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Biochimica et Biophysica Acta 1757 (2006) 562–566
BIOCHIMICA ET BIOPHYSICA ACTA
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S-nitrosothiol inhibition of mitochondrial complex I causes a reversible increase in mitochondrial hydrogen peroxide production

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Received 13 December 2005; received in revised form 24 January 2006; accepted 22 February 2006
Available online 23 March 2006

Abstract

We found that reversible inactivation of mitochondrial complex I by S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) in isolated rat heart mitochondria resulted in a three-fold increase in H_2O_2 production, when mitochondria were respiring on pyruvate and malate, (but not when respiring on succinate or in the absence of added respiratory substrate). The inactivation of complex I and the increased H_2O_2 production were present in mitochondria washed free of SNAP or NO, but were partially reversed by light or dithiothreitol, treatments known to reverse S-nitrosation. Specific inhibition of complex I with rotenone increased H_2O_2 production to a similar extent as that caused by SNAP. The results suggest that S-nitrosation of complex I can reversibly increase oxidant production by mitochondria, which is potentially important in cell signalling and/or pathology.

Keywords: Mitochondria; Reactive oxygen species; Nitric oxide

1. Introduction

There is increasing interest in mitochondria as major sources of cellular oxidants (reactive oxygen species, ROS, and reactive nitrogen species, RNS), which may play physiological or pathological roles. Physiologically, mitochondrial ROS and RNS have been suggested to regulate mitochondrial functions [1–3], and cell proliferation and differentiation [1,4]. Pathologically, mitochondrial ROS and RNS have been implicated in aging [5], diabetes [6], neurodegeneration [7,8], and cell death [9–12]. Most mitochondrial ROS are thought to be derived from superoxide produced from either complex I or III [13–17]. Subsequent dismutation of superoxide, particularly by mitochondrial superoxide dismutase, results in hydrogen peroxide production. However, if or how mitochondrial ROS production is regulated is poorly understood.

Nitric oxide (NO), peroxynitrite ($ONOO^-$) and S-nitrosothiols (SNO) can inactivate complex I probably by several different mechanisms (reviewed in [18]). However, part of this inactivation can be reversed by reduced thiols or light, treatments that are known to breakdown S-nitrosothiols [3,19,20], suggesting that inactivation of the complex is due to S-nitrosation of complex I protein. This is supported by the fact that S-nitrosothiols can reversibly inactivate complex I even in conditions where there is little or no free NO released [19], suggesting that inactivation occurs by transnitrosation. Low molecular weight S-nitrosothiols, such as S-nitrosoglutathione (GSNO) or S-nitroso-*N*-acetyl-penicillamine (SNAP), can directly transfer the NO^+ group to reduced thiols (transnitrosation), including protein thiols, and this may be an important means by which NO regulates proteins. Inactivation of complex I by NO, $ONOO^-$ or SNO has been suggested to contribute to the pathology of sepsis [21], Parkinson's disease [22], and heart failure [23].

Because SNO reversibly inactivate complex I, and because specific inhibitors of complex I can increase mitochondrial ROS production, we were interested whether SNO inactivation of complex I would result in increased mitochondrial ROS production. It is not obvious how SNO inactivation of complex I will effect ROS production as the inactivation may occur

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doi:10.1016/j.bbabio.2006.02.014

upstream, downstream or at ROS production sites within complex I. Thus, we tested the effect of SNO on ROS production in isolated mitochondria.

2. Methods

Rat heart mitochondria were isolated using standard procedures of homogenization and differential centrifugation [24]. Mitochondria (1 mg protein/ml) were incubated in a stirred and thermostated (37 °C) 1 ml chamber in the presence/absence of 1 mM SNAP (Alexis). The incubation buffer contained 120 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.2 and either 1 mM pyruvate+1 mM malate or 5 mM succinate. After 30 min incubation mitochondria were spun down in an Eppendorf microcentrifuge (13,000 rpm, 5 min), washed 3 times with incubation buffer and resuspended in 1 ml of incubation buffer for Amplex Red assay of hydrogen peroxide production or frozen for complex I assay. In some experiments, SNAP-treated and washed mitochondria were suspended in 1 ml of ice-cold incubation buffer and illuminated by a Fiber Optic Illuminator (WPI, 150 W halogen bulb) for 40 min or incubated in the presence of 1 mM DTT for 30 min. After treatment with DTT, mitochondria were washed twice with incubation buffer to remove residual DTT. In experiments where PTIO was used, 1 mM PTIO was added together with 1 mM SNAP to the mitochondrial incubation mixture.

For determination of complex I activity, pellets were resuspended in 10 mM KH_2PO_4 (pH 8) and freeze–thawed at least 3 times. The activity of complex I was detected spectrophotometrically from the rate of decrease of NADH absorbance at λ=340 nm, ε=6.81/mM/cm in the presence of added coenzyme Q_1 (Sigma). For determination of complex II–III activity, the rate of cytochrome c reduction was measured at 550 nm (ε=19 mM^{-1} cm^{-1}) in broken mitochondria with succinate as described in [25]. Mitochondria (0.025 mg, freeze–thawed 3 times) were incubated in 1 ml 25 mM KH_2PO_4, pH 7.2 for 10 min in the presence of 20 mM succinate, 2 μM rotenone and 2 mM NaN_3. The reaction was started by the addition of 10 μM cytochrome c.

Production of hydrogen peroxide by mitochondria was measured using Amplex Red (Molecular Probes). Mitochondria were incubated in the incubation buffer (see above) supplemented with 1 μM Amplex Red and 1 U/ml horseradish peroxidase and the rate of hydrogen peroxide production was determined by following the increase in fluorescence (excitation at 560 nm, emission at 587 nm) at room temperature measured with a Shimadzu RF-1501 spectrofluorimeter. That the Amplex Red oxidation by mitochondria was due to H_2O_2 was verified by adding catalase, which reduced this oxidation by more than 80%. In the absence of mitochondria, there was no significant rate of Amplex Red oxidation in the presence of pyruvate plus malate, succinate or rotenone.

For measurement of reduced glutathione (GSH) levels, control or SNAP-treated mitochondria (0.5 mg protein) were spun down (13,000 rpm, 5 min), washed with incubation medium, suspended in 200 μl 50 mM Tris buffer (pH 7.4) and freezed/thawed 3 times. Then, monochlorobimane (Molecular Probes) was added to the extract to a final concentration of 100 μM along with glutathione S-transferase (1 U/ml, Sigma). After 60 min incubation at room temperature the fluorescence (excitation at 380 nm and emission at 520 nm) was measured in a BMG Fluostar Optima plate reader. 0–50 μM GSH was used for a calibration curve.

Mitochondrial respiration was measured using a Clark-type oxygen electrode in the incubation buffer additionally supplemented with 5 mM KH_2PO_4 and 1 mM ADP. Respiratory control index measured as ratio of State 3/State 2 respiratory rates with 1 mM pyruvate plus 1 mM malate as substrates was 9.3±1.2.

Data are presented as means±standard error of at least 4 separate experiments. Statistical analysis was performed using Student's t test. Differences were regarded to be statistically significant at $p<0.05$.

3. Results and discussion

It has previously been shown that exposure of isolated mitochondria to S-nitrosating NO donors (such as GSNO or SNAP) results in rapid inactivation of complex I probably due to its transnitrosation [19,26]. We tested whether SNAP-treated mitochondria have higher rate of ROS production by treating isolated rat heart mitochondria with 1 mM SNAP and then assaying mitochondrial H_2O_2 production by Amplex Red assay. Note that after treating mitochondria with SNAP for 30 min the mitochondria were washed three times, so that there was no SNAP present during the assay. As can be seen in Fig. 1A, mitochondria oxidizing pyruvate plus malate produce low levels of H_2O_2. After treatment of mitochondria with 1 mM SNAP complex I activity was decreased by about 60% (Fig. 1B), and the rate of H_2O_2 production increased 2.8 fold (Fig. 1A). When SNAP-treated mitochondria were exposed to light, a treatment known to destroy S-nitrosothiols, there was partial recovery of complex I activity and return to low rates of hydrogen peroxide production by mitochondria (Fig. 1), suggesting that S-nitrosation of the complex may be responsible for the ROS production. Similarly to SNAP, rotenone, a selective inhibitor of complex I, caused a 3.9 fold increase in ROS production by mitochondria (Figs. 1 and 2). Interestingly, inhibition of complex I and stimulation of ROS production was observed even when mitochondria were incubated with SNAP in the presence of PTIO—a scavenger of NO that removes free NO (Fig. 1). This suggests that inhibition was induced by a transnitrosation reaction (i.e. transfer of an NO^+ group from SNAP to complex I) rather than being mediated by free NO released from the NO donor.

Fig. 1C summarizes the data on complex I activity and H_2O_2 production obtained in mitochondria under these various experimental conditions. There was a negative correlation (r^2=0.85) between complex I activity and H_2O_2 generation: the lower activity of complex I (reduced by treatment with SNAP, rotenone, SNAP plus light) the higher the rate of H_2O_2 production. This suggests that inhibition of complex I activity by S-nitrosation may be the cause for increased ROS production by mitochondria.

In separate series of experiments, we tested whether dithiothreitol (DTT) would reverse the SNAP-induced increase in ROS production, as DTT is known to reverse S-nitrosation and reverse the SNO-induced inhibition of complex I [19]. In these experiments, SNAP-treatment decreased complex I activity from 150±24 nmol/min/mg in control to 63±9 in SNAP treated mitochondria ($P<0.01$), and subsequent 1 mM DTT treatment increased the rate back to 108±16 ($P<0.01$). In the same mitochondria, SNAP treatment increased H_2O_2 production from 30±3 pmol/min/mg in control to 159±17 after SNAP treatment ($P<0.05$), and subsequent exposure to DTT decreased the rate back to 88±11 ($P<0.05$). Thus DTT reversed both the SNAP-induced inhibition of complex I and the increased ROS production, consistent with both being due to S-nitrosation of complex I. The fact that DTT did not completely reverse the SNAP-induced inhibition of complex I and stimulation of ROS production suggests either that SNAP also inhibits complex I by mechanisms other than S-nitrosation or that DTT does not completely reverse the S-nitrosation.

Control, untreated mitochondria respiring on endogenous substrates produced low levels of H_2O_2, and this rate was stimulated 2.5 fold by the addition of pyruvate plus malate or 4

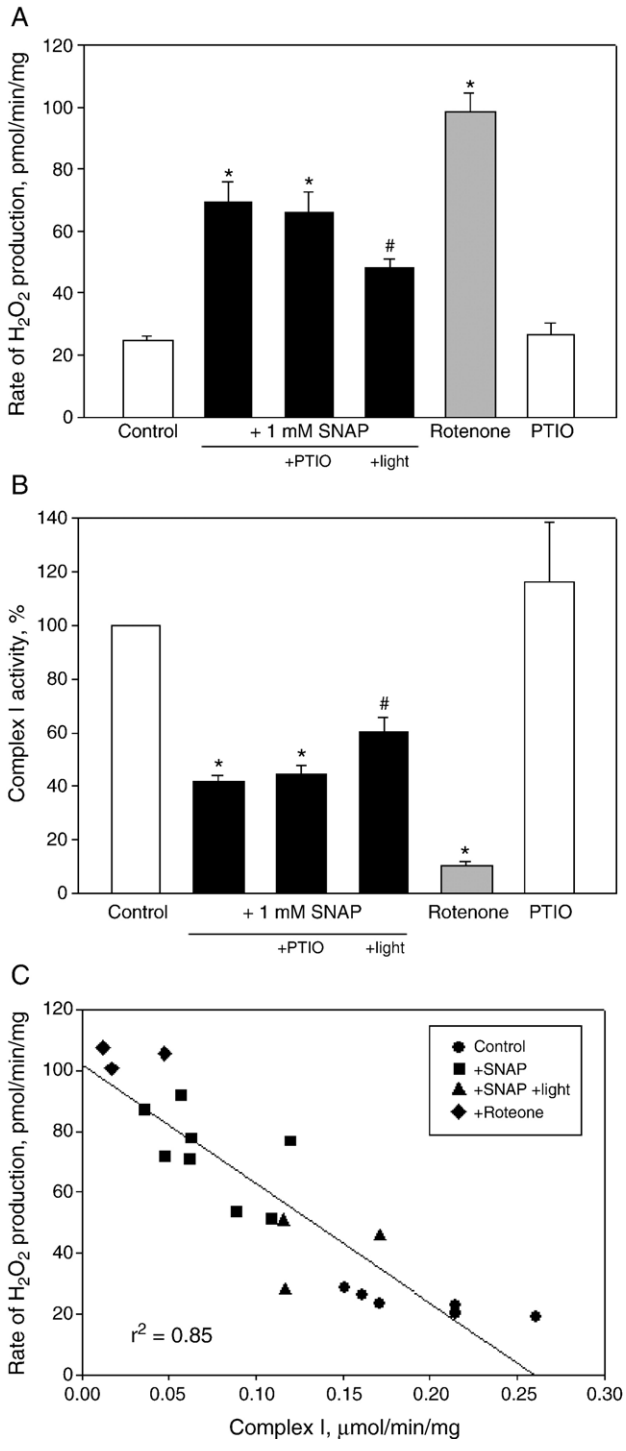

Fig. 1. Effect of SNAP on H_2O_2 production and complex I activity in isolated heart mitochondria. Heart mitochondria were treated with 1 mM SNAP for 30 min in the absence or presence of 1 mM PTIO (indicated as+PTIO) or with 2 μM rotenone for about 5 min, then washed with buffer and used for measurement of H_2O_2 production by Amplex Red assay (A) or complex I activity (B). Where indicated, SNAP-treated mitochondria were exposed to light for 40 min (indicated as +light). C—correlation between rate of H_2O_2 production and complex I activity in isolated heart mitochondria. Each point represents mean of 2–3 repeats of measurements of H_2O_2 production rate and complex I activity under particular conditions (untreated (control), treated with SNAP, SNAP plus light, or rotenone) in 3–6 separate mitochondrial preparations. Correlation coefficient (r^2=0.85) was calculated using SigmaPlot software. *Statistically significant difference from control (P<0.01), #Statistically significant difference from SNAP-treated mitochondria (P<0.05).

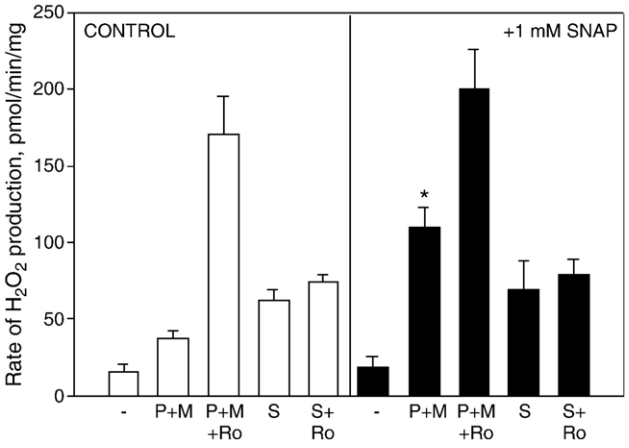

Fig. 2. Effect of exogenous substrates on H_2O_2 production by isolated heart mitochondria. During measurement of H_2O_2 production by Amplex Red assay, control or SNAP-treated mitochondria were incubated in the absence (−) or presence of added substrates: P+M − 1 mM pyruvate plus 1 mM malate, P+M+Ro – 1 mM pyruvate plus 1 mM malate plus 2 μM rotenone, S − 5 mM succinate, S+Ro − 5 mM succinate and 2 μM rotenone. *Statistically significant difference (P<0.05) between the SNAP-treated and control mitochondria in the presence of the same substrate combination.

fold by the addition of succinate (Fig. 2). The rate of H_2O_2 generation in the absence of exogenous substrates or in the presence of succinate (±rotenone) was not changed by SNAP treatment, but the rate in the presence of pyruvate plus malate was substantially increased (Fig. 2). This indicates that the H_2O_2 production of SNAP-treated mitochondria was related to complex I function, rather than actions on other respiratory chain complexes, decreased H_2O_2 removal or to any residual SNAP associated with the mitochondria. We also tested whether SNAP treatment had any effect on complex II–III activity, but SNAP had no significant effect (rates in nmol/min/mg of 36±7 in control and 33±6 in SNAP-treated mitochondria).

Exposure of mitochondria to SNAP also induced depletion of mitochondrial GSH (and possibly other reduced thiols): after 20 min incubation with 1 mM SNAP, the content of reduced thiols decreased from 8.3±1.8 nmol/mg protein in control to 4.3±1.1 nmol/mg protein in SNAP-treated mitochondria. In principle, therefore, SNAP-induced ROS production might result from glutathione depletion decreasing H_2O_2 removal by mitochondrial glutathione peroxidase. However, in practice, this level of glutathione depletion has little effect on mitochondrial ROS production [27]. And the findings that light reversed the SNAP induced H_2O_2 production and that SNAP had no effect on the rates of H_2O_2 production in the presence of succinate or absence of substrates are not consistent with the H_2O_2 production resulting from glutathione depletion. The decrease in mitochondrial glutathione might result from transnitrosation or the increased mitochondrial ROS production.

Mitochondrial ROS production comes mainly from complexes I and III [13–17]. Because SNAP inactivates complex I and a specific complex I inhibitor, rotenone, stimulates ROS to a similar extent as SNAP, it seems likely that SNAP-induced ROS production comes from complex I.

SNAP did not inhibit complex II–III activity and had no effect on ROS production when mitochondria were respiring on succinate, indicating that SNAP had no relevant effect on the rest of the respiratory chain. In mitochondria from some tissues reversed electron transfer through complex I when respiring on succinate results in very high rates of ROS production [15], however in heart mitochondria we found that preincubating the mitochondria in the absence of substrate resulted in a relatively low rate of ROS production in the presence of succinate (Fig. 2). The site(s) within complex I from which ROS are derived is still controversial, and has been attributed to either FMN, iron–sulphur centres or bound quinones [14–17,28,29]. Our finding that SNAP and rotenone stimulate complex I ROS production to a similar extent (for similar complex I inhibition, Fig. 1C) is consistent with both SNAP and rotenone inhibiting complex I electron transfer downstream of a ROS generation site, which due to inhibition of electron transfer becomes fully reduced and therefore has increased ROS production.

NO itself has previously been found to acutely stimulate ROS production by mitochondria, probably by inhibiting complexes III or IV or reacting with ubiquinol, but this effect is immediately reversed when the NO is removed [1,30–32]. S-nitrosation of complex I may provide a longer lasting means of modifying ROS production.

Superoxide, produced by mitochondria, may react rapidly with NO to produce peroxynitrite, which may further inhibit complex I, potentially resulting in a vicious circle contributing to pathology [32]. Hydrogen peroxide, produced by mitochondria, might also synergise with NO to induce cell death [30], or alternatively may regulate protein kinase pathways [33,34], transcription [35] and/or cell proliferation [4,35]. In conclusion, our results suggest that S-nitrosothiol-induced nitrosation and inhibition of complex I, causes a reversible increase in mitochondrial ROS production. This might be important in cellular signalling, aging or death.

Acknowledgement

The work was supported by the British Heart Foundation.

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