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Biochimica et Biophysica Acta 1777 (2008) 397–403

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Diphenyleneiodonium acutely inhibits reactive oxygen species production by mitochondrial complex I during reverse, but not forward electron transport

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ARTICLE INFO

Article history:
Received 12 October 2007
Received in revised form 26 February 2008
Accepted 3 March 2008
Available online 18 March 2008

Keywords:
Mitochondria
Superoxide
Diphenyleneiodonium
Hydrogen peroxide
Reactive oxygen species

ABSTRACT

We investigated the effects of diphenyleneiodonium (DPI) on superoxide production by complex I in mitochondria isolated from rat skeletal muscle. Superoxide production was measured indirectly as hydrogen peroxide production. In a conventional medium containing chloride, DPI strongly inhibited superoxide production by complex I driven by reverse electron transport from succinate. In principle, this inhibition could be explained by an observed decrease in the mitochondrial pH gradient caused by the known chloride–hydroxide antiport activity of DPI. In a medium containing gluconate instead of chloride, DPI did not affect the pH gradient. In this gluconate medium, DPI still inhibited superoxide production driven by reverse electron transport, showing that the inhibition of superoxide production was not dependent on changes in the pH gradient. It had no effect on superoxide production during forward electron transport from NAD-linked substrates in the presence of rotenone (to maximise superoxide production from the flavin of complex I) or antimycin (to maximise superoxide production from complex III), suggesting that the effects of DPI were not through inhibition of the flavin. We conclude that DPI has the novel and potentially very useful ability to prevent superoxide production from the site in complex I that is active during reverse electron transport, without affecting superoxide production during forward electron transport.

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1. Introduction

The production of superoxide and other reactive oxygen species by mitochondria plays an important role in a variety of signalling pathways and pathological disorders and possibly in ageing [1–3]. Therefore it is of great importance to understand the sites, topology, mechanisms and regulation of reactive oxygen species production by mitochondria. Although several superoxide-producing enzymes within mitochondria have been identified [4], the major sites are believed to be within complexes I and III of the electron transport chain. Within complex III, the main site is most likely centre '*o*' on the positive side of the membrane [5]. This site can produce superoxide at high rates when centre '*i*' is inhibited by antimycin [6]. However, the major site of superoxide production within complex I is uncertain. The flavin, iron–sulphur centres N1a or N2, iron–sulphur centres in general and the ubiquinone (Q)-reduction site have all been proposed as sites of superoxide production [7–16].

The rate of superoxide production from complex I can vary greatly depending on the conditions employed. In isolated de-energised complex I, the dominant site of superoxide production is the flavin [14,16]. In intact mitochondria there are three modes of superoxide production from complex I [11,17]. The first occurs during forward electron transport from NADH (generated by pyruvate plus malate or pyruvate plus glutamate); the superoxide production rate is low, because the redox centres in the complex are relatively oxidised. The second mode occurs when the redox centres become reduced following inhibition of the Q-reducing site of complex I with rotenone, or inhibition of downstream electron transport at complex III with stigmatellin. Under these conditions, superoxide is produced at a modest rate, probably from the flavin. However, the maximum rate of superoxide production from complex I in the third mode is several-fold greater than this rate from the flavin, so the dominant role of the flavin in the isolated de-energised complex reflects only a minor component of superoxide production by energised complex I in intact mitochondria [11]. The third mode occurs when electrons enter the electron transport chain at the level of ubiquinone (e.g. during succinate oxidation at complex II) and the superoxide production rate is high [8,9,11,17–19]. This high rate is inhibited by the complex I inhibitor rotenone, showing that the superoxide is produced at complex I during reverse electron transport from succinate. It is very sensitive to the pH gradient across the mitochondrial inner membrane (ΔpH) (suggesting that it is intimately related to the proton-pumping function of complex I) and it can be strongly inhibited by compounds that decrease ΔpH, such as nigericin [11]. This third mode of superoxide production can also be measured during forward electron transport, but only when the mitochondria have a large ΔpH in the presence of particular inhibitors of the Q-reduction site, such as myxothiazol or piericidin, that presumably poise complex I in a state similar to that achieved during reverse electron transport [17]. This

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doi:10.1016/j.bbabio.2008.03.005

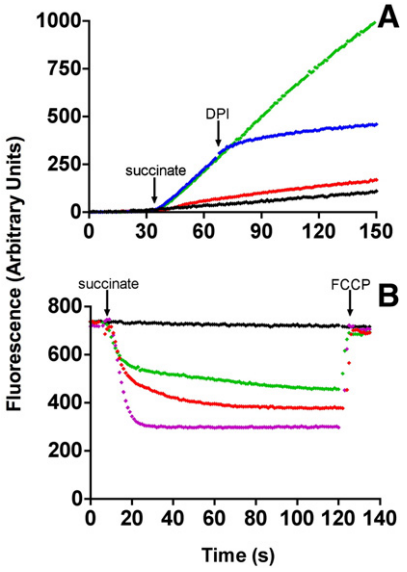

Fig. 1. Effect of DPI on hydrogen peroxide production (A) and membrane potential (B) in mitochondria oxidising succinate in chloride-containing medium. A is a typical trace of resorufin fluorescence over time: green, no DPI; red, DPI added at $t=0$ s; blue, DPI added at ~70 s; black, rotenone added at $t=0$ s. B is a typical trace of safranin O fluorescence over time: black, no additions; green, no DPI; red, DPI added at $t=0$ s; purple, nigericin added at $t=0$ s. The medium (pH 7.2 and 37 °C) contained 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA, 1 μg ml^{-1} oligomycin, and 120 mM potassium chloride. The succinate and DPI concentrations were 5 mM and 5 μM, respectively. A also contained 50 μM amplex red, 4 U horseradish peroxidase ml^{-1} and 30 U SOD ml^{-1}. B also contained 5 μM safranin O. The rotenone and FCCP concentrations were 2 μM each, the nigericin concentration was 0.1 μM.

indicates that the Q-reduction site itself (or a site upstream that is connected to the Q-reduction site by functional interactions such as conformational changes) is the site of superoxide production.

The hypoglycaemic agent, diphenyleneiodonium (DPI) has been used to probe the sites of superoxide production in mitochondria [9,10,19,20]. The logic behind these studies is that DPI has been shown to bind covalently to reduced flavin groups of various enzymes, to bind complex I, and to inhibit the reduction of complex I iron–sulphur centres by NADH [21–23]. Thus, when DPI was found to inhibit superoxide production in intact mitochondria, the inference was that the superoxide must have originated from the flavin site within complex I. However, none of these studies examined whether the flavin was indeed inhibited by DPI when superoxide production became inhibited, or whether other potential sites of superoxide production were affected by DPI. This is important, because DPI is known to have other effects on mitochondria. In particular, it is known to catalyse chloride–hydroxide antiport across membranes [24], which will affect ΔpH and any reactions (including superoxide production dependent on reverse electron transport) that depend on ΔpH.

In the present study, we investigate the effects of DPI on superoxide production from complex I during reverse electron transport in intact mitochondria. We conclude that DPI has the ability to inhibit superoxide production with minimal effects on the overall electron transport activity of complex I, under conditions in which it does not affect ΔpH and there is no indication that it reacts with the flavin of complex I.

2. Experimental procedures

2.1. Materials

Diphenyleneiodonium sulphate (DPI) was from Toronto Research Chemicals, Canada. Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) was from Invitrogen UK. Potassium chloride was from BDH UK. HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulphonic acid) was from Fisher UK. All other chemicals were from Sigma.

2.2. Isolation of mitochondria

Mitochondria from skeletal muscle of female Wistar rats (aged between 5 and 8 weeks) were isolated as described [25] with modification. Briefly, tissue was removed from hind limb and lower back, chopped with scissors and minced with a scalpel blade. After digestion with protease, the tissue was homogenised and the mitochondria were isolated by differential centrifugation. In experiments using chloride-free incubation medium, the final wash and resuspension of mitochondria was performed with chloride-free medium, with 120 mM potassium gluconate replacing potassium chloride.

2.3. Measurement of mitochondrial hydrogen peroxide production

Superoxide production rate was assessed by measurement of hydrogen peroxide generation rate, determined fluorometrically by measurement of oxidation of amplex

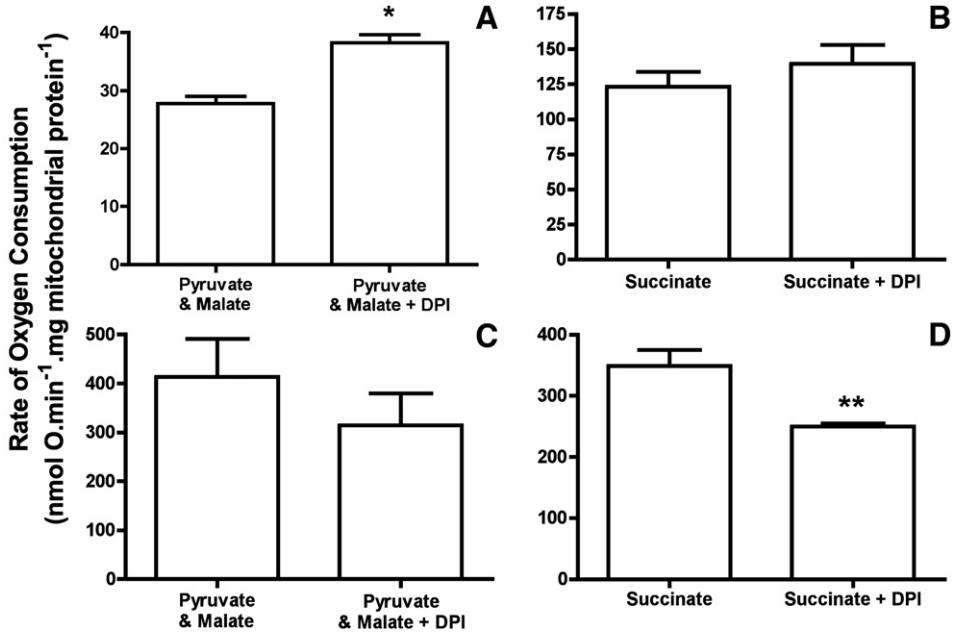

Fig. 2. Effect of DPI on mitochondrial oxygen consumption rates in chloride-containing medium. The medium contained 120 mM potassium chloride, 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA (pH 7.2 and 37 °C) with 1 μg ml^{-1} oligomycin. The DPI concentration was 5 μM. The succinate concentration was 5 mM, the pyruvate and malate concentrations were 2.5 mM each. Panels A and B show rates of state II respiration, panels C and D show uncoupled rates obtained by adding FCCP to a final concentration of 2 μM. Significant difference: *($p<0.005$) and **($p<0.05$). Values are means ± S.E.M. where $n=4$ separate mitochondrial preparations.

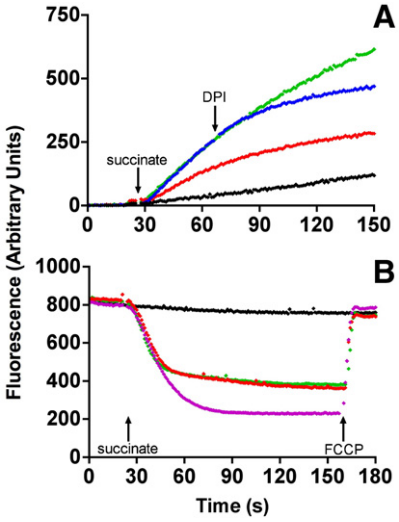

Fig. 3. Effect of DPI on hydrogen peroxide production (A) and membrane potential (B) in mitochondria oxidising succinate in chloride-free medium. A shows typical traces of resorufin fluorescence over time: green, no DPI; red, DPI added at $t=0$ s; blue, DPI added at ~70 s; black, rotenone added at $t=0$ s. Panel B shows typical traces of safranin O fluorescence over time: black, no additions; green, no DPI; red, DPI added at $t=0$ s; purple, nigericin added at $t=0$ s. The medium (pH 7.2 and 37 °C) contained 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA, 1 μg ml^{-1} oligomycin, and 120 mM potassium gluconate. The succinate and DPI concentrations were 5 mM and 5 μM, respectively. A also contained 50 μM amplex red, 4 U horseradish peroxidase ml^{-1} and 30 U SOD ml^{-1}. B also contained 5 μM safranin O. The rotenone and FCCP concentrations were 2 μM each, the nigericin concentration was 0.1 μM.

red to fluorescent resorufin coupled to the enzymatic reduction of H_2O_2 by horseradish peroxidase. Mitochondria were incubated at 0.35 mg mitochondrial protein ml^{-1} in standard buffer containing 3 mM HEPES, 1 mM EGTA, 0.3% BSA (w/v) (pH 7.2 and 37 °C) and either 120 mM potassium chloride or 120 mM potassium gluconate. All incubations also contained 50 μM amplex red, 4 U ml^{-1} horseradish peroxidase, 30 U ml^{-1} superoxide dismutase (SOD) and 1 μg ml^{-1} oligomycin. The reaction was initiated by addition of respiratory substrates and the increase in fluorescence at an excitation of 563 nm and emission of 587 nm was followed on a Shimadzu RF5301 spectrofluorometer. Appropriate correction for background signals, and standard curves generated using known amounts of H_2O_2 were used to calculate the rate of H_2O_2 production in nmol min^{-1} mg mitochondrial $protein^{-1}$. Essentially all the superoxide from complex I is generated on the matrix side of the inner membrane, then converted by endogenous processes to H_2O_2 which leaks out and is measured in the assay. Inclusion of exogenous SOD in the assay medium ensures that all superoxide produced in the inter-membrane space (by complex III) is converted to hydrogen peroxide [26].

2.4. Assessment of mitochondrial membrane potential

Mitochondrial membrane potential was followed using the potential-sensitive dye safranin O [27]. Mitochondria were incubated at a concentration of 0.35 mg mitochondrial protein ml^{-1} in standard buffer with 5 μM safranin O and 1 μg ml^{-1} oligomycin. The change in fluorescence at an excitation of 533 nm and an emission of 576 nm was followed over time (a decrease in fluorescence indicating an increase in mitochondrial membrane potential). The membrane potential was dissipated at the end of each run by addition of 2 μM FCCP.

2.5. Measurement of reverse electron transport

The rate of reverse electron transport from succinate to NAD^+ was assessed by monitoring the rate of NADH formation fluorometrically at excitation and emission wavelengths of 365 and 450 nm respectively [28]. Incubation conditions were 0.35 mg mitochondrial protein ml^{-1} in standard buffer with 1 μg ml^{-1} oligomycin.

2.6. Measurement of mitochondrial oxygen consumption

Oxygen consumption was measured using a Clark-type electrode. Coupled ("state II") respiration was initiated by addition of respiratory substrates, then uncoupled respiration was initiated by addition of FCCP. Incubation conditions were 0.35 mg mitochondrial protein ml^{-1} in standard buffer with 1 μg ml^{-1} oligomycin. Respiration rates were measured for approximately 120 s after addition of substrate.

2.7. General incubation protocol

In all experiments the sequence and timing of additions were as follows. Initially buffer was added to the cuvette or respiration chamber followed by the addition of mitochondria within 10 s. Then, for measurements of hydrogen peroxide and membrane potential, the detection system was added within 10 s. Then inhibitors and/or effectors (e.g. DPI, rotenone, FCCP, nigericin) were added within another 10 s to start the experiment and this was taken as time=zero. Substrates (succinate, pyruvate and malate) were then added within 30 s, and the experiment continued for 2 to 3 min. In some experiments as described in the text, compounds were added in-line after addition of substrate. The various detection systems employed (HRP+amplex red; safranin O) did not affect reverse electron transport with succinate or respiration rates with any substrate and/or inhibitor combination.

2.8. Statistics

Values are given as means±standard error with n being the number of separate mitochondrial preparations. The significance of differences between means was assessed by either unpaired Student's t test, or by ANOVA with Tukey's multiple comparisons test. p values <0.05 were taken to be significant.

3. Results

3.1. Effects of DPI on superoxide production and respiration rate in chloride-containing medium

As previously reported by several different groups [13,18,19,30,31], mitochondria oxidising succinate produced superoxide at a relatively high, linear rate that was strongly inhibited by rotenone (Fig. 1A), showing that it was produced from complex I by reverse electron transport. Fig. 1A shows that 5 μM DPI also markedly inhibited the high rate, whether DPI was added before succinate or 30 s later, showing that under these conditions DPI almost completely prevents superoxide production from complex I during reverse electron transport. The rates (measured between 20 and 80 s) in nmol H_2O_2 min^{-1} mg mitochondrial $protein^{-1}$ were: succinate, 4.35±0.62 and succinate+DPI, 0.67±0.16 ($n=3$ separate mitochondrial preparations, $p<0.005$).

However, since DPI is a chloride–hydroxide anion exchanger [24], it will lower ΔpH in chloride-containing media. Superoxide production by complex I is very sensitive to ΔpH [17], so the inhibition of superoxide production seen in Fig. 1A might have been caused by this indirect effect of DPI. Fig. 1B shows the effect of DPI on ΔpH. Addition of succinate to energise the mitochondria caused an increase in protonmotive force, expressed mostly as membrane potential but partly as ΔpH. In the presence of nigericin, which exchanges potassium for protons, ΔpH was collapsed, so all of the protonmotive force was expressed as membrane potential, which therefore increased. DPI also increased the membrane potential, showing that it too decreased ΔpH, potentially explaining its inhibition of superoxide production.

Previous studies showed that in chloride-containing medium, DPI enhances coupled respiration slightly and inhibits uncoupled respiration with either succinate or NADH-generating substrates [29]. We confirmed these findings. During coupled respiration, DPI enhanced the oxygen consumption rate with pyruvate plus malate (and perhaps with succinate) (Fig. 2A, B), presumably due to a slight uncoupling effect. Under fully uncoupled conditions the oxygen consumption rates with succinate (and possibly pyruvate plus malate) were inhibited (Fig. 2C, D), showing that DPI inhibits electron transport at a site other than complex I in this medium. These findings show that in our hands, in a chloride-containing medium, DPI affected mitochondrial respiration as previously reported.

3.2. DPI inhibits succinate-supported superoxide production from complex I in chloride-free medium

To avoid the confounding effects of changes in ΔpH catalysed by DPI, we switched to a chloride-free medium for all subsequent experiments. In potassium gluconate-containing medium, using mitochondria washed free of chloride, the rates of superoxide production were not quite linear (Fig. 3A). Nevertheless, the effect of DPI was clear: the rate of superoxide production by mitochondria oxidising succinate was decreased by approximately 80% after 60 s, whether the DPI was

added before or after succinate (Fig. 3A). This finding is consistent with previous studies [9,10,19], which also found strong inhibition of succinate-supported superoxide production by DPI in chloride-free media. The rates (measured between 20 and 80 s) in nmol H_2O_2 min^{-1} mg mitochondrial $protein^{-1}$ are shown in Fig. 5C.

Fig. 3B confirms that in chloride-free medium, as predicted, DPI did not affect ΔpH. In this medium, DPI, unlike nigericin, did not lower ΔpH and allow more of the protonmotive force to be expressed as membrane potential. The inhibition of superoxide production by DPI seen in Fig. 3A was therefore not caused by changes in ΔpH.

3.3. DPI does not uncouple in chloride-free medium

DPI had no significant uncoupling effect on mitochondria oxidising either pyruvate plus malate or succinate in chloride-free medium, since it did not significantly increase the rates of coupled respiration (Fig. 4A, B). Lack of uncoupling by DPI is also shown by the lack of effect of DPI on membrane potential during oxidation of pyruvate plus malate (Fig. 4D) or succinate (Fig. 3B). DPI also had no effect on membrane potential in the presence of nigericin (not shown), showing that it did not affect protonmotive force. Thus the inhibition of superoxide production by DPI seen in Fig. 3A was not caused by uncoupling.

3.4. DPI does not inhibit forward electron transport rate in chloride-free medium

In agreement with previous observations [29], DPI had no effect on the rate of uncoupled respiration with pyruvate plus malate as substrate (Fig. 4C), showing that DPI did not affect the overall rate of forward electron transport through complex I or through the remainder of the electron transport chain in chloride-free medium. As expected, rotenone was an effective inhibitor of coupled or uncoupled respiration on pyruvate plus malate (Fig. 4A, C).

3.5. DPI does not inhibit reverse electron transport rate significantly in chloride-free medium

The rate of formation of intramitochondrial NADH in the presence of succinate is a measure of the rate of reverse electron transport through complex I. Panels A and B of Fig. 5 show that this process was not significantly affected by 5 μM DPI (incubation time of about 10 s before addition of succinate), although, as expected, it was strongly inhibited by 2 μM rotenone. However, there may be an effect of DPI on reverse electron transport that is too small to be significant. Thus the inhibition of superoxide production by DPI seen in Fig. 3A may be accompanied by a very small inhibition of reverse electron transport rate through complex I.

DPI takes about 60 s to fully inhibit superoxide production during reverse electron transport (Fig. 3A). We therefore allowed a longer incubation of mitochondria with DPI for ~80 s before the addition of succinate; however this did not result in any inhibition of reverse electron transport (Fig. 5A inset).

Figs. 5A and B also show that lowering the rotenone concentration to 100 nM resulted in very small non-significant effects on reverse electron transport rate, similar to DPI. Yet at 100 nM, rotenone inhibited superoxide production to the same extent as DPI (and 2 μM rotenone) as shown in Fig. 5C. Therefore DPI may behave acutely like a low concentration of a known Q-site inhibitor.

3.6. DPI does not affect superoxide production from the flavin of complex I during forward electron transport in chloride-free medium

To test whether DPI had subtle inhibitory effects at the flavin of complex I that would decrease superoxide production from this site, we examined superoxide production during forward electron transport. During oxidation of pyruvate plus malate, the rate of mitochondrial superoxide production was low and was unaffected by DPI (Fig. 6A). Addition of the complex I inhibitor, rotenone,

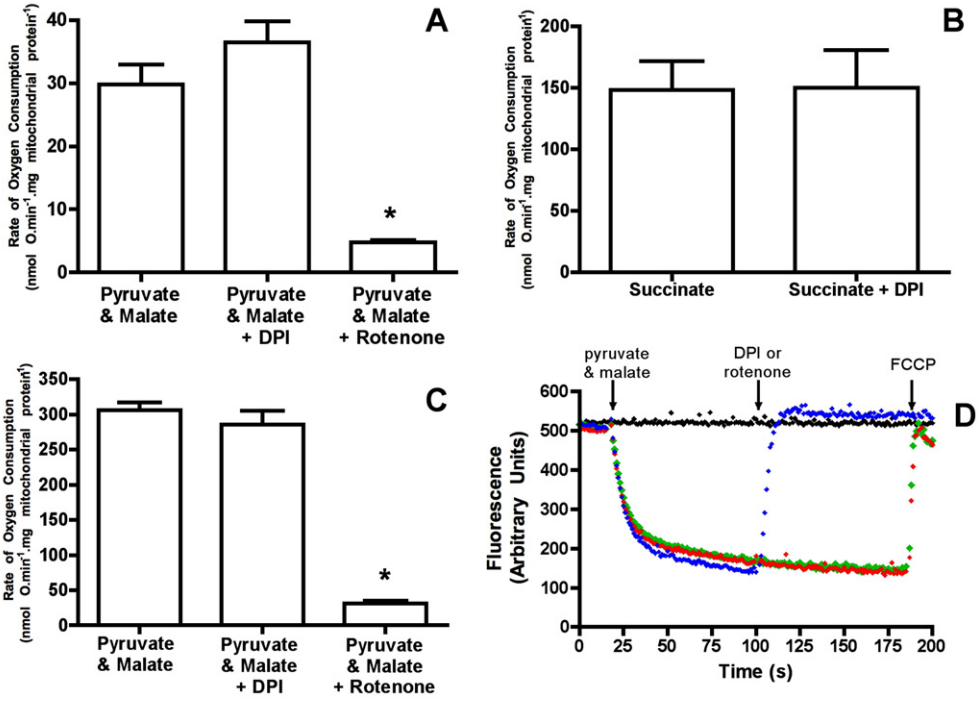

Fig. 4. Effect of DPI and rotenone on oxygen consumption rate and membrane potential in mitochondria oxidising succinate or pyruvate and malate. The medium contained 120 mM potassium gluconate, 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA (pH 7.2 and 37 °C) with 1 μg ml^{-1} oligomycin. The DPI and rotenone concentrations were 5 μM and 2 μM respectively. Succinate was added to a concentration of 5 mM, pyruvate and malate were added to final concentrations of 2.5 mM each. Panels A ($n=5$) and B ($n=3$) show rates of state II respiration (A = pyruvate and malate, B = succinate). Panel C ($n=3$) shows rates of uncoupled respiration for pyruvate and malate. The values are means ± S.E.M. where n = the number of separate mitochondrial preparations. *Significant ($p<0.001$) effect of rotenone vs. pyruvate and malate. Panel D shows typical traces of safranin O fluorescence over time: black, no additions; green, no DPI; red, DPI added at $t=100$ s; blue, rotenone added at $t=100$ s. The safranin O concentration was 5 μM.

increased the superoxide production rate, presumably because a superoxide-producing site within complex I becomes more reduced. Rotenone acts at the Q-reducing site within complex I [30], so the increase in superoxide production under these conditions is either from the rotenone inhibition site itself or from sites upstream of the rotenone inhibition site, i.e. most probably from the flavin. Panels B and C of Fig. 6 show that addition of DPI did not affect the superoxide production rate with pyruvate plus malate in the presence of 2 mM rotenone. The same result was found for two other complex I Q-reduction site inhibitors, piericidin and myxothiazol, or if DPI was added before rotenone (not shown). Thus DPI does not inhibit superoxide production from the flavin site of complex I during forward electron transport in these experimental conditions.

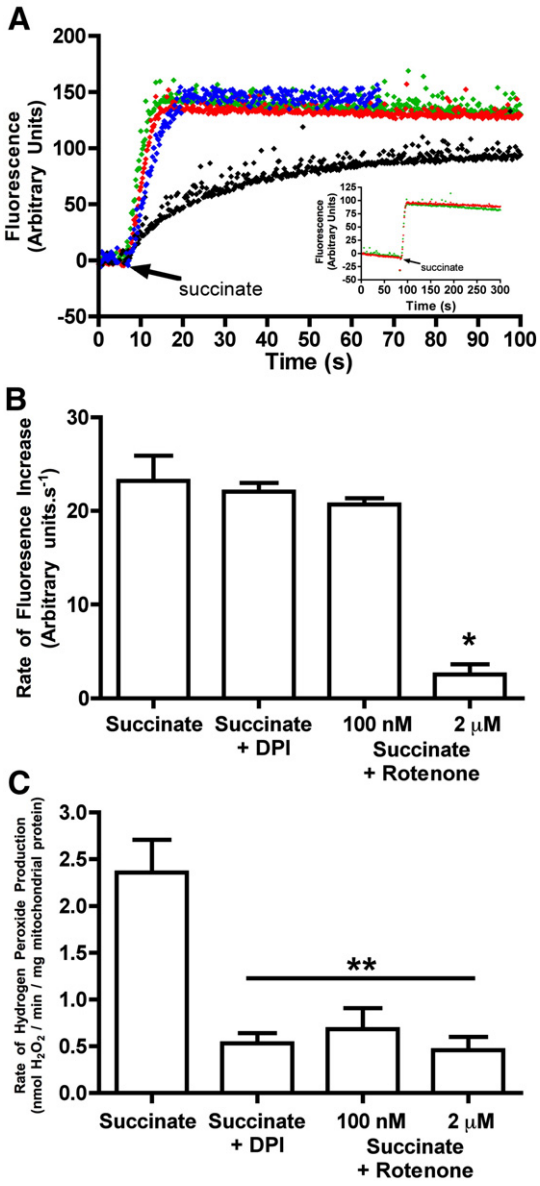

Fig. 5. Effect of DPI on reverse electron transport rate and hydrogen peroxide production rate. The medium contained 120 mM potassium gluconate, 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA (pH 7.2 and 37 °C) with 1 μg ml^{-1} oligomycin. The DPI concentration was 5 μM. Succinate was added to a final concentration of 5 mM. Panel A shows typical raw traces of NADH fluorescence over time: green, no DPI; red, DPI added at $t=0$ s; blue, 100 nM rotenone added at $t=0$ s; black, 2 μM rotenone added at $t=0$ s. Panel A inset (delayed addition of succinate): green, no DPI; red, DPI added at $t=0$ s. Panel B shows rates of NADH fluorescence increase between 1 and 5 s after succinate addition, the values are means ± S.E.M. where $n=3$ separate mitochondrial preparations. *Significant ($p<0.001$) difference vs. succinate. Panel C shows rates of hydrogen peroxide production, $n=3$ separate mitochondrial preparations. **Significant ($p<0.01$) difference vs. succinate (ANOVA).

The uptake of DPI into the mitochondrial matrix (where the flavin of complex I is situated) may depend on the membrane potential (because DPI is a cation). In the inhibited state (pyruvate and malate plus rotenone) the membrane potential is low, potentially explaining the lack of effect of DPI on superoxide production under these conditions. Therefore, we induced a potential by adding succinate under conditions of inhibited forward electron transport (pyruvate and malate plus rotenone). In this state, electrons from succinate cannot enter complex I by reverse electron transport, because rotenone is present, but they can pass through complexes III and IV, pumping protons and generating membrane potential, while electrons from pyruvate and malate still feed into complex I to generate superoxide. Fig. 6C shows that superoxide production during forward electron transport increases under these conditions when succinate is added, as previously reported [17]. However, DPI still did not affect the rate of superoxide production, thus it is not the lack of a membrane potential that prevents DPI inhibition of superoxide production during forward electron transport.

3.7. DPI does not affect superoxide production from the Q_o site of complex III during forward electron transport in chloride-free medium

To test the possibility that effects of DPI at the flavin of complex I are prevented by inhibition of the Q-reducing site of complex I by rotenone, we examined superoxide production in the presence of antimycin, an inhibitor of centre *i* in complex III, during forward electron transport from pyruvate plus malate. Under these conditions there is high superoxide production from centre *o* of complex III and the flavin site of complex I, but no direct inhibition of the Q-reducing site of complex I. Panels B and C of Fig. 6 show that the rate of superoxide production in the presence of antimycin was high, and was unaffected by DPI. DPI also had no effect when added at the beginning of the run (not shown). Thus the lack of effect of DPI on superoxide production from the flavin of complex I in our conditions is not caused by interference from an inhibited Q-reduction site.

The observation that DPI has no effect on hydrogen peroxide production under the conditions of Fig. 6 also shows that DPI has no general, non-specific inhibitory effect on superoxide production from intact mitochondria, and that it does not interfere with conversion of matrix superoxide to hydrogen peroxide, or with the detection of that hydrogen peroxide by our fluorescent assay system.

4. Discussion

DPI is often described as a covalent inhibitor of flavin redox activity, and in particular, as the most prominent membrane-permeant inhibitor of the flavin site of complex I. Early studies with DPI showed it to have chronic hypoglycaemic properties, and its mechanism of action was suggested to involve inhibition of NADH oxidation in mitochondria [24,31,32]. Subsequently, DPI was found to be a broad-spectrum flavoprotein inhibitor, and it is best known for inhibiting NADPH oxidase via slow formation of a covalent flavin-DPI adduct [22]. When DPI is incubated with isolated complex I, it slowly inhibits both the oxidation of NADH and the reduction of the iron–sulphur centres, suggesting that it inhibits at a site upstream of these centres, i.e. at the complex I flavin [23]. This evidence, along with observations that DPI inhibits superoxide formation during reverse electron transport, has been used to support the argument that superoxide production is from the flavin in complex I during reverse electron transport [9,10,19,20]. However, we demonstrate here that the acute inhibition of mitochondrial superoxide production from complex I by DPI is exclusive to reverse electron transport, as no inhibition during forward electron transport was observed. Therefore, we cannot draw the conclusion that in intact mitochondria, DPI is acting acutely as a simple flavin inhibitor.

Where we have repeated earlier work from other laboratories, our experimental results do not disagree with that work. In our hands, as

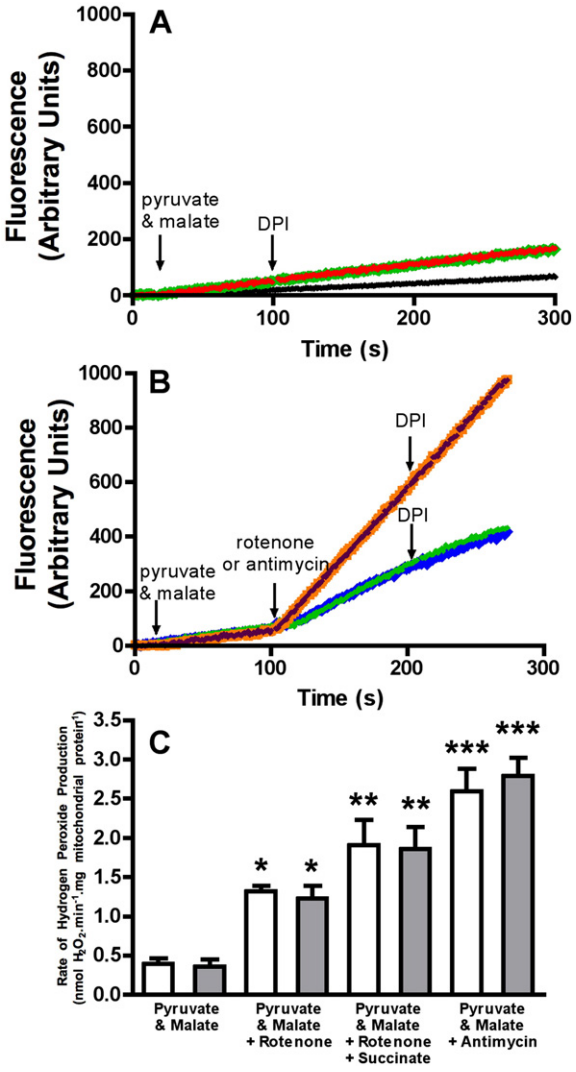

Fig. 6. Effect of DPI on rate of hydrogen peroxide production by mitochondria oxidising pyruvate and malate. The medium contained 120 mM potassium gluconate, 3 mM HEPES, 1 mM EGTA and 0.3% (w/v) BSA (pH 7.2 and 37 °C), with 1 μg ml^{-1} oligomycin, 50 μM amplex red, 4 U horseradish peroxidase ml^{-1}, 30 U SOD ml^{-1}. The concentrations of DPI, rotenone and antimycin were 5 μM, 2 μM and 1 μM respectively. Pyruvate and malate were added to final concentrations of 2.5 mM each, succinate was used at a concentration of 5 mM. Panel A shows typical raw traces of resorufin fluorescence over time: black, no additions; green, no DPI; red, DPI added at $t=100$ s. Panel B also shows typical raw traces resorufin fluorescence over time: green, rotenone added at 100 s; blue, rotenone added at 100 s+DPI added at 200 s; orange, antimycin added at 100 s; purple, antimycin added at 100 s+DPI added at 200 s. Panel C shows rates of hydrogen peroxide production: white bars: no DPI, grey bars: +DPI. *Significant ($p<0.05$) effect of rotenone, **significant ($p<0.05$) effect of rotenone and succinate, ***significant ($p<0.001$) effect of antimycin (ANOVA). Values are means±S.E.M. where $n=3$ separate mitochondrial preparations.

expected [29], DPI acutely inhibited uncoupled respiration in a chloride-based medium. However, in chloride-free medium, the effects of DPI on uncoupled respiration were negligible. This is because inhibition depends on the chloride–hydroxide exchange catalysed by DPI [29]. In chloride-free medium, the rate of superoxide production during succinate oxidation was markedly inhibited by DPI (in agreement with previous reports) [9,10,19]. Some workers have incubated isolated complex I for 60–70 min with DPI to inhibit NADH oxidation [14]. It may be that in our relatively short mitochondrial incubations in chloride-free medium, DPI had insufficient time to bind at the flavin, and instead we observed only more rapid effects of DPI at a different site. This could have been misinterpreted as inhibition at the flavin in longer incubations or in the presence of chloride. It is clear from our results that in chloride-free medium during succinate oxidation DPI can markedly lower superoxide production in less than 1 min. There are no apparent effects on reverse electron transport within the same time frame, although they may be too small to be significant. In addition, during forward electron transport, superoxide production, membrane potential and respiration rate were also unaffected during short incubation times of around 1 min. These effects dissociate the action of DPI on superoxide production rate from any potential inhibitory action at the flavin (or any other rate-limiting site) of complex I.

Very high rates of superoxide production in mitochondria are obtained during succinate oxidation. Under these conditions, the superoxide could theoretically be coming from any site or sites, such as complex II, complex III or even from enzymes of the citric acid cycle. However the addition of the complex I specific inhibitor rotenone markedly diminishes the high rate of superoxide production seen during succinate oxidation. The simplest explanation for this is that nearly all of the high superoxide production rate originates at complex I via reverse electron transport from QH_2 to NAD^+. In our system, DPI can also markedly lower the high rate of superoxide production during succinate oxidation, but (unlike rotenone) without apparent inhibition of overall electron transport by complex I itself. This does not mean that other sites apart from complex I are responsible for the majority of superoxide production, but indicates that the relevant site in complex I limits superoxide production but not the overall electron transport rate. Our results show that DPI lowers the same high rate that rotenone does, but at a step that is not strongly rate-limiting for overall electron transport.

It seems that during reverse electron transport in chloride-free medium, DPI acutely decreases the concentration of the intermediate within complex I that reacts with oxygen to produce superoxide. This intermediate cannot be the flavin site in complex I, because superoxide production from the flavin during forward electron transport in the presence of rotenone or antimycin was not inhibited, and because the rate of forward electron transport through the complex was unaffected by DPI in this medium. There is some evidence that indicates that DPI can act at the Q-binding site of complex I, as one study found that DPI inhibited NADH-Q but not NADH-ferricyanide oxidoreductase activity [21]. Our results support this notion as DPI behaved in a very similar way to a low concentration (100 nM) of rotenone, a known Q-site inhibitor.

In isolated complex I, a high NADH/NAD^+ ratio (thus a highly reduced flavin) enhances the binding of DPI to the flavin [23]. Thus one explanation for the lack of effect of DPI on superoxide production during forward electron transport is that the flavin is not reduced enough compared to reverse electron transport. Our own unpublished observations and those of Hansford et al. [33] do not support this idea: during succinate oxidation, the NAD(P)H pool (and by inference the flavin) is less reduced than during oxidation of pyruvate and malate in the presence of rotenone. Therefore enhanced binding of DPI to the flavin would be expected with pyruvate, malate and rotenone along with inhibition of superoxide production, but this is clearly not the case (Fig. 6).

Our studies show that DPI may be a useful tool in investigating the production of reactive oxygen species by isolated mitochondria, as it lowers superoxide production during reverse electron transport without inhibiting electron transport. However, recent studies show that DPI has a wide variety of cellular effects, such as inhibition of redox metabolism, loss of glutathione and induction of apoptosis [34–36], in addition to its expected effects on cellular pH gradients. In whole cells, therefore, its usefulness as an agent to decrease mitochondrial superoxide production is likely to be severely limited.

Although we have not identified the precise site of action of DPI in our experiments, they show that inhibition of the production of mitochondrial reactive oxygen species by DPI cannot be used to assert that the site of reactive oxygen species production is the complex I flavin, without, as a minimum requirement, a simultaneous demonstration of flavin-site inhibition. Such flavin-site inhibition has not been demonstrated in the published studies that link superoxide production to the flavin site using DPI [9,10,19,20], so these reports

cannot be taken as strong evidence for a flavin-site origin of superoxide during reverse electron transport through complex I. Also, the inhibitory effect of DPI on the complex I flavin has only been demonstrated in the isolated enzyme [23]. In whole mitochondria there may be several factors, such as the high concentration of matrix NADH plus NAD, that prevent DPI from inhibiting at the flavin and allow secondary inhibitory sites to be revealed. The main site of superoxide production in energised complex I remains uncertain, and further work is needed to verify the exact site(s) of production in intact mitochondria and whole cells.

Acknowledgements

AJL is supported by a Research into Ageing Fellowship, MDB is supported by the Medical Research Council and the Wellcome Trust (066750/B/01/Z). We thank Judy Hirst and Alexander Galkin for their useful discussions.

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