F₁F₀-ATPase, early target of the radical initiator 2,2'-azobis-(2-amidinopropane) dihydrochloride in rat liver mitochondria *in vitro*

Frédéric BEAUSEIGNEUR*, Marc GOUBERN†, Marie-France CHAPEY†, Joseph GRETTI*, Catherine VERGELY‡, Marcelline TSOKO*, Jean DEMARQUOY*, Luc ROCHETTE‡ and Pierre CLOUET*§

* Nutrition Cellulaire et Métabolique, EA DRED 1867, BP 138, 21004 Dijon Cedex, France, † Nutrition et Sécurité Alimentaire, INRA EPHE, 78352 Jouy-en-Josas, France and ‡ LPPCE, Facultés de Médecine et de Pharmacie, 21000 Dijon, France

This study was designed to determine which enzyme activities were first impaired in mitochondria exposed to 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), a known radical initiator. EPR spin-trapping revealed generation of reactive oxygen species although malondialdehyde formation remained very low. With increasing AAPH concentrations, State-3 respiration was progressively depressed with unaltered ADP/O ratios. A top-down approach demonstrated that alterations were located at the phosphorylation level. As shown by inhibitor titrations, ATP/ADP translocase activity was unaffected in the range of AAPH concentrations used. In contrast, AAPH appeared to exert a deleterious effect at the level of F₁F₀-ATPase,

comparable with dicyclohexylcarbodi-imide, which alters F₀ proton channel. A comparison of ATP hydrolase activity in uncoupled and broken mitochondria reinforced this finding. In spite of its pro-oxidant properties, AAPH was shown to act as a dose-dependent inhibitor of cyclosporin-sensitive permeability transition initiated by Ca²⁺, probably as a consequence of its effect on F₁F₀-ATPase. Resveratrol, a potent antiperoxidant, completely failed to prevent the decrease in State-3 respiration caused by AAPH. The data suggest that AAPH, when used under mild conditions, acted as a radical initiator and was capable of damaging F₁F₀-ATPase, thereby slowing respiratory chain activity and reducing mitochondrial antioxidant defences.

INTRODUCTION

Much evidence is emerging that free radicals play a crucial role in cellular aging and in a range of degenerative diseases that accompany aging [1,2]. Peroxidation, which is a normal process in tissues, causes proteins and polyunsaturated fatty acids to be damaged [3,4]. Mitochondria are good models for peroxidation studies because (a) they exchange oxygen continuously to meet the steady requirement of the respiratory chain, (b) part of this oxygen gives rise to superoxide anion (O₂⁻) which is likely to diffuse within peroxidizable structures, (c) these organelles contain a series of enzymes and coenzymes bound sequentially in the respiratory chain in such a way that the damage of one affects the activity of the whole sequence, thereby rendering a possible protein alteration more sensitive.

In experimental studies, organic pro-oxidants have often been used to reproduce pro-oxidant effects of free radicals occurring within the cell, because they are not metabolized by catalase. A polymerization agent, 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), the decomposition of which is modulated by temperature and which is believed to be a carbon-centred radical [5–7], has been used in this respect. Both *in vivo* and *in vitro* dysfunctions of mouse liver mitochondria, such as impaired ATP synthesis and swelling, have been observed after AAPH administration [7]. Therefore this study was designed to determine more precisely the early target effects of AAPH treatment on mitochondrial functions *in vitro*. The data provide an explanation for the depression of State-3 respiration observed [7] and address the question of the link between swelling and mitochondrial permeability transition [8].

EXPERIMENTAL

Materials

Fatty acid-free albumin (fraction V) was from Paesel-Lorei (Frankfurt, Germany). AAPH was from Interchim (Montluçon, France). Carboxyatractyloside (CATR), tri-*n*-butyltin and venturicidine were gifts from Dr. F. Haraux (CNRS, Saclay, France) and cyclosporin A was kindly provided by Sandoz (Ruel, France). Dicyclohexylcarbodi-imide (DCCD), 5,5'-dimethyl-1-pyrroline *N*-oxide (DMPO), 2,4-dinitrophenol (DNP), resveratrol, tetraphenylphosphonium (TPP⁺) and other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The chemicals from Prolabo (Paris, France) and Merck (Darmstadt, Germany) were of analytical grade.

Female Wistar rats (200–300 g) were purchased from Dépré (Saint-Doulchard, France). They had free access to tap water and standard laboratory chow (AO3 pellets from UAR, Villemoisson-sur-Orge, France). They were killed by decapitation.

Preparation of mitochondrial fractions

Isolation of purified fractions

A crude mitochondrial fraction of rat liver was first prepared by differential centrifugation in a medium containing 250 mM sucrose, 10 mM Tris/HCl (pH 7.4), 1 mM EGTA and 1% albumin [9,10]. Microsomal and peroxisomal particles, which are also involved in peroxidative processes, were removed by step-

Abbreviations used: AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; CATR, carboxyatractyloside; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DCCD, dicyclohexylcarbodi-imide; DMPO, 5,5'-dimethyl-1-pyrroline *N*-oxide; DNP, 2,4-dinitrophenol; ΔΨ, mitochondrial inner-membrane potential; Lubrol, polyoxyethylene-9-lauryl ether (polidocanol); MDA, malondialdehyde; resveratrol, *trans*-3,4',5-trihydroxystilbene TPP⁺, tetraphenylphosphonium.

§ To whom correspondence and reprint requests should be addressed.

gradient centrifugation at 10000 *g* for 10 min on three sequential 4 ml layers of 18, 30 and 60 % Percoll in 300 mM sucrose/20 mM Tes, pH 7.4. Purified mitochondria were collected at the 30–60 % interface and washed twice by sedimentation in 150 mM KCl buffered with 10 mM Tris/HCl, pH 7.4.

Treatment of fractions with AAPH

Mitochondrial fractions were exposed to AAPH in the presence of 0–50 μ M resveratrol for 5 min at 30 °C (15 mg of protein/ml) and then used immediately in respiratory medium (see below) at 25 °C (0.5 mg of protein in 1.5 ml) to obtain a minimum steady state of peroxidation and to lower AAPH concentration so as to limit its direct reaction with oxygen [7].

Measurements related to respiration

Oxygen consumption

Measurements were performed at 25 °C using a Clark-type electrode (Hansatech, Kings Lynn, Norfolk, U.K.) in the medium of Hafner et al. [11], slightly modified to contain 100 mM KCl, 20 mM sucrose, 20 mM glucose, 10 mM KH_2PO_4 , 5 mM MgCl_2 , 1 mM EGTA and 0.1 % fatty acid-free albumin (pH 7.4). Respiratory control and ADP/O ratios were defined and accurately calculated using total oxygen consumption [12] since State-4 respiration (succinate oxidation titrated by malonate) was very low at membrane potentials corresponding to State 3 (not shown).

F_1F_0 -ATPase activity

ADP phosphorylation and ATP hydrolysis were directly determined from the uptake and production respectively of H^+ ions as described by Nishimura et al. [13], using a fast sensitive pH electrode in the medium used for oxygen consumption experiments. The change in pH was used to calculate the amount of ATP consumed or produced by titrating the suspension with HCl after each run, as described by Nishimura et al. [13]. ATP hydrolysis was initiated by adding either a classical uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 3.5 μ M) or a detergent (0.025 % Lubrol), in the presence of 1 mM MgATP [14].

Transmembrane potential

The time course of the mitochondrial inner-membrane potential ($\Delta\Psi$) was monitored by the distribution of TPP^+ (5 μ M) between the mitochondrial matrix and the incubation medium with a laboratory-made TPP^+ -selective electrode [15]. Membrane potential was corrected to take into account the activity coefficient of TPP^+ in the matrix as described by Rottenberg [16].

Mitochondrial permeability transition

Swelling of mitochondria (0.6 mg of protein) was monitored at 520 nm in 2 ml of a medium containing 250 mM sucrose, 0.3 mM sodium phosphate, 10 μ M EGTA and 5 μ M rotenone (pH 7.1) at 25 °C (low concentrations of phosphate and EGTA improved succinate oxidation and reproducibility). State-4 respiration was initiated with 7 mM succinate for 3 min, then CaCl_2 (55 nmol) was added, which corresponded to zero time on the recorder chart. To facilitate comparisons, mitochondrial swelling was followed by the change in inverse absorbance (linearly related to mitochondrial volume) as described by Beavis et al. [17].

EPR

Spin-trapping studies were performed by using the spin trap DMPO [18] after purification by double distillation, at a final concentration of 100 mM. EPR spectra were recorded at 303 K using a Bruker ESP 300 E-X Band spectrometer with a ER 4103 cavity and an aqueous flat cell.

Other measurements

Malondialdehyde (MDA) was quantified as thiobarbituric acid-reactive substances by the procedure of Quintanilha and Packer [19]. Protein concentration of mitochondrial fractions was determined by the method of Smith et al. [20].

RESULTS

Peroxidation

The EPR spin-trapping technique using the spin trap DMPO enabled us to show that AAPH was capable of generating free radicals in a time- and concentration-dependent manner under the same conditions as those applied to mitochondria (composition of medium, temperature and duration of exposure) (Figure 1). However, MDA formation, as estimated by thiobarbituric acid-reactive substances, was negligible when mitochondria were treated with the peroxidant over a wide range of concentrations (up to 7 μ mol of AAPH/mg of protein) (not shown).

Oxygen consumption

In mitochondria pretreated for 5 min with AAPH (Figure 2), the rate of oxygen consumption with succinate was unchanged in State-4 respiration and in the DNP-uncoupled state over the range of AAPH concentrations used. However, in the presence of ADP (State 3), oxygen consumption progressively fell when the concentration of AAPH exceeded 0.5 μ mol/mg of protein,

Figure 1 Transfer of hydroxyl radicals from AAPH to the spin trap DMPO

The mixture contained 100 mM DMPO and the indicated concentrations of AAPH in 150 mM KCl/10 mM Tris/HCl pH 7.4. Values (means $\pm$ S.E.M.; $n = 3$) are expressed as arbitrary units corresponding to peak height measurements of DMPO-OH at the indicated times. ○, Control; △, 5 mM AAPH; ▲, 15 mM AAPH. These values are in the range of concentrations actually used with mitochondria in the following experiments.

Figure 2 Effects of pretreatment of mitochondria with AAPH on respiratory parameters in the presence of succinate

Oxygen consumption was monitored using a Clark-type electrode. Results are given as nmol of O_2 consumed/min per mg of protein. Mitochondria were previously exposed to the indicated amounts of AAPH. ■, State 4; ●, State 3; ▲, DNP-stimulated respiration; ○, ADP/O ratio. Data are representative of experiments using three different mitochondrial preparations.

without affecting ADP/O ratios. This indicated that there was no impaired coupling (Figure 2). Comparable results were obtained with glutamate/malate as substrates (not shown). Resveratrol, a

Figure 3 Effects of pretreatment with AAPH on force/flux relationships in rat liver mitochondria

Inner-membrane potentials and oxygen consumption were monitored with a TPP⁺-selective electrode and a Clark-type electrode respectively. In (A) succinate oxidation was titrated by malonate during State-3 respiration (0.5 mM ADP in presence of a regenerating system using excess hexokinase in the presence of 20 mM glucose). In (B) respiratory chain was titrated by CCCP in the presence of oligomycin (1 μg/mg of protein) to inhibit the phosphorylating system. ○, Control; △, 0.3 μmol of AAPH/mg of protein; ▲, 0.45 μmol of AAPH/mg of protein. Data are representative of experiments using three different mitochondrial preparations.

Figure 4 Inhibitory effect of CATR on the entry of ADP into mitochondria pretreated with AAPH

Results are given as percentages of the rate of phosphorylation in the absence of inhibitor (%V_p), using a pH electrode as indicated in the Experimental section. ○, Control; △, 0.3 μmol of AAPH/mg of protein; ▲, 0.9 μmol of AAPH/mg of protein. Data are representative of experiments using three different mitochondrial preparations.

potent antioxidant [21,22], did not prevent the observed decrease in State-3 respiration (not shown).

To confirm the specific inhibition of State-3 respiration by AAPH, we established the relationships between membrane potential of protonmotive force-generating and -consuming (ATP synthesis) reactions. Under our incubation conditions (medium with a high phosphate content), $-\Delta pH$ was low (6–8 mV) and did not vary with conditions. Thus membrane potential was the main component representative of protonmotive force. Malonate titration of State-3 respiration, which modulates potential generated by succinate oxidation, showed that AAPH decreases the rate of the protonmotive force consumers at any given membrane potential (Figure 3A). In contrast, titration of respiration with an uncoupler (CCCP) in the presence of oligomycin indicated no difference in the elasticity of the electron-transport chain to membrane potential (Figure 3B). Similar results were obtained by titrating the native proton leak across the membrane by malonate in the presence of oligomycin, which was very low and led to a comparable leak at a membrane potential corresponding to State 3 (not shown). Thus Figure 3 clearly shows that reactions related to ATP synthesis were damaged early by AAPH.

Rate of phosphorylation

Since an altered rate of entry of ADP into the mitochondrial matrix may affect the rate of phosphorylation, the possible inhibition of the ATP/ADP antiporter was investigated by monitoring ATP synthesis in the presence of increasing concentrations of the specific inhibitor CATR [23]. Figure 4 clearly shows that no cumulative inhibitory effect of CATR could be demonstrated in mitochondria pretreated with 0.3 μmol of AAPH/mg by reference to untreated particles. With 0.9 μmol of AAPH, the initial inhibitory effect was slightly more pronounced, indicating some increased control of phosphorylation by the ATP/ADP antiporter.

A possible impairment of the F_1F_0 -ATPase complex, which may also affect ATP synthesis, was studied using tri-n-butyltin

Figure 5 Inhibitor effect of tri-*n*-butyltin and venturicidine on F_1F_0 -ATPase of mitochondria pretreated with AAPH or exposed to DCCD during the measurement

The rate of phosphorylation was expressed as a percentage of the value in the absence of inhibitor (%Vp), by using a pH electrode as indicated in the Experimental section. ○, Control; △, 0.3 μ mol of AAPH/mg of protein; ▲, 0.9 μ mol of AAPH/mg of protein; □, 0.15 nmol of DCCD/mg of protein. Data are representative of experiments using three different mitochondrial preparations.

and venturicidine, two specific inhibitors that block proton flux in the F_o subunit [24–26]. Figure 5 shows the titration of phosphorylation rate with tri-*n*-butyltin and venturicidine. Comparable displacements of titration curves were observed, showing more efficient inhibition in mitochondria pretreated with AAPH. Similar displacements of curves were observed when DCCD (a modifier that binds covalently to subunit 9 involved in proton translocation [27,28]) was used at 10 nmol/mg of protein of control mitochondria to ensure the same phosphorylation rate as for mitochondria treated with 0.3 μ mol of AAPH/mg of protein (Figure 5). These results show that the early decay in phosphorylating activity observed in AAPH-treated mitochondria involved the F_1F_0 -ATPase and not the ATP/ADP antiporter.

Rate of hydrolysis by the oligomycin-sensitive F_1F_0 -ATPase

To confirm this inference more directly, the oligomycin-sensitive ATPase activity was measured in either intact or broken mitochondria. In mitochondria, the non-ionic detergent Lubrol was used to disrupt the membrane instantaneously. This immediately stopped the kinetic control exerted by the ATP/ADP antiporter or the phosphate carrier [29]. Indeed, the control exerted by the ATP/ADP antiporter was particularly high in rat liver mitochondria as shown by comparing CCCP- (Figure 6A) and Lubrol- (Figure 6B) induced ATP hydrolysis. ATPase activity was

Figure 6 Time course of ATP hydrolysis on de-energization monitored by pH

Effects of AAPH pretreatment were compared with those of CATR (1 μ g/mg of protein) or oligomycin (1 μ g of protein added in the incubation medium 5 min before hydrolysis). ATP hydrolysis was initiated by either 3.5 μ M CCCP (A) or 0.025% Lubrol (B). Before hydrolysis in the presence of 1 mM MgATP, mitochondria were conditioned so that they experienced State-4/State-3 transition with 75 nmol of ADP. Curve a, control; curve b, plus CATR; curve c, plus oligomycin; curves d and e, 0.45 and 0.6 μ mol of AAPH/mg of protein respectively. Data are representative of experiments using three different mitochondrial preparations.

Figure 7 Ca^{2+} -induced swelling of AAPH-pretreated mitochondria

Zero time corresponded to the addition of 55 nmol of Ca^{2+} . Absorbance was recorded at 520 nm. Curve a, control; curves b and c, 0.9 and 1.2 μ mol of AAPH/mg of protein respectively; curve d, plus 1 μ mol of cyclosporin A/mg of protein; curve e, plus 0.03 μ mol of *t*-butyl hydroperoxide/mg of protein. Data are representative of experiments using three different mitochondrial preparations.

shown to decline rapidly after dissipation of protonmotive force, as previously observed [14].

In Lubrol-dissociated mitochondria, AAPH acted like oligomycin by decreasing ATP hydrolysis, whereas CATR had no effect. This confirmed unequivocally that AAPH is a potent inhibitor of the F_1F_o -ATPase.

Mitochondrial swelling

Assays were performed by adding Ca^{2+} in the presence or absence of peroxidants. Swelling induced by the amount of Ca^{2+} added was totally inhibited by cyclosporin A (Figure 7). In contrast with the mitochondrial swelling that occurred with 30 nmol of *t*-butyl hydroperoxide (a pro-oxidant that durably triggers permeability transition [30,31]), no promoting effect of AAPH could be shown. Surprisingly, AAPH acted more as a dose-dependent inhibitor of permeability transition initiated by Ca^{2+} (Figure 7), in spite of its pro-oxidant properties (Figure 1).

DISCUSSION

Our results clearly show that early changes observed in AAPH-treated mitochondria were due to (a) a decrease in phosphorylation rate linked to impaired F_1F_o -ATPase activity, (b) an inhibition of permeability transition in spite of production of reactive oxygen species.

Free radicals are increasingly assumed to play a crucial role in cellular aging and associated degenerative diseases [1,2]. Some of these processes were ascribed to mitochondrial decay [32]. Therefore a knowledge of the effects of various pro-oxidants on mitochondrial functions may be useful in understanding the possible relation between mitochondrial failure and aging.

The decrease in oxidative phosphorylation without inactivation of the respiratory chain observed in AAPH-treated mitochondria is consistent with the report of Kanno et al. [7]. Our findings of the steady native proton leak through the inner mitochondrial membrane and the constant ADP/O ratios provide evidence that the inner membrane retained its permeability properties. Therefore the events underlying such a decrease in oxidative activity were related to the activity of enzymes controlling the phosphorylation of ADP. A top-down approach confirmed that alterations were located at the phosphorylation level. Such an approach has been used successfully to determine the site of action of agents, such as glucagon, and physiopathological states, such as hypothyroidism, on oxidative phosphorylation in liver mitochondria [33,34].

In isolated liver mitochondria, modulation of ATP synthesis is highly dependent on modifications of the degree of control exerted by ADP transport [35,36]. However, as assessed by the similar inhibition of phosphorylation by CATR in treated and control mitochondria, AAPH did not appear to act primarily by altering the entry of ADP at the translocase level.

In contrast, the specific and cumulative inhibition of phosphorylation by tri-*n*-butyltin or venturicidine, two specific superstoichiometric inhibitors that block proton flux through the F_o subunit at the F_1F_o -ATPase level, strongly suggest that the site of phosphorylation was impaired in AAPH-treated mitochondria. A comparison of ATP hydrolase activity in uncoupled and disrupted mitochondria strongly confirmed this conclusion.

Under the mild conditions used in the present studies, AAPH was able to transfer hydroxyl radicals to DMPO molecules. However, owing to lack of MDA production from mitochondrial phospholipids, peroxidative products generated from AAPH were likely to act primarily in the aqueous region [4,37]. This would explain why two potent hydrophobic antiperoxidants, resveratrol (our study) and α -tocopherol [7], did not protect

mitochondria from AAPH. Thus AAPH appeared to act through the generation of free radicals although one cannot exclude the possibility of a reaction between the imine ends of AAPH and channel proteins (similar to the reaction with DCCD [38], which is somewhat related to AAPH).

It is well established that many pro-oxidants in the presence of Ca^{2+} trigger a characteristic membrane disruption and mitochondrial swelling known as the cyclosporin-sensitive mitochondrial permeability transition [39]. However, in our experiments, not only did AAPH fail to induce such an effect, but it inhibited Ca^{2+} -sensitive pore opening. Taking into account the fact that oligomycin and DCCD are potent inhibitors of both F_1F_o -ATPase and permeability transition [8,40], the attenuated opening of the transition pore by Ca^{2+} in the presence of AAPH may also be related to an inhibitory effect on F_1F_o -ATPase.

Since the decay in phosphorylation rate is thought to lower the resistance of mitochondria to pro-oxidants [41,42], AAPH used under more severe conditions (i.e. parenteral injection or longer exposure) would be expected to induce lipoperoxidation with significant MDA production, thus resulting in mitochondrial swelling [7] or rupture of erythrocytes [43]. These previous observations, which were obtained under much harsher conditions than those used in the present study, probably result from non-specific destabilization of biological membranes.

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