The redox state of endogenous pyridine nucleotides can determine both the degree of mitochondrial oxidative stress and the solute selectivity of the permeability transition pore

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Abstract Acetoacetate, an NADH oxidant, stimulated the ruthenium red-insensitive rat liver mitochondrial Ca^{2+} efflux without significant release of state-4 respiration, disruption of membrane potential ($\Delta\psi$) or mitochondrial swelling. This process is compatible with the opening of the currently designated low conductance state of the permeability transition pore (PTP) and, under our experimental conditions, was associated with a partial oxidation of the mitochondrial pyridine nucleotides. In contrast, diamide, a thiol oxidant, induced a fast mitochondrial Ca^{2+} efflux associated with a release of state-4 respiration, a disruption of $\Delta\psi$ and a large amplitude mitochondrial swelling. This is compatible with the opening of the high conductance state of the PTP and was associated with extensive oxidation of pyridine nucleotides. Interestingly, the addition of carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone to the acetoacetate experiment promoted a fast shift from the low to the high conductance state of the PTP. Both acetoacetate and diamide-induced mitochondrial permeabilization were inhibited by exogenous catalase. We propose that the shift from a low to a high conductance state of the PTP can be promoted by the oxidation of NADPH. This impairs the antioxidant function of the glutathione reductase/peroxidase system, strongly strengthening the state of mitochondrial oxidative stress.

Key words: Rat liver mitochondrion; Permeability transition pore; NAD(P)H redox state; Calcium ion; Oxidative stress

1. Introduction

Mitochondrial permeability transition pore (PTP) is characterized as a Ca^{2+}-dependent, cyclosporin A-sensitive proteinaceous pore located in the inner mitochondrial membrane, whose permeability to large molecules, osmotic support and even to small proteins gradually increases [1,2]. The Ca^{2+}-induced PTP is enhanced by a variety of compounds that include inorganic phosphate, protonophores, thiol reagents, fatty acids, thyroid hormones and others [1–3]. Most of these compounds, designated as inducers, like the pyridine nucleotides (PN) oxidants, are capable of enhancing Ca^{2+}-induced oxidative stress because they exhaust mitochondrial GSH and NADPH, substrates of the antioxidant enzymes glutathione peroxidase/reductase, thus favoring accumulation of H_2O_2 [2]. It should be mentioned that mitochondrial Ca^{2+} overload itself [4–9] induces the generation of reactive oxygen species. Regarding PTP induction by PN oxidation, further studies on this mechanism indicated that the oxidation of NADPH was more important than the oxidation of NADH to promote Ca^{2+} release from mitochondria [10,11]. Considering that under high membrane potentials the energy-linked transhydrogenase maintains NADP in the reduced state, even when NAD is almost completely oxidized [12], we hypothesized that the extent of mitochondrial membrane potential ($\Delta\psi$) could be a key in this mechanism [11]. Indeed, manipulation of NADP redox state as a function of $\Delta\psi$ values in rat liver mitochondria (RLM) energized by tetramethyl-*p*-phenylenediamide/ascorbate in the presence of acetoacetate, provided evidence that $\Delta\psi$ and the activity of the energy-linked transhydrogenase were key elements in determining the redox state of NADP and thus of Ca^{2+} retention and release from mitochondria [11].

The participation of reactive oxygen species in Ca^{2+}-induced PTP was demonstrated by experiments showing that catalase, ebselen or the thiol-specific antioxidant enzyme prevent the disruption of membrane potential and swelling caused by the cation alone or in the presence of inducers such as *t*-butyl hydroperoxide, inorganic phosphate, protonophores and fatty acids [3,4,6–8]. In addition, no PTP opening occurs in the absence of molecular O_2. Indeed, literature data show that PTP opening is induced by exogenous reactive oxygen species-generating systems such as menadione, 5-aminolevulinic acid and xanthine/xanthine oxidase, in the presence of Ca^{2+} (for a recent review, see [2]).

Since the discovery that cyclosporin A is a potent inhibitor of the PTP, the permeability transition has been proposed to be a regulated pore possibly involved in physiological and pathophysiological mitochondrial functions [13–15]. Furthermore, electrophysiological studies suggest that it occurs in several substate levels [16–18] which, in intact mitochondria, seem to correspond to different conductance conformations that operate with different selectivity and functions [14]. It has been proposed that under its low conductance conformation, the PTP would not be related to mechanisms of cell death and does not impair mitochondrial functions [14], playing a role in the regulation of cellular Ca^{2+} homeostasis by acting as a Ca^{2+}-releasing channel [14]. In this regard, the

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Abbreviations: $\Delta\psi$, mitochondrial membrane potential; PTP, mitochondrial permeability transition pore; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; PN, pyridine nucleotides; RR, ruthenium red

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objective of the present work was to analyze the nature of two forms of pore opening in isolated RLM induced by Ca^{2+} plus prooxidants: first, a form of low conductance induced by Ca^{2+} plus acetoacetate, which occurs without a large decrease in membrane potential and swelling [19,20], and second, a form of high conductance induced by Ca^{2+} plus diamide that is associated with membrane potential disruption and large amplitude swelling [4,21].

2. Materials and methods

2.1. Isolation of RLM and standard incubation procedure

RLM were isolated by conventional differential centrifugation from the livers of adult female Wistar rats fasted overnight. The experiments were carried out at 30°C in a standard medium containing 250 mM sucrose, 3.0 mM HEPES (2-hydroxyethyl-1-piperazineethanesulfonic acid), pH 7.1, 20 mM K^+-acetate, 1.0 mM $MgCl_2$, 5.0 μM rotenone, 2.0 mM succinate, 0.2 mM K_2HPO_4, 0.12 mM ADP and 2.5 μM oligomycin. Other additions are indicated in the figure legends. The results shown are representative or averages of at least three experiments using different mitochondrial preparations.

2.2. Measurements of mitochondrial transmembrane electrical potential ($\Delta\psi$)

The mitochondrial membrane potential ($\Delta\psi$) was estimated by measuring the fluorescence changes of safranin O (5.0 μM), using a model F-4010 Hitachi spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operated at excitation and emission wavelengths of 495 nm and 586 nm, respectively, and slit widths of 5 nm [22].

2.3. Determination of Ca^{2+} movement

Variations in the concentration of free extramitochondrial Ca^{2+} were followed by measuring the changes in the absorbance spectrum of arsenazo III (40 μM), using an SLM Aminco DW2000 spectrophotometer (SLM Instruments, Inc., Urbana, IL, USA) set at the wavelength pair 675–685 nm.

2.4. Determination of the NAD(P) redox state

The oxidation or reduction of PN in the mitochondrial suspension was followed in a Hitachi F-4010 spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 366 and 450 nm, respectively, with slit widths of 5 nm.

2.5. Oxygen uptake measurements

Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments, OH, USA) in 1.3 ml of standard reaction medium (30°C), in a sealed glass cuvette equipped with a magnetic stirrer.

2.6. Materials

Acetoacetate, ADP, antimycin A, arsenazo III, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), catalase, cyclosporin A, dithiothreitol, EGTA, HEPES, oligomycin, rotenone, safranin O, succinate, ruthenium red (RR) were obtained from Sigma Chemical Company (St. Louis, MO, USA).

3. Results

The results presented in Fig. 1 show the effects of acetoacetate and diamide on the redox state of endogenous PN, respiration and Ca^{2+} handling by mitochondria incubated in a reaction medium in which stimulation of Ca^{2+} release by acetoacetate can occur without loss of respiratory control [20]. The a traces correspond to the control system without acetoacetate and diamide, the b traces are those of systems containing acetoacetate and the c traces correspond to the systems containing diamide. Additions of mitochondria to both systems resulted in increased fluorescence at 366–450 nm (Fig. 1A), indicating the presence of PN in the reduced state. In the control experiment (a), this increase in fluorescence was larger

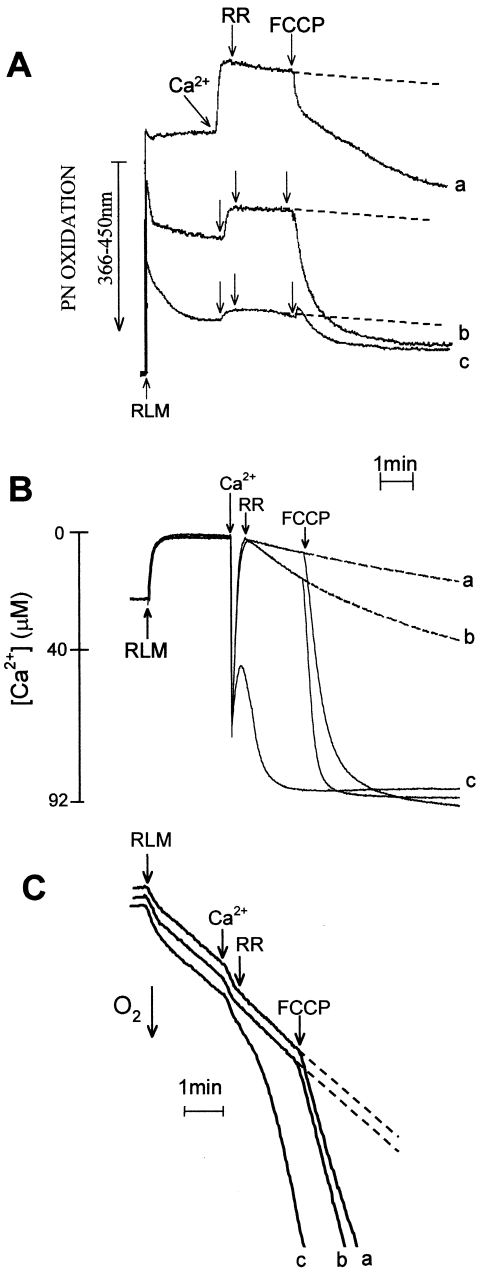

Fig. 1. The solute selectivity of mitochondrial membrane permeabilization, induced by prooxidants, depends on the extent of oxidation of the PN. The effects of acetoacetate and diamide were studied on the redox state of PN (A), Ca^{2+} transport (B) and oxygen uptake (C) of intact isolated mitochondria. RLM (1.0 mg/ml) were added to standard reaction medium as described in Section 2. Ca^{2+} (80 nmol/mg) was added 2.5 min after mitochondria addition. RR (0.5 μM) and 0.4 μM FCCP were added where indicated. Traces b and c represent experiments carried out in the presence of acetoacetate (1.0 mM) and diamide (1.0 mM), respectively. Traces a correspond to control experiments (no acetoacetate or diamide present).

and sustained during the first 2.5 min; had an intermediate value and decreased initially in the presence of acetoacetate (b); and was much smaller and not sustained in the presence of diamide (c). During this period of 2.5 min, the contaminant Ca^{2+} (about 12 μM) present in the incubation medium was taken up and retained by mitochondria (Fig. 1B) and respiration proceeded at state 4 (Fig. 1C) under all experimental

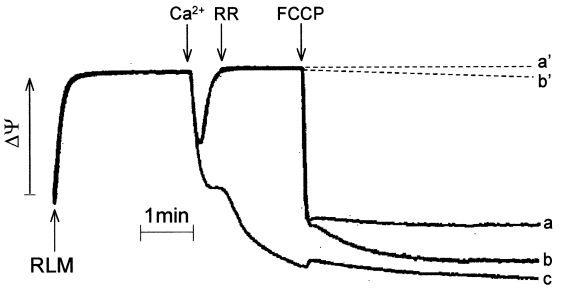

Fig. 2. Effect of acetoacetate and diamide on the membrane potential of intact RLM. The experiments were carried out in the same conditions described for Fig. 1A in standard reaction medium containing 5 μM safranin O. The dotted traces a′ and b′ correspond to the experimental conditions of traces a and b, respectively, without FCCP addition.

conditions. The subsequent addition of a high concentration of Ca^{2+} (80 μM), at minute 2.5, was followed by the immediate uptake of the cation (Fig. 1B), and an increase in both the rate of oxygen consumption (Fig. 1C) and NAD(P)H fluorescence (Fig. 1A). A similar increase in NAD(P)H fluorescence during mitochondrial Ca^{2+} uptake has previously been observed [23]. This increase in fluorescence was much larger in the control experiment, reflecting the larger content of reduced PN owing to the absence of oxidants. When Ca^{2+} uptake was essentially complete, the rate of respiration returned to the controlled rate in a and b, but not in c. At this moment, RR was added to all systems to prevent Ca^{2+} cycling, allowing for determination of the rates of net Ca^{2+} efflux. In the a system (control), only very low net release of Ca^{2+} took place, less than 1.5 nmol/min/mg. In the b system (acetoacetate present), Ca^{2+} release occurred at a rate of about 8 nmol/min/mg and was paralleled by a very low increase in the controlled state-4 rate of respiration, but in c (diamide present), the rate of Ca^{2+} release was very fast (larger than 60 nmol/min/mg) and state-4 respiration was totally released.

To ascertain whether the state-4 respiration in the presence of acetoacetate was actually a controlled state, FCCP was added to all systems after a period of net Ca^{2+} release induced by RR. There was an immediate and very large acceleration of both Ca^{2+} release (Fig. 1B) and oxygen consumption (Fig. 1C). FCCP-induced Ca^{2+} release was faster in the acetoacetate containing experiment (b) than in the control experiment (a). In the presence of cyclosporin A, the rate of Ca^{2+} release was similar in both conditions (results not shown). The respiratory control was about 5 in both systems, indicating that $NADP^+$-stimulated Ca^{2+} release can occur in highly coupled mitochondria. The FCCP-induced uncoupling was followed by different degrees of NAD(P)H oxidation. In a, despite the absence of exogenous oxidants, the oxidation was certainly caused by both a decrease in matrix Ca^{2+} [23] and an increased rate of respiration. In b, the collapse of $\Delta\psi$ favored NADPH oxidation by acetoacetate, due to the arrest of the $\Delta\psi$ driven energy-linked NADP transhydrogenase reaction [12]. The low effect of FCCP addition observed in trace c is related to the highly oxidized state of PN that preceded the inclusion of the protonophore.

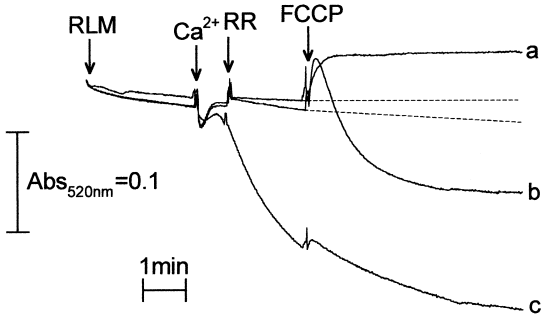

Fig. 3. Effect of acetoacetate, diamide and FCCP on the changes in volume of intact RLM. The experiments were carried out in the same conditions described for Fig. 1A. The dotted lines correspond to the experiments of traces a and b, without FCCP addition.

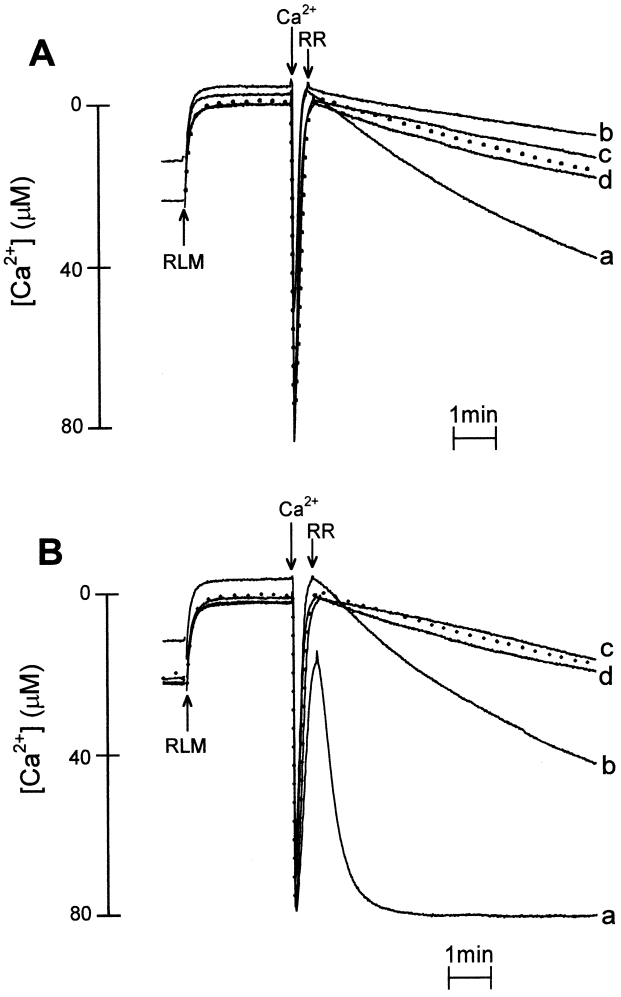

Fig. 4. Acetoacetate and diamide-induced PTP opening is mediated by reactive oxygen species. RLM (1 mg/ml) were added to standard reaction medium containing 1.0 mM acetoacetate (A) or 1.0 mM diamide (B) in the presence of 2 μM catalase (traces b), 1.0 μM cyclosporin A (traces c), 5 mM dithiothreitol (traces d) or no other additions (traces a). Dotted lines correspond to control experiments (no acetoacetate or diamide present).

The experiment depicted in Fig. 2 supports the interpretation that Ca^{2+} addition caused only a transient decrease in $\Delta\psi$ in systems a and b, but collapsed $\Delta\psi$ in the presence of diamide. Also in agreement with this interpretation are the results of Fig. 3, showing that under the conditions in which mitochondria retained a high $\Delta\psi$ in the presence of acetoacetate, no large amplitude swelling occurred. However, killing of the energy-linked transhydrogenase by FCCP was followed by

a sharp decrease in absorbance of the mitochondrial suspension (swelling), which was paralleled by an increased rate of Ca^{2+} release (b) comparable to that observed in the experiment done in the presence of diamide.

The above results allow for at least two different interpretations with respect to the mechanisms leading to the different effects of acetoacetate and diamide in mitochondrial coupling, Ca^{2+} movements and swelling. First, it might be possible that diamide is opening a high and acetoacetate a low conductance PTP. Second, Ca^{2+} release induced by these compounds might be taking place via totally different routes. In this regard, we hypothesized that the differences may be related to the different degrees of oxidative stress induced by these prooxidants. Diamide oxidizes both NAD(P)H and thiols [2,4,21]. Acetoacetate oxidizes predominantly NADH and NADPH is maintained reduced at the expense of the energy-linked transhydrogenase [24]. Evidence has been presented [25] that, under the latter conditions, less thiols are oxidized in the inner mitochondrial membrane.

These interpretations were addressed by experiments using PTP inhibitors that act by different mechanisms: cyclosporin A, that seems to displace cyclophylin D from the adenine nucleotide translocator [26], catalase, which eliminates H_2O_2, and dithiothreitol, which reduces dithiols. Interestingly, the experiment of Fig. 4 shows that the net Ca^{2+} release induced by both acetoacetate and diamide, under the conditions of Fig. 1B, are highly sensitive to all of these inhibitors. This indicates that both compounds open the PTP under the present experimental conditions and that the different substates of the mitochondrial permeability seem to be related to different degrees of mitochondrial oxidative stress.

4. Discussion

Available data provide evidence that PTP inhibitors such as cyclosporin A and trifluoperazine protect cells from liver, heart and other tissues against injuries [15,27–29]. Moreover, the detection of different substates of the PTP [16–18] has led some workers to propose that it can operate with different selectivities, possibly functioning as a Ca^{2+}-releasing channel in the low conductance state [14]. Such interpretations of the properties of the PTP recall earlier data from this laboratory showing that mitochondrial Ca^{2+} release stimulated by the oxidized state of NAD(P) could apparently occur with maintenance of respiratory control [10,11], as was observed in the acetoacetate experiments presented here (Fig. 1). The possible difference in the nature of such a low conductance pore opened by acetoacetate and that of a high conductance pore opened by diamide, was assessed by analyzing their sensitivity to cyclosporin A, dithiothreitol and exogenous catalase.

The sensitivity of both pores to cyclosporin identified them as the PTP. The involvement of reactive oxygen and dithiols production in the mechanism of pore formation was confirmed by the sensitivity of both to catalase and dithiothreitol, respectively. These results strongly suggest that the low conductance state of the pore is a precursor of the high conductance state and that the shift from low to high conductance induced by FCCP was mediated by NADPH oxidation. This interpretation is supported by the extensive swelling that followed NADPH oxidation upon FCCP addition in the acetoacetate experiment (Fig. 3). When acetoacetate or diamide were absent, the elimination of $\Delta\psi$ by FCCP was followed by the release of state-4 respiration, and Ca^{2+} release certainly occurred via the reverse of the uniporter, given its insensitivity to the PTP inhibitors. In these conditions, the oxidation of NAD(P)H was only discrete when compared to the very fast oxidation observed in the experiment done in the presence of acetoacetate. This is in agreement with previous data [11,24] showing that at high $\Delta\psi$, acetoacetate oxidizes predominantly NADH, because the energy-linked transhydrogenase maintains NADPH reduced and the reactive oxygen generated is scavenged by reducing equivalents supplied to the glutathione reductase/peroxidase system. This is equivalent to saying that $\Delta\psi$ is the ultimate defense weapon against mitochondrial oxidative stress and that the conductance of the PTP can be a function of its extent. It should be stressed, however, that a high mitochondrial membrane potential facilitates mitochondrial O_2^-/H_2O_2 production [30,31], but that a mild uncoupling, under which the energy-linked transhydrogenase is still active, decreases O_2^-/H_2O_2 production and protects mitochondria [30,31].

Regarding the possible biological roles of the different conductance states of the PTP analyzed here, if they occur in situ, it should be considered that a slow but continuous decrease in $\Delta\psi$ and swelling take place and are paralleled by an increased rate of reactive oxygen production in the acetoacetate experiments. This argues against a possible physiological role of the Ca^{2+} release that occurs under these conditions. It should be remembered that mitochondria possess specific carriers to mediate Ca^{2+} release at these rates under physiological conditions [32]. Since mitochondrial oxidative stress associated with reduced mitochondrial energy output is an important factor in mitochondrial mediated cell death [15,33], one could speculate that the different states of the PTP conductance may determine the time course in oxidative phosphorylation arrest, a factor that determines whether necrotic cell death or apoptosis follows PTP opening.

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