The assumption that nitric oxide inhibits mitochondrial ATP synthesis is correct

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Abstract The assumption that reversible inhibition of mitochondrial respiration by nitric oxide ($NO^{\bullet}$) represents inhibition of ATP synthesis is unproven. $NO^{\bullet}$ could theoretically inhibit the oxygen consumption with continued ATP synthesis, by acting as an electron acceptor from cytochrome *c* or as a terminal electron acceptor in stead of oxygen. We report here that $NO^{\bullet}$ does reversibly inhibit brain mitochondrial ATP synthesis with a time course similar to its inhibition of respiration. Whilst such inhibition was largely reversible, there appeared to be a small irreversible component which may theoretically be due to peroxynitrite formation, i.e. as a result of the reaction between $NO^{\bullet}$ and superoxide, generated by the mitochondrial respiratory chain.

Key words: Brain mitochondria; Nitric oxide; ATP; Oxidative stress; Neurodegeneration

1. Introduction

In early bioenergetic research, the interaction of molecules such as nitric oxide ($NO^{\bullet}$) with the mitochondrial respiratory chain proved useful in elucidating the molecular mechanism of oxidative phosphorylation [1]. However, it was not until the discovery that cells possess a family of $NO^{\bullet}$ synthetic enzymes [2] that such molecular interactions were considered to have physiological relevance.

It is reported that $NO^{\bullet}$ reversibly inhibits the mitochondrial respiration in synaptosomes [3,4], skeletal muscle mitochondria [5], brain mitochondria and sub-mitochondrial particles [6], liver mitochondria [7,8], heart mitochondria [9,10] and sub-mitochondrial particles [10] and cultured astrocytes [11]. In addition, $NO^{\bullet}$ decreases membrane potential in liver mitochondria [8], ATP/ADP ratio in synaptosomes [4] and creatine phosphate (PCr) NMR signal in intact beating guinea pig heart [12].

The putative role of $NO^{\bullet}$ as a physiological regulator of cellular energy metabolism rests on the assumption that $NO^{\bullet}$ inhibition of mitochondrial oxygen consumption is representative for the inhibition of mitochondrial ATP synthesis. However, there is no direct evidence for this. None of the above studies examined mitochondrial ATP synthesis. The observations that $NO^{\bullet}$ reduces the ATP/ADP ratio in synaptosomes [3] and the PCr NMR signal in isolated guinea pig heart [12], may be due to increased ATP consumption and not necessarily due to a decreased ATP production by inhibited mitochondria.

It is possible that $NO^{\bullet}$ may act as a substitute electron acceptor at cytochrome *c* oxidase (COX), or that $NO^{\bullet}$ may directly oxidise cytochrome *c* by passing COX. Thus, proton pumping and ATP synthesis could continue despite a decreased oxygen consumption. It was therefore the aim of this study to directly examine the effects of $NO^{\bullet}$ on ATP synthesis in isolated brain mitochondria.

2. Materials and methods

All chemicals were of analytical grade from Sigma (Poole, Dorset, UK) except Ficoll 400 which was from Amersham Pharmacia (Little Chalfont, Buckinghamshire, UK) and cytochrome *c* from Roche Products (Lewes, East Sussex, UK). $NO^{\bullet}$ solution was prepared by bubbling water (3 ml) with oxygen free N_2 for 10 min, followed by bubbling with $NO^{\bullet}$ gas (Aldrich, Poole, Dorset, UK) for 15 min. The resulting saturated solution was assumed to contain 1.9 mM $NO^{\bullet}$ [13]. The solution was prepared fresh daily, kept sealed until use and rebubbled with $NO^{\bullet}$ every 30 min throughout the experimental procedures (1–2 h). Degraded $NO^{\bullet}$ was prepared by leaving this solution for 1 week with occasional agitation and opening to air.

Brain mitochondria were prepared from male rats of the Wistar strain, 300–350 g in weight, as previously described [14]. Protein was quantified by the method of Lowry et al. [15] with bovine serum albumin as standard.

Mitochondria (0.125 mg protein) were incubated in plastic 1.5 ml tubes in a final volume of 500 μl of respiration buffer containing sucrose (25 mM), mannitol (75 mM), KCl (199 mM), Tris-phosphate (10 mM), fat-free BSA (1 mg/ml), glutamate (10 mM), malate (2.5 mM) and ADP (5 mM). Optionally present was KCN (2 mM). $NO^{\bullet}$ solution (1 μl, final concentration 4 μM) or degraded $NO^{\bullet}$ were added after 3 min and incubations allowed to run for a maximum of 15 min, mixing and aerating tubes every 3 min. A time course was constructed by performing individual incubations for each time point.

At the end of incubations, reactions were quenched by the addition of 10% perchloric acid (125 μl) to each tube, mixing, addition of 1 M KH_2PO_4 (200 μl), mixing and immediate transfer of the tube to liquid N_2. Samples were stored at −80°C (< 2 days) and analysis of the ATP content was performed by luminometry [16]. No significant degradation of ATP occurred over the storage period.

Cytochrome *c* was reduced by incubation with ascorbate, then purified using a gel filtration column (Pharmacia PD-10). Wavelength spectra were obtained using a Uvikon 930 spectrophotometer (Kontron Instruments). Reduced cytochrome *c* was incubated at 20 μM in 10 mM phosphate buffer (equilibrated with the air), pH 7.2, with a single addition of $NO^{\bullet}$ solution (final concentration 80 μM).

3. Results

Fig. 1 shows the effects of $NO^{\bullet}$, degraded $NO^{\bullet}$ and KCN on rat brain mitochondrial ATP synthesis. Addition of $NO^{\bullet}$

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(4 μM) at 3 min caused a rapid inhibition of the ATP synthesis. After a further 7–12 min, the rate of ATP synthesis had recovered to 72% of the control rate (no NO•). Degraded NO• had no effect on the mitochondrial ATP synthesis. Addition of KCN (2 mM) at 3 min caused a rapid irreversible inhibition of the ATP production. This inhibition was not ameliorated by co-addition of NO• at the same time as KCN.

Fig. 2 shows the effects of NO• exposure on the wavelength spectra of initially reduced cytochrome *c*. The addition of NO• (80 μM) caused a decrease in the peak at 550 nm, indicating oxidation of cytochrome *c*. Sequential additions of intermediate concentrations of NO• caused intermediate reductions in the peak at 550 nm (data omitted for clarity).

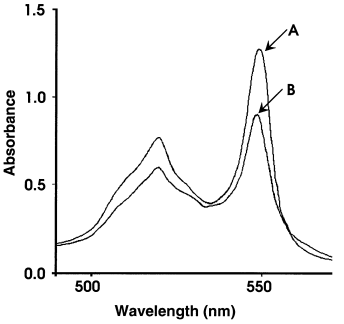

Fig. 2. Absorbance spectrum of cytochrome *c* (20 μM). A: fully reduced form, B: following addition of NO• (80 μM). Spectra were measured as detailed in Section 2.

4. Discussion

The recent surge for publications regarding reversible inhibition of mitochondrial oxygen consumption by NO• [3–12], together with the reported discovery of a putative NO• synthase activity in mitochondria [17–21] has led to the common assumption that NO• may be a physiological regulator of cellular energy metabolism. However, most NO• inhibition studies in mitochondria have only examined the mitochondrial oxygen consumption and there is no direct evidence that NO• inhibits the mitochondrial ATP synthesis. In this study we have used isolated brain mitochondria to investigate the effects of NO• on the mitochondrial ATP synthesis.

The results in Fig. 1 show that NO• does indeed inhibit the mitochondrial ATP synthesis. The time course of the inhibition bears remarkable similarity to that seen for NO• inhibition of mitochondrial oxygen consumption [4,5,7–9,11]. Degraded NO• had no effect on the ATP synthetic rate, suggesting the effects of NO• were direct and not due to its breakdown products.

In Fig. 2, the characteristic α peak at 550 nm in the absorption spectrum of reduced cytochrome *c* is decreased following exposure to NO•, suggesting the direct oxidation of cytochrome by NO• or one of the products generated from NO• under aerobic conditions, e.g. NO_2 and N_2O_3. In respiring mitochondria, such a reaction could theoretically bypass COX, allowing continued proton pumping by complexes I and III of the respiratory chain and continued ATP synthesis at decreased stoichiometry. However the data in Fig. 1 show that NO• is unable to recover any ATP synthesis in mitochondria that have their cytochrome oxidase blocked by cyanide, suggesting that such a bypass does not occur in intact mitochondria. In addition, the high concentrations of NO• required to bring about cytochrome *c* oxidation in this experiment (80 μM: four times the cytochrome *c* concentration) are unlikely to be encountered by cytochrome *c* in mitochondria in vivo, even in pathological situations.

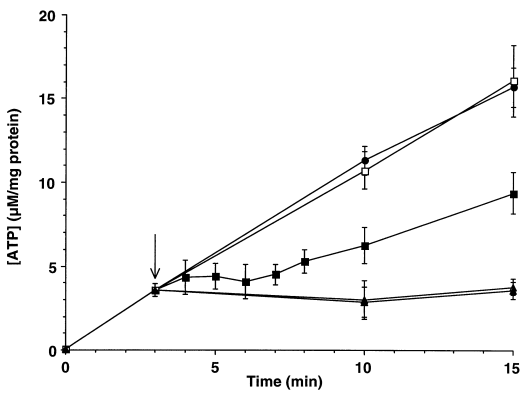

Fig. 1. The effects of NO• on rat brain mitochondrial ATP synthesis. Mitochondria were incubated at 0.25 mg protein/ml as detailed in Section 2 (●) with the following additions after 3 min (as indicated by arrow): 4 μM NO• (■), degraded NO• (□), 2 mM KCN (▲) and KCN plus NO● (Δ). Data are means ± S.E.M. of 5 independent experiments.

It is interesting to note that the NO• inhibition of mitochondrial ATP synthesis observed in Fig. 1 is not fully reversible during the time course and experimental conditions studied. Closer examination of oxygen electrode data in previously published studies shows that oxygen consumption in mitochondria isolated from heart [9] and liver [7,8] and mitochondria within isolated synaptosomes [3] and cultured astrocytes [11] is also not 100% recoverable on a similar time scale. These results taken together suggest that even at the low NO• concentrations required to inhibit mitochondrial respiration and ATP synthesis, there may be a secondary irreversible inhibition. Clementi et al. recently reported that long term exposure of cells to NO• leads to the irreversible inhibition of mitochondrial complex I, which the authors suggest may be nitrosothiol-mediated [22]. Irreversible damage to the mitochondrial respiratory chain may also occur as a result of peroxynitrite generation. It is known that mitochondria are a source of superoxide ($O_2^{\bullet -}$) within eukaryotic cells [23] and it has been demonstrated that this $O_2^{\bullet -}$ may react with NO• to make peroxynitrite ($ONOO^-$) [24], which is widely reported to cause irreversible damage to mitochondria [9,14,24–27].

In summary we have shown here that, as previously assumed from indirect evidence, NO• inhibits the mitochondrial ATP synthesis. This inhibition is not fully reversible and the irreversible portion may theoretically involve $ONOO^-$. Thus, it is reasonable to assume that in pathological conditions in which overproduction of NO• is thought to play a role, cellular energy metabolism and functions that rely on ATP may be compromised. Such effects may be of particular relevance to tissues such as the brain that rely heavily on oxidative phosphorylation for their energy requirements.

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