically. In the absence of mitochondria, the formation of NO+ detected by the electrode was minimal, whereas on addition of the organelle a progressive increase in NO+ at a rate of 1.3 ± 0.4 nM/s (mean ± S.E.M. n = 3) was detected after approx. 1–20 s (Figure 1A). A steady-state concentration of NO+ was reached after a period of 4–5 min and maintained for 10 min, after which the signal decreased gradually back to baseline (results not shown). Oxyhaemoglobin (10 μM) was added to the chamber once an approximation to a steady state was achieved, and this resulted in an immediate decrease
in the signal (Figure 1A). A similar NO\textsuperscript{•} profile was observed when AS was added to SMPs (results not shown), precluding a mechanism of NO\textsuperscript{•} generation involving components of the matrix, such as Mn superoxide dismutase. The decomposition products of AS did not result in any detectable production of NO\textsuperscript{•} when added to the mitochondria. Decay of AS under these conditions was measured spectrophotometrically, and the rate of decomposition was 1.27 ± 0.069 μM/min in buffer alone (n = 3) and 1.48 ± 0.097 μM/min in the presence of mitochondria (200 μg/ml).

Since haemoglobin cannot distinguish HNO from NO\textsuperscript{•}, chemiluminescence detection was used as an independent method of verifying NO\textsuperscript{•} formation. In these experiments, the solution containing the mitochondria was stirred gently, with a stream of He gas flowing over the sample to carry any NO\textsuperscript{•} formed into the chemiluminescence detector. Using these conditions and the extremely sensitive detection that chemiluminescence offers, AS concentrations as low as 1 μM resulted in NO\textsuperscript{•} formation of approx. 70 pmol over a time period of approx. 1 min from non-respiring mitochondria, while no NO\textsuperscript{•} was detected in the absence of the organelle.

The conversion of HNO into NO\textsuperscript{•} was dependent on the concentrations of both AS and mitochondria. Consecutive additions of AS (0–20 μM) were made to a suspension of mitochondria (1 mg/ml). The steady-state concentration of NO\textsuperscript{•} generated is plotted against the cumulative concentration of AS. Line B was not decreased by the presence of respiratory inhibitors such as cyanide, rotenone or antimycin A, or the iron chelator HBED \(\text{N},\text{N}-\text{di-(2-hydroxybenzyl)ethylmediamine-N,N'-diacetic acid} \) (results not shown). Taken together, these data demonstrate that non-respiring mitochondria are capable of converting HNO into NO\textsuperscript{•} by a process that requires only components of the mitochondrial inner membrane and does not require O\textsubscript{2}.

**Inhibition of mitochondrial respiration by HNO**

In order to determine whether HNO may inhibit components of the respiratory chain, the effects of AS on respiration were investigated. Mitochondria (1 mg/ml) were incubated with or without AS (40 μM) for 10 min at 37 °C in the O\textsubscript{2} electrode chamber, and then either glutamate (15 mM) and succinate (10 mM) or glutamate (15 mM) plus malate (5 mM) were added as indicated by the arrows, and oxygen consumption was monitored. In mitochondria exposed to AS, state 3 respiration was inhibited by approx. 40% (Figure 2B). However, in the presence of glutamate plus malate, neither state 3 nor state 4 respiration was significantly affected from 0.1 to 1 mg/ml also resulted in the detection of increasing concentrations of NO\textsuperscript{•}. To test whether the production of NO\textsuperscript{•} was O\textsubscript{2} dependent, the mitochondria were incubated with succinate and ADP in an oxygen electrode and allowed to consume O\textsubscript{2} until the chamber became anoxic. AS (20 μM) was then added, and the formation of NO\textsuperscript{•} was essentially identical with that found in the presence of oxygen (open symbol in Figure 1C). Furthermore, the production of NO\textsuperscript{•} was not decreased by the presence of respiratory inhibitors such as cytochrome c, rotenone or antimycin A, or the iron chelator HBED \(\text{N},\text{N}-\text{di-(2-hydroxybenzyl)ethylmediamine-N,N'-diacetic acid} \) (results not shown). Taken together, these data demonstrate that non-respiring mitochondria are capable of converting HNO into NO\textsuperscript{•} by a process that requires only components of the mitochondrial inner membrane and does not require O\textsubscript{2}.

![Figure 1](image1.png)  
**Figure 1** Mitochondria convert HNO into NO\textsuperscript{•}  
(A) Representative NO\textsuperscript{•} electrode trace of AS (20 μM) added to 1 mg/ml mitochondria (solid line) or buffer alone (dotted line). Diversehaemoglobin (Hb; 10 μM) was added as indicated by the arrow. (B) Consecutive additions of AS (0–20 μM) were made to a suspension of mitochondria (1 mg/ml). The steady-state concentration of NO\textsuperscript{•} generated is plotted against the cumulative concentration of AS. (C) AS (20 μM) was added to mitochondria (0–1 mg/ml) and the NO\textsuperscript{•} concentration was monitored. The steady-state NO\textsuperscript{•} concentration is plotted as a function of mitochondrial concentration. Mitochondria were given succinate (15 mM) and ADP (0.5 mM) to support state 3 respiration, and allowed to consume oxygen until the chamber became anoxic. AS (20 μM) was added to the chamber and the NO\textsuperscript{•} concentration was measured (C).

![Figure 2](image2.png)  
**Figure 2** HNO inhibits succinate-supported respiration  
(A) Mitochondria (1 mg/ml) were incubated in the presence (solid line) or absence (dotted line) of AS (40 μM) for approx. 10 min at 37 °C and then succinate (15 mM) and ADP (0.5 mM) were added as indicated by the arrows, and oxygen consumption was monitored. (B) Quantification of the results shown in (A), showing state 3 and 4 respiratory rates in the presence (■) and absence (□) of AS. (C) Quantification of traces similar to those in (A), showing state 3 and 4 respiratory rates in the presence of AS (40 μM); □, untreated controls. Data are means ± S.E.M. of three experiments; *P < 0.05 compared with control.
by AS treatment (Figure 2C). Since the polarographic assay of complex I also involves other components of the respiratory chain, the activity of the isolated complex was also measured spectrophotometrically. Under these conditions, complex I activity was decreased by approx. 20% by AS treatment (control, 143 ± 4.7 nmol/min per mg; AS-treated, 116 ± 9.7 nmol/min per mg; means ± S.E.M., n = 3, P < 0.05). It is important to note that, at high O2 levels and low levels of NO (approx. 250–275 nM), inhibition of cytochrome c oxidase is minimal [28]. However, under physiological conditions, significant inhibition of complex IV by NO generated from HNO is likely. Decomposed AS had no effect on respiration using either substrate (results not shown). These results indicate that the predominant site of the HNO-dependent inhibition of respiration is complex II, unlike NO*-mediated inhibition, which occurs at complex IV.

**Inhibition of complex II by HNO**

In order to confirm the site of HNO-dependent inhibition, the activity of complex II was measured directly. Mitochondria were incubated with AS (40 µM), the NO* donor Papa (10 µM, yielding a concentration of NO in excess of that formed by AS), or ONOO− (40 µM) for 15 min at 37 °C and centrifuged to remove any remaining RNS. The activities of complexes I, II and IV and of citrate synthase, a matrix enzyme resistant to oxidative damage, were then measured spectrophotometrically. The activity of the matrix enzyme citrate synthase was found to be unchanged by any of the treatments, indicating that they did not cause mitochondrial rupture and loss of matrix components (Figure 3C). Complex II activity in mitochondria exposed to AS was inhibited by 60% in comparison with that in untreated mitochondria, while neither Papa nor decomposed AS had any effect. This inhibition was dependent on the concentration of AS (Figure 4A). Under aerobic conditions the decomposition of AS may potentially produce a product similar to ONOO−, a species that has been shown to inhibit complex II [20,29]. To confirm that the changes observed after AS treatment were not due to the formation of ONOO−, mitochondria were treated with ONOO− (40 µM), AS (40 µM) or Papa (10 µM), subjected to electrophoresis, Western blotted, and probed with an anti-nitrotyrosine antibody (Figure 4B). While ONOO−-treated mitochondria showed significant levels of nitrotyrosine, this modification was not detected in samples treated with AS or Papa.

**Treatment with HNO results in a loss of glutathione equivalents**

HNO has been shown to modify thiols, and since the mitochondrial matrix contains high concentrations of thiols, including millimolar levels of glutathione, the effect of HNO on mitochondrial thiol status was tested. Mitochondria (1 mg/ml) were incubated with AS (40 µM), Papa (10 µM) or ONOO− (40 µM) as described above and the glutathione concentration measured. In mitochondria exposed to AS, GSH + GSSG concentrations were significantly decreased (0.66 ± 0.24 nmol/mg of protein) in comparison with untreated mitochondria (3.04 ± 0.31 nmol/mg), whereas Papa, ONOO− and decomposed AS did not significantly affect the GSH + GSSG concentration.

In the next series of experiments, chemiluminescence was used to measure the products of the reaction of RNS with mitochondria, in order to determine the stable modifications of proteins and low-molecular-mass mitochondrial components formed by HNO, NO* and ONOO−. After 15 min incubations of mitochondria (1 mg/ml) with AS (40 µM), Papa (10 µM) or SIN-1 (3-morpholinosydnonimine hydrochloride; 50 µM), which generates NO and O2− to form ONOO−, each sample was divided...
into three aliquots for the determination of NO$_2^-$, RSNO and iron-nitrosyl (Fe-NO) content. In both the control and SIN-1-treated mitochondria, <1 µM NO oxidation products were detected, and the concentrations of RSNO and Hg$^{2+}$-resistant species were below the level of detection. In contrast, in the samples treated with Papa or AS, all three species could be detected. Although the concentrations of NO$_2^-$ formed in the samples treated with AS and Papa were similar, the concentrations of RSNO and Hg$^{2+}$-resistant species were different (Table 1). Levels of RSNO were in the low picomolar range, and were 4-fold higher in the NO-treated samples than in those treated with AS.

### Table 1 RNS mediate mitochondrial thiol modifications

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Content (pmol/mg of protein)</th>
<th>NO$_2^-$</th>
<th>RSNO</th>
<th>HgCl$_2$-resistant species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.087±0.01</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Papa (10 µM)</td>
<td></td>
<td>1.42±0.21</td>
<td>0.297±0.053</td>
<td>0.107±0.047</td>
</tr>
<tr>
<td>AS (40 µM)</td>
<td></td>
<td>2.41±0.78</td>
<td>0.07±0.01</td>
<td>0.013±0.01</td>
</tr>
<tr>
<td>SIN-1 (50 µM)</td>
<td></td>
<td>2.07±0.03</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Consistent with the inhibition being due to a thiol modification, glutathione reversed the inhibition of respiration induced by AS partially at a concentration of 1 mM and fully at a concentration of 5 mM (Figure 5B). Control experiments demonstrated that glutathione alone at these concentrations had no effect on activity (results not shown).

### HNO-dependent inhibition of complex II is prevented by malonate and reversed by glutathione

Complex II consists of four subunits, and contains three Fe–S clusters, a cytochrome b, and separate coenzyme Q and substrate binding sites. The active site, located in the 70 kDa subunit of the enzyme, contains a flavoprotein which catalyses the oxidation of succinate. This site also contains thiol groups that are required for the activity of the enzyme, and modification of these critical thiols results in inhibition of the complex. However, these thiols can be protected from modification, and hence the enzyme from inactivation, by the binding of substrate or inhibitors [30]. To determine whether AS inhibited complex II by modifying thiols in the active site of the enzyme, mitochondria were treated with succinate (15 mM) or with competitive inhibitors of succinate, i.e. malonate (1 mM) or oxaloacetate (1 mM). The mitochondria were then exposed to AS (40 µM) for 15 min at 37 °C, centrifuged to remove the inhibitor and excess AS, and complex II activity was measured (Figure 5A). Interestingly, the AS-dependent decrease in complex II activity was prevented in the mitochondria pretreated with malonate, while succinate and oxaloacetate had little effect on AS-dependent inhibition.

There is evidence that HNO may react with thiols to form intermediates such as the N-hydroxysulphenamide, which in the presence of other thiols, such as glutathione, can result in the formation of a mixed disulphide [31]. In order to determine whether thiol modification contributes to the AS-dependent inhibition of complex II activity, samples were prepared in SMPs in the presence of AS, followed by its removal and incubation of the samples with the thiol reductants dithiothreitol and glutathione for 20 min at 22 °C. We found that dithiothreitol interfered with the complex II assay in SMPs in a non-specific manner, but that glutathione was compatible with activity measurements. Consistent with the inhibition being due to a thiol modification, glutathione reversed the inhibition of respiration induced by AS partially at a concentration of 1 mM and fully at a concentration of 5 mM (Figure 5B). Control experiments demonstrated that glutathione alone at these concentrations had no effect on activity (results not shown).

### Treatment with AS modifies reactive cysteines on proteins in mitochondria

A proteomics approach was used to investigate further the stable modifications formed by HNO. Mitochondria were treated with AS in the absence or presence of malonate. The protein thiols in the samples were then tagged with biotin using the reagent BIAM. Under these conditions, HNO-dependent modification of thiols is reflected in decreased BIAM labelling relative to controls. The samples were then initially separated on a 10–18% (w/v) polyacrylamide gradient SDS gel, followed by Western blotting and probing with streptavidin–HRP. As seen in Figure 6(A), treatment with AS resulted in a significant decrease in BIAM labelling on a number of different proteins. As a control, labelling was also performed in the presence of malonate, which again showed a substantial decrease in BIAM labelling after AS treatment. These data rule out the possibility that malonate is a scavenger of the species derived from HNO which lead to inhibition of complex II. Samples were also analysed on a two-dimensional format, with separation in the first dimension on isoelectric focusing strips, followed by a second-dimension resolution on a 10–18% gradient SDS/PAGE. Analysis of the silver-stained gels from control samples using PD QUEST revealed approx. 130 spots (Figure 6B), of which approx. 30% matched with those from the AS-treated samples (Figure 6D). Treatment with AS resulted in a significant decrease in BIAM labelling (Figure 6D) when compared with controls (Figure 6C). Approx. 88 spots were detected in the control samples, of which only 62 spots were matched to AS-treated samples.

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DISCUSSION

NO\(^{•}\) is thought to regulate mitochondrial function by binding transiently to the binuclear oxygen binding site in cytochrome c oxidase. In contrast, other RNS, such as N\(_2\)O\(_3\) and ONOO\(^{•}\), alter mitochondrial function through the irreversible modification of proteins. Some evidence exists for the metabolism of NO\(^{•}\) to HNO within the mitochondria [32,33], and in addition HNO may be formed in vivo by the decomposition of RSNO or by nitric oxide synthase under some conditions [34,35]. However, the role of HNO in the regulation of mitochondrial function has not been investigated previously.

In the first series of experiments, we demonstrated that intact isolated mitochondria are able to convert HNO into NO\(^{•}\) via a mechanism that is saturable but independent of electron transfer or the presence of O\(_2\). It has been suggested previously that mitochondria are capable of converting HNO into NO\(^{•}\) through mechanisms involving ubiquinone or ferricytochrome c, but this appears unlikely from the present results [27]. For example, the generation of NO\(^{•}\) from HNO by SMPs, which are devoid of cytochrome c, demonstrates that a component within the inner membrane is at least partly responsible for the oxidation of HNO. Furthermore, NO\(^{•}\) production from anoxic mitochondria in the presence of substrate, a condition in which all components of the respiratory chain are reduced, is not consistent with a role for ubiquinone as an electron acceptor. Since the formation of NO\(^{•}\) is a saturable process with respect to the mitochondrial, it seems most likely that a number of components of the inner membrane, including a broad range of electron acceptors, may serve this function, including perhaps protein thiols. Alternatively, one of the reaction products of thiols with HNO is thought to be an intermediate which can yield hydroxylamine [36]. It is possible that either the N-hydroxysulphenamide or hydroxylamine itself can be converted into NO\(^{•}\) by mitochondria. In support of this argument, it has been shown that hydroxylamine can be converted into NO\(^{•}\) by haemoglobin [37]. Although we estimate the yield of NO formation from mitochondria exposed to HNO to be of the order of 4–5\%, this may be sufficient to activate the soluble guanylate cyclase pathway and thus explain the ‘NO-like’ properties of AS.

In addition to the formation of NO\(^{•}\), we found that AS inhibited mitochondrial respiration supported by succinate as a substrate. These data suggest that complex II is the primary site of inhibition of mitochondrial respiration by HNO, with a minor effect on complex I. These data were confirmed by the specific measurement of succinate dehydrogenase activity and complex I activity spectrophotometrically. Complex IV inhibition by NO\(^{•}\) is independent of the substrate used to support respiration, unlike the HNO-mediated inhibition of complex II. Previous studies have shown that complex II activity can be disrupted by the modification of critical thiols in the active site of the enzyme. Furthermore, binding of a competitive inhibitor to the substrate binding site can prevent modification of this critical thiol residue [30,38]. It was found that the HNO-dependent inhibition of complex II was prevented by malonate, suggesting that this inhibition is mediated through the modification of a cysteine residue in the active site of the enzyme.

It is recognized that HNO may mediate many of its biological actions through the modification of thiols [9,12,31]. It is thought that HNO reacts directly with thiol groups to initially form an N-hydroxysulphenamide. This intermediate can then react with other thiols in the system to form mixed disulphides and hydroxylamine. In the absence of other thiols, a rearrangement to sulphinamide occurs [31,36]. This is interesting, since the formation of a disulphide can be reversed by excess low-molecular-mass thiols such as glutathione, while the formation of sulphynamide is irreversible under these conditions [31]. Indeed, the finding that the AS-dependent inhibition of complex II is reversed by glutathione, which is consistent with the idea that a mixed disulphide or N-hydroxysulphenamide is formed in the presence of HNO and mitochondria.

Interestingly, unlike ONOO\(^{•}\) at these concentrations, AS depleted total mitochondrial glutathione, which would be consistent with the reactions outlined above. The spectrophotometric assay used in these studies detects both oxidized and reduced glutathione, and lack of a reaction indicates that the glutathione is converted into a product distinct from either form. The products formed from AS, assessed by chemiluminescence, were similar to those formed following the treatment of mitochondria with Papa, except the NO donor resulted in higher concentrations of both RSNO and the Hg\(^{2+}\)-resistant species when compared with AS treatment. It is most likely that the nitrosated species formed on exposure to AS were derived from the production of NO. It is important to note that, relative to the total thiol content (protein plus free thiol) of the mitochondria (30–40 nmol/mg of protein), the RSNO content represents a very small fraction (of the order of 0.006\%), again making it unlikely that S-nitrosation contributes significantly to the inhibition of complex II by AS.

Using a proteomics approach, we determined the effects of AS on the mitochondrial thiol subproteome. As expected, the
modification of protein thiols was extensive. In these studies we found that the complex II 70 kDa subunit, which contains the reactive thiol, did not appear on the second dimension of proteins from mitochondria treated with AS, although it was unchanged in one-dimensional SDS/PAGE as assessed by Western blotting (result not shown). For this reason we cannot confirm that the only modification induced by AS in complex II was at this protein thiol. It seems likely that a more extensive analysis of mitochondrial function would reveal other defects in metabolic pathways in the organelle beyond the effects on complex I and II reported here. Indeed, it has been shown that HNO is a potent inhibitor of mitochondrial aldehyde dehydrogenase, again through a mechanism involving thiol modification of a critical cysteine residue in the protein.

Mitochondria have several functions within the cell, one of which is the production of ROS (reactive oxygen species). Defects in complex II have been shown to lead to increased ROS production and increased susceptibility to apoptosis [39,40], and this may contribute to the mechanism by which HNO mediates cytotoxicity in cells. Furthermore, inhibition of complex II in brain mitochondria has been associated with the development of Huntington’s disease [41]. The depletion of mitochondrial glutathione by HNO may play an important role in mitochondrial signalling and dysfunction. It has been shown recently that specific thiols on complex I can be glutathionylated, resulting in increased production of ROS from the mitochondria [42].

The interactions of HNO with mitochondria also have implications in ischaemia/reperfusion injury. It has been shown that HNO administered during reperfusion is detrimental to the cell, while treatment with HNO before the ischaemic episode results in myocardial protection [12,15]. This protective effect is similar to the effect seen with diazoxide, a mitochondrial K<sub>ATP</sub> channel opener, and is thought to be mediated via the modification of thiols. It is interesting to speculate that the generation of NO<sup>•</sup> by the mitochondrion under conditions of low oxygen tension and turnover is protective, whereas inhibition of complex II is detrimental. In addition, an increased production of mitochondrial ROS has been shown to protect the myocardium by initiating adaptive pathways involving NO<sup>•</sup>, and this may be an additional potential mechanism by which HNO mediates its protective effect [43].

In summary, HNO and NO<sup>•</sup> have been shown to elicit distinct responses in a number of biological systems. The present study demonstrates that this also holds true in the mitochondrion, where NO<sup>•</sup> acts discretely and reversibly at cytochrome c oxidase, while HNO has more widespread effects on the organelle, including the depletion of glutathione and the inhibition of complexes I and II. In conclusion, the mitochondrion may then serve as a target for HNO, and also as a ‘detoxification system’, since NO<sup>•</sup> has been shown to be less cytotoxic than HNO, and in some cases to be cytoprotective.

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