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Stimulation of Ca^{2+} Release from Rat Liver Mitochondria by the Dithiol Reagent α-Lipoic Acid

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ABSTRACT. Rat liver mitochondria contain a Ca^{2+}-specific release pathway stimulated by Ca^{2+}-dependent hydrolysis of oxidized intramitochondrial pyridine nucleotides to ADP ribose and nicotinamide. We have previously shown that NAD^+ hydrolysis and subsequent Ca^{2+} release are inhibited by cyclosporine A and that they are only possible when some critical thiols are cross-linked or oxidized, e.g. by phenylarsine oxide, gliotoxin, or peroxynitrite. We now report that the antioxidant α-lipoic acid stimulates Ca^{2+} release from intact mitochondria, i.e. with preservation of the mitochondrial membrane potential and without large-amplitude swelling. The release stimulated by α-lipoic acid is inhibited by cyclosporine A and is more effective when the pyridine nucleotides are oxidized. The results strongly suggest that α-lipoic acid stimulates the Ca^{2+}-specific release pathway from intact mitochondria by oxidizing some vicinal thiols, thereby stimulating hydrolysis of oxidized pyridine nucleotides. These observations further corroborate that intact rat liver mitochondria contain a specific Ca^{2+} release pathway stimulated by modification of vicinal thiols. Prolonged stimulation of Ca^{2+} release by lipoic acid followed by its re-uptake (Ca^{2+} "cycling") may contribute to the detrimental, prooxidant-like effects seen with higher concentrations of lipoic acid. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;12:1815–1820, 1996.

KEY WORDS. intactness; membrane potential; sulfhydryls; pyridine nucleotide hydrolysis; glutathione; cyclosporine A

The maintenance of mitochondrial Ca^{2+} homeostasis is important for regulation of several intramitochondrial enzymes, e.g. pyruvate dehydrogenase, NAD^+-isocitrate-dehydrogenase, and 2-oxoglutarate-dehydrogenase [1, 2]. Mitochondrial Ca^{2+} transport also contributes to cellular Ca^{2+} regulation. Given their large storage capacity, mitochondria provide a safety device against potentially harmful Ca^{2+} flooding of the cytosol [3]. In addition, they play an important role in shaping physiological Ca^{2+} transients and in the maintenance of cytosolic Ca^{2+} homeostasis [4, 5].

Uptake and release of mitochondrial Ca^{2+} occur via different pathways [6]. As a consequence, Ca^{2+} can be "cycled" across the mitochondrial inner membrane [7]. Ca^{2+} uptake is driven by $\Delta\Psi$.† Ca^{2+} release occurs either with preservation of $\Delta\Psi$ and is specific for Ca^{2+}, or when $\Delta\Psi$ collapses and the inner mitochondrial membrane becomes nonspecifically leaky. In rat liver mitochondria, the specific pathway operates when oxidized intramitochondrial pyridine nucleotides are hydrolyzed in a Ca^{2+}-specific manner to ADP ribose and nicotinamide (reviewed in [8]). NAD^+ hydrolysis is inhibited by cyclosporine-bound cyclophilin [9] and is only possible when some vicinal thiols are cross-linked, either by oxidation [10, 11] or by reaction with phenylarsine oxide [12].

α-Lipoic acid (1,2-dithiolane-3-pentanoic acid) and its reduced form, dihydrolipoic acid (6,8-dimercaptooctanoic acid), have powerful antioxidant properties [13]. Endogenous α-lipoic acid, covalently bound as lipoamide, is a cofactor of mitochondrial dehydrogenase complexes. It is, therefore, unavailable as an antioxidant [14]. However, exogenous lipoic acid may be effective as an antioxidant and has attracted interest in the treatment of complications related to diabetes and neurodegenerative disorders [13, 14].

We now report that the hydrophobic bifunctional thiol reagent α-lipoic acid stimulates Ca^{2+} release from intact rat liver mitochondria, i.e. under preservation of $\Delta\Psi$, by activating the specific Ca^{2+} release pathway independent of the mitochondrial glutathione status.

MATERIALS AND METHODS

Materials

Lipoic acid (D,L-α-lipoic acid) was from Fluka, Buchs, Switzerland, and dihydrolipoic acid (D,L-6,8-thioctic acid) came

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† *Abbreviations:* CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CSA, cyclosporine A; $\Delta\Psi$, electrical potential across the inner mitochondrial membrane, negative inside; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid.

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from Sigma, Buchs, Switzerland. They were dissolved in ethanol prior to use [15]. CSA was a gift of Sandoz Pharma Preclinical Research, Basel, Switzerland. It was stored in solid form at −20°C and dissolved in ethanol immediately prior to use. All other chemicals were purchased from standard suppliers, and were of the highest purity commercially available.

Methods

ISOLATION OF MITOCHONDRIA. The isolation of rat liver mitochondria was performed by differential centrifugation [16]. The protein content was determined by the Biuret method with bovine serum albumin as standard.

DEPLETION OF MITOCHONDRIAL GLUTATHIONE. Depletion of mitochondrial glutathione *in vivo* was performed as described [17]. Briefly, after overnight starvation, the rats were injected i.p. with 250 mg of phorone (dissolved in sunflower oil) per kilogram of body weight. Mitochondria were isolated 3 hr thereafter.

STANDARD INCUBATION PROCEDURE. Mitochondria (2 mg of protein/mL) were incubated at 25°C with continuous stirring and oxygenation in 3 mL of 210 mM mannitol, 70 mM sucrose, and 5 mM Hepes, pH 7.2.

DETERMINATION OF Ca^{2+} UPTAKE AND RELEASE BY MITOCHONDRIA. The standard incubation procedure was followed. After addition of rotenone (5 μM) and K^+-succinate (2.5 mM), mitochondria were loaded with Ca^{2+}. Its movement across the inner mitochondrial membrane was monitored at 675–685 nm in the presence of 50 μM arsenazo III with an Aminco DW2A spectrophotometer. Ca^{2+} was added to give a total load as indicated in the figure legends, and its uptake was allowed to proceed for 2–3 min.

DETERMINATION OF THE MITOCHONDRIAL MEMBRANE POTENTIAL. Mitochondria were incubated according to the standard procedure in the presence of 10 μM safranine. After addition of rotenone (5 μM) and K^+-succinate (2.5 mM), mitochondria were loaded with Ca^{2+}. $\Delta\Psi$ was determined with an Aminco DW2A spectrophotometer at 511–533 nm [18]. Other compounds were added as indicated in the figure legends.

SPECTROPHOTOMETRIC ANALYSIS OF MITOCHONDRIAL PYRIDINE NUCLEOTIDES. The standard incubation procedure was followed. After addition of rotenone (5 μM) and K^+-succinate (2.5 mM), the absorption of mitochondrial pyridine nucleotides was determined with an Aminco DW2A spectrophotometer at 340–370 nm [9]. Other compounds were added as indicated in the figure legends.

MEASUREMENT OF MITOCHONDRIAL SWELLING. Swelling of mitochondria incubated according to the standard procedure was monitored continuously as change in OD_{540}.

RESULTS

Stimulation by Lipoic Acid of Ca^{2+} Release

Lipoic acid stimulated Ca^{2+} release from rat liver mitochondria in a dose-dependent manner (Fig. 1A). It was as effective (results not shown) in inducing Ca^{2+} release from glutathione-depleted mitochondria [17] as from glutathione-adequate mitochondria with respect to both the kinetics and the extent of the lipoic acid-induced Ca^{2+} release. This ruled out a significant involvement of the enzyme cascade glutathione peroxidase/glutathione reductase/energy-dependent transhydrogenase [19]. CSA (1 μM) completely inhibited Ca^{2+} release induced by 1 mM lipoic acid (Fig. 1B). This figure also shows that CSA partially inhibited Ca^{2+} release induced by the mitochondrial Ca^{2+} uptake inhibitor ruthenium red alone, indicating that this "spontaneous" Ca^{2+} release occurred via the same mechanism. Dihydrolipoic acid (1 mM) stimulated Ca^{2+} release to the same extent as lipoic acid, but only after a lag phase (Fig. 1C).

Intactness of Mitochondria during Lipoic Acid-Induced Ca^{2+} Release

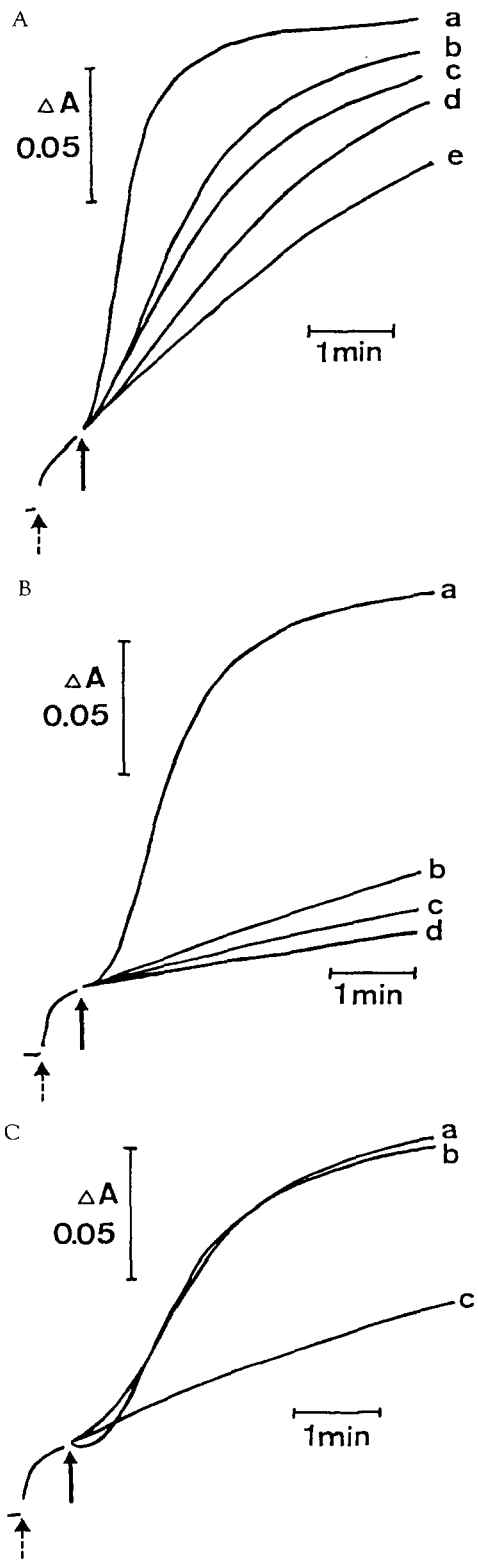
A useful parameter for assessing the intactness of the inner mitochondrial membrane during and after Ca^{2+} release is the determination of $\Delta\Psi$ [20, 21]. Upon addition of lipoic acid to mitochondria loaded with Ca^{2+}, $\Delta\Psi$ changes depended on whether or not mitochondria were allowed to continuously take up and release Ca^{2+} (Fig. 2). Thus, $\Delta\Psi$ soon decreased after addition of 1 mM lipoic acid in the absence of EGTA (Fig. 2, trace d) but increased in its presence (Fig. 2, trace a), i.e. under conditions that prevented Ca^{2+} cycling. The increase in $\Delta\Psi$ was prevented by 1 μM CSA (Fig. 2, trace c), reflecting the inhibition of the release of previously accumulated Ca^{2+} [22]. Also, no lipoic acid-induced large-amplitude swelling of mitochondria occurred under these conditions, provided Ca^{2+} cycling was prevented by the addition of either EGTA or ruthenium red (results not shown).

Fate of Mitochondrial Pyridine Nucleotides

Intact rat liver mitochondria release Ca^{2+} via the specific pathway when their oxidized pyridine nucleotides are hydrolyzed [3]. CSA inhibits mitochondrial Ca^{2+} release by preventing NAD^+ hydrolysis [9]. To gauge their hydrolysis [9, 23] we determined spectrophotometrically to what extent the lipoic acid-dependent oxidation of pyridine nucleotides was reversible. Lipoic acid induced a decrease in pyridine nucleotide absorption at 340–370 nm in Ca^{2+}-loaded mitochondria in the absence (Fig. 3, trace b) but only marginally in the presence of 1 μM CSA (Fig. 3, trace a). In glutathione-deficient mitochondria [17], the decrease in pyridine nucleotide absorption induced by lipoic acid and its inhibition by CSA are the same as in glutathione-adequate mitochondria (not shown).

The hydrolysis of oxidized pyridine nucleotides is a prerequisite for Ca^{2+} release from intact mitochondria [24]. Therefore, we tested whether the oxidation state of the intramitochondrial pyridine nucleotides influences the lipoic acid-stimulated Ca^{2+} release. Indeed, oxidation of the

pyridine nucleotides in succinate-energized mitochondria by acetoacetate in the presence of the complex-I inhibitor rotenone strongly stimulated Ca^{2+} release, whereas the addition of β-hydroxybutyrate, which keeps the mitochondrial pyridine nucleotides reduced, prevented Ca^{2+} release induced by lipoic acid (not shown).



DISCUSSION

α-Lipoic acid is an essential cofactor in α-keto-acid dehydrogenase complexes, e.g. pyruvate or α-ketoglutarate dehydrogenase. At physiological concentrations, lipoic acid is mainly taken up by the liver in a carrier-mediated process. At higher concentrations, diffusion may become the major pathway of uptake [25]. Both lipoic acid and its reduced form, dihydrolipoic acid, have potent antioxidant properties. Lipoic acid was reported to scavenge hypochlorous acid and hydroxyl, ascorbyl, chromanoxyl, or peroxyl radicals [13–15, 26]; to protect cultured neurones against hypoxia-, glutamate-, or iron-induced injury [27]; and to protect against cerebral ischemia/reperfusion injury in gerbils [28]. Dihydrolipoic acid inhibited thymocyte apoptosis antioxidatively [29], but was also reported to have prooxidant activities, probably due to its ability to reduce iron ions and to generate reactive sulphur-containing radicals [13]. The protective effects of dihydrolipoic acid may involve "recycling" of other antioxidants such as glutathione, ascorbate, or vitamin E, rather than direct scavenging of reactive oxygen species by dihydrolipoic acid itself [13, 30].

In addition, lipoic and dihydrolipoic acids influence NMDA receptor activity *in vitro* by modifying the sulfhydryl groups of its redox modulatory site [31]. Because specific Ca^{2+} release from intact mitochondria is only possible when vicinal thiols are oxidized [10, 11] or cross-linked [12], we determined whether lipoic acid can induce Ca^{2+} release from mitochondria.

Lipoic acid stimulates Ca^{2+} release from rat liver mitochondria. When Ca^{2+} cycling is prevented by blocking its re-uptake with ruthenium red or by chelating extramitochondrial Ca^{2+} with EGTA, mitochondria remain intact during and after Ca^{2+} release, as judged by $\Delta\Psi$ and swelling

FIG. 1. Lipoic acid-stimulated Ca^{2+} release from rat liver mitochondria. (A) Mitochondria were incubated according to the standard procedure. After addition of rotenone and K^+-succinate, they were loaded with 50 nmol of Ca^{2+}/mg of protein. At the *dashed arrow*, ruthenium red (2 nmol/mg of protein), and at the *solid arrow*, 1 mM (trace a), 500 μM (trace b), 250 μM (trace c), or 100 μM (trace d) lipoic acid were added; trace e: vehicle alone. The results shown are a typical experiment out of three. (B) Mitochondria were incubated according to the standard procedure in the absence (traces a and b) or presence (traces c and d) of 1 μM CSA. After addition of rotenone and K^+-succinate, they were loaded with 35 nmol of Ca^{2+}/mg of protein. At the *dashed arrow*, ruthenium red (2 nmol/mg of protein), and at the *solid arrow*, 1 mM lipoic acid (traces a and d) or its vehicle (traces b and c) were added. The results shown are a typical experiment out of three. (C) Mitochondria were incubated according to the standard procedure. After addition of rotenone and K^+-succinate, they were loaded with 40 nmol of Ca^{2+}/mg of protein. At the *dashed arrow*, ruthenium red (2 nmol/mg of protein), and at the *solid arrow*, 1 mM lipoic acid (trace a), 1 mM dihydrolipoic acid (trace b), or their vehicle (trace c) were added. The results shown are a typical experiment out of four.

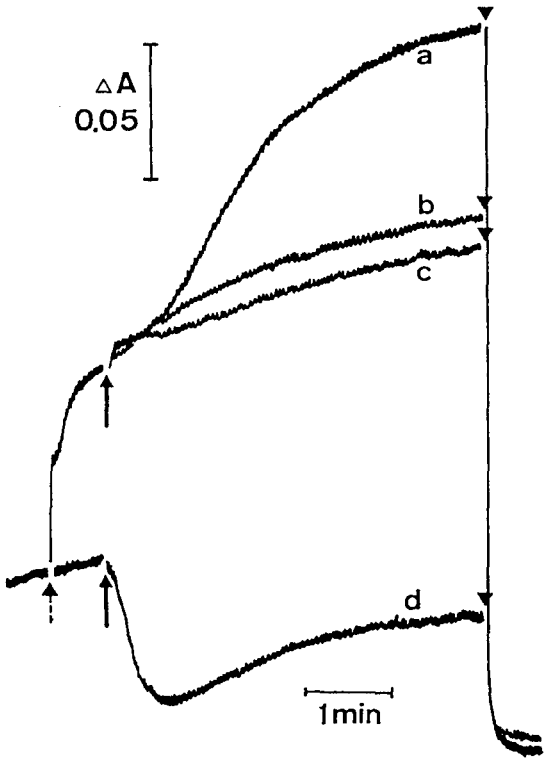


FIG. 2. Lipoic acid-induced alterations of the mitochondrial membrane potential. Mitochondria were incubated according to the standard procedure in the presence of 10 μM safranine and in the absence (traces a, b, and d) or presence (trace c) of 1 μM CSA. They were energized with K^+-succinate in the presence of rotenone. Three minutes after the addition of Ca^{2+} (40 nmol/mg of protein), 5 mM EGTA (*dashed arrow*) (traces a to c) and 30 sec later, 1 mM lipoic acid (traces a, c, and d) or its vehicle (trace b) were added (*solid arrows*). At the *arrowheads*, the uncoupler carbonyl-cyanide-3-chloro-phenylhydrazone (1 μM) was added. The results shown are a typical experiment out of three.

measurements. Consistent with this, the action of lipoic acid is completely blocked by 1 μM CSA, which is known to inhibit intramitochondrial NAD^+ hydrolysis and subsequent Ca^{2+} release via the Ca^{2+}-specific release pathway [9–12]. Because the stimulation of Ca^{2+} release is independent of the mitochondrial glutathione status, it is evident that lipoic acid affects an event distal to the oxidation of pyridine nucleotides by the glutathione/pyridine nucleotide-enzyme cascade, which is engaged in Ca^{2+} release induced by *t*-butylhydroperoxide [19].

Hydrolysis of oxidized pyridine nucleotides is a prerequisite for Ca^{2+} release from intact mitochondria. Accordingly, Ca^{2+} release induced by lipoic acid is accelerated in the presence of acetoacetate, i.e. under conditions where the intramitochondrial NAD^+ content is increased. It should be noted here that between 14 and 43% of pyridine nucleotides are oxidized in Ca^{2+}-loaded mitochondria in the presence of rotenone and succinate [10, 19]. The existence of an NAD^+ glycohydrolase in the inner mitochondrial membrane was first proposed and documented by us [23, 32]. The recently reported identification [33] of an NAD^+ hydrolyzing enzyme on the outer side of the inner mitochondrial membrane does not disprove NAD^+ hydrolysis in the matrix of intact mitochondria. We envisage that lipoic acid activates the NAD^+ hydrolyzing enzyme in the inner mitochondrial membrane. Accordingly, gliotoxin [10] or peroxynitrite [11] stimulates pyridine nucleotide hydrolysis (as judged directly by nicotinamide release) intramitochondrially, because it is not paralleled by sucrose entry into mitochondria, i.e. the inner mitochondrial membrane continuously retains its impermeability towards low molecular weight solutes.

These results strongly suggest that lipoic acid stimulates Ca^{2+} release from intact rat liver mitochondria by oxidizing some critical thiols other than glutathione in such a way that hydrolysis of oxidized pyridine nucleotides is achieved. Such thiols may be located directly on a protein catalyzing pyridine nucleotide hydrolysis, e.g. an NAD^+ glycohydrolase, or on an endogenous regulator of such an enzyme. Similarly, the NAD^+ glycohydrolase activity of pig brain NADase [34] or of CD38 [35], a human leukocyte cell surface antigen, is inhibited by the reduction of an essential protein disulfide group. Whether the mitochondrial thiol residues are directly involved in the regulation of the hydrolase activity or whether the disulfide bridges play an essential role in maintaining a monomeric, catalytically active structure, as shown for CD38 [36], remains to be established.

According to the above-mentioned mechanism, dihydrolipoic acid, which is unable to oxidize dithiols, should not stimulate Ca^{2+} release from mitochondria via the specific release pathway. However, mitochondria contain enzymes able to establish an equilibrium between the oxidized and the reduced forms of various dithiols [37]. In addition, di-

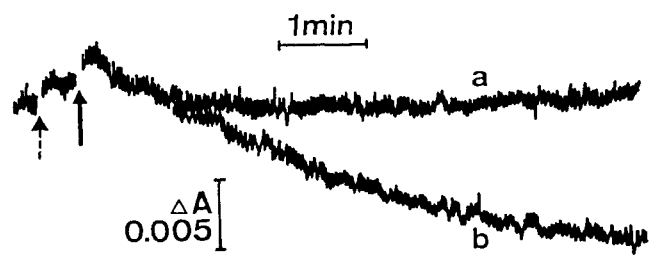


FIG. 3. Lipoic acid-induced oxidation and re-reduction of mitochondrial pyridine nucleotides. Mitochondria were incubated according to the standard procedure in the presence (trace a) or absence (trace b) of 1 μM CSA. Changes in the redox state of mitochondrial pyridine nucleotides were monitored at 340–370 nm. After addition of rotenone and K^+-succinate, mitochondria were loaded with 40 nmol of Ca^{2+}/mg of protein. Three minutes after Ca^{2+} uptake, 5 mM EGTA (*dashed arrow*) and 30 sec later, 1 mM lipoic acid (*solid arrow*) were added. The results shown are a typical experiment out of three.

hydrolipoic acid was reported to be oxidized to lipoic acid by reducing mitochondrial ubiquinone [38]. Therefore, the lag phase seen before the onset of Ca^{2+} release induced by dihydrolipoic acid may originate from its oxidation to lipoic acid, which in turn stimulates the specific Ca^{2+} release. Along this line, the stimulation of the mitochondrial Ca^{2+} release and subsequent Ca^{2+} cycling may be the cause of the detrimental effects of higher concentrations of dihydrolipoic acid in a model of heart ischemia/reperfusion injury [38]. Low levels of lipoic acid have predominantly antioxidative properties [14, 26]. Because mitochondrial Ca^{2+} release is stimulated by various prooxidants (reviewed in [8], it can be speculated that lipoic acid also indirectly modifies intracellular Ca^{2+} homeostasis by altering the intracellular redox status and/or by changing the concentration of different intracellular antioxidants [14, 26, 30].

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