BioFactors 17 (2003) 297–306
IOS Press

Redox regulation of mitochondrial permeability transition: Effects of uncoupler, lipoic acid and its positively charged analog LA-plus and selenium[1]

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1. Introduction

1.1. Mitochondria and ROS

Oxidative phosphorylation is arguably the most important energy transudation process in eukaryotes. In eukaryotes, oxidative phosphorylation occurs in the mitochondria. The chemiosmotic theory of mitochondrial energy production suggests the formation of a proton-motive force that represents the capacity to generate energy-rich ATP molecules [36,37]. Over 90% of the oxygen consumption in vivo has been attributed to mitochondrial respiration [3].

Mitochondria are considered the main source of ROS in eukaryotic cells [6,7,9,10]. The realization that energy consumption by mitochondria can generate oxygen radicals has linked the free radical theory of aging to mitochondrial functionality [21,35]. Mitochondria from post-mitotic cells use O_2 at a high rate and may release oxygen radicals that overwhelm cellular antioxidant defenses [52]. Indeed, mitochondria are the major source of superoxide anion production in cells. During the transfer of electrons to molecular oxygen, an estimated 1 to 5% of electrons in the respiratory chain "leak" to form superoxide radicals [6, 10,52].

[1]This article also appeared in *Thiol Metabolism and Redox Regulation of Cellular Functions,* A. Pompella, G. Bánhegyi and M. Wellman-Rousseau, eds, IOS Press, Amsterdam.
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1.2. Cell death

The last decade has witnessed a major improvement of our understanding of the fundamental mechanisms that lead to cell death [1,17,38,42,59,61]. Efforts have been made to classify cellular death into two major categories: necrosis and apoptosis or programmed cell death [20,41,51]. It should be noted, however, that death processes sharing the characteristics of apoptosis and necrosis has been evident as well [62]. Somatic cells are capable of self-destruction by activating an intrinsic death program, which is usually turned on when cells are no longer needed or have become seriously damaged. This biological death cascade, which has been termed apoptosis, is often associated with specific morphological and biochemical characteristics [59]. Recently it has been suggested that mitochondria is involved in the redox regulation of cell death and aging processes [67].

1.3. Mitochondrial control of cell death

One of the hallmarks of apoptosis is the early and temporal dissipation of the mitochondrial membrane potential [30,31,47,48]. Several mechanisms are known by which mitochondria play a role in cell death, and their effects may be inter-related [5,18,25,27,28]. These mechanisms are: i) disruption of electron transport, oxidative phosphorylation, and ATP production. The cellular level of ATP has been shown to determine the fate of the apoptotic process [50]; ii) release of proteins that trigger activation of caspase-family proteases (cytochrome c, AIF) which in turn are responsible for the execution of the apoptotic process. Mitochondria are known to serve as a pool for factors that can initiate and exacerbate the apoptosis; and iii) alteration of cellular reduction-oxidation (redox) potential because of excess production of ROS [32,33,39,60–62].

MPT is triggered by an increase in the inner mitochondrial membrane's permeability most easily observed after matrix Ca^{2+} accumulation [19]. Although MPT can be favored by a large series of heterogeneous compounds and conditions, it is generally agreed that it is mediated by the opening of a cyclosporin A (CsA)-sensitive complex channel [55,68]. Evidence that pore opening is voltage-controlled in intact isolated mitochondria is largely based on the effects of the protonophoric uncoupler carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone (FCCP), whereas other uncouplers have not been reported to induce MPT [4].

ROS (e.g., t-butyl hydroperoxide, peroxynitrite) have been suggested to facilitate the process of MPT pore opening [13,14,28,66]. The mechanism of action has been suggested to involve cross-linking of critical thiols in the MPT pore region. In addition, elevation in the production of endogenous mitochondrial ROS was demonstrated in Pi-induced MPT [26]. However, it is still not clear whether ROS are the effectors of MPT or are simply the released product of several PT inducers. In a recent publication, induction of MPT was shown not to be facilitated by ROS as demonstrated by several antioxidants, which were unable to prevent MPT [49].

2. Mitochondrial depolarization and oxidative stress

Exposure of rat liver mitochondria loaded with calcium to 250 μM of t-butyl hydroperoxide promoted extensive swelling (Fig. 1). FCCP added to the rat liver mitochondria after exposure to the oxidant prevented mitochondrial swelling in a dose-dependent fashion. Depolarization of the mitochondrial membrane prevented oxidant-induced swelling by closing the MPT-pore as was evaluated by PEG500 contraction assay. FCCP afforded protection when added after exposure to the oxidant. Therefore,

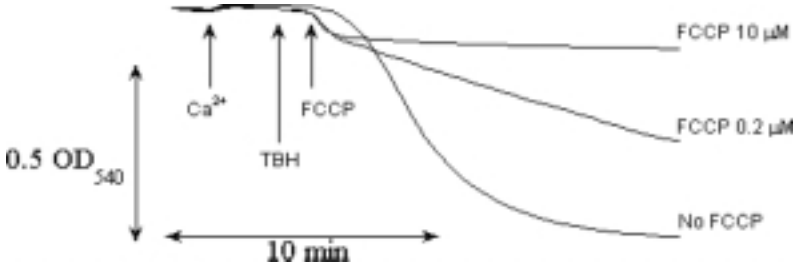


Fig. 1. Protective effect of FCCP against oxidant (t-butyl hydroperoxide; TBH) challenge of rat liver mitochondria. Swelling effect: FCCP was added after exposure to 250 μM TBH.

attenuated membrane potential under oxidative stress may permit the mitochondria to regulate swelling by releasing calcium. Introducing a small amount of FCCP together with an oxidant exacerbated calcium release. This observation implies that mitochondria exposed to oxidative stress are more receptive to low membrane potential-dependent calcium release. It is therefore plausible that the level of mitochondrial polarization governs the thiol oxidation status of the MPT pore. In this way, the state of mitochondrial polarization may regulate MPT pore opening.

3. Regulation of mitochondrial permeability transition

Exposing mitochondria to various thiol-containing compounds has been suggested to facilitate high amplitude swelling [54] of isolated mitochondria.On the other hand, thiol-antioxidants were reported to prevent Pi induced MPT pore opening and to protect against high amplitude swelling [27,29]. It is therefore important to investigate the specific effect of each thiol compound and to elucidate whether or not it is capable of affecting mitochondrial function. R-α-Lipoic acid (R-LA) is a naturally occurring compound present as a co-factor in a number of mitochondrial enzymes that are involved in metabolism and energy production.

α-Lipoic acid (LA) was first isolated in 1951 by Reed and colleagues. LA is an eight-carbon compound containing two sulfur atoms in a dithiolane ring structure. The two enantiomers of LA are the S form and the R form (Fig. 2). In its free form, LA is considered to be a powerful antioxidant, functioning as a reactive oxygen species scavenger [43–46].

Previously it has been demonstrated that active (reduced) α-dihydrolipoic acid (DHLA) can potentiate apoptosis in Jurkat T-cells treated with anti CD95 antibodies [56]. The intracellular events, which were potentiated by LA in these cells undergoing apoptosis, were increased cytosolic calcium and loss of mitochondrial membrane potential. We developed a water soluble positively charged (at pH 7.4) analogue of lipoamide, LA-plus (Fig. 3) [57]. This two sulfur-bearing antioxidant has been investigated in the context of affecting MPT.

3.1. Uptake of LA-plus and LA into mitochondria

Exposure of isolated liver or brain mitochondria to 150 μM LA-plus or LA resulted in more of the total amount (sum of oxidized and reduced forms) of LA-plus relative to LA being taken up. More of the reduced form of LA-plus (DHLA-plus) than DHLA was found in liver as well as brain mitochondria indicating that reduction of LA-plus is far more efficient than LA (Fig. 4). This is consistent with the enzyme kinetics that we [61] and others [24] have reported indicating that the LA-plus analogue is

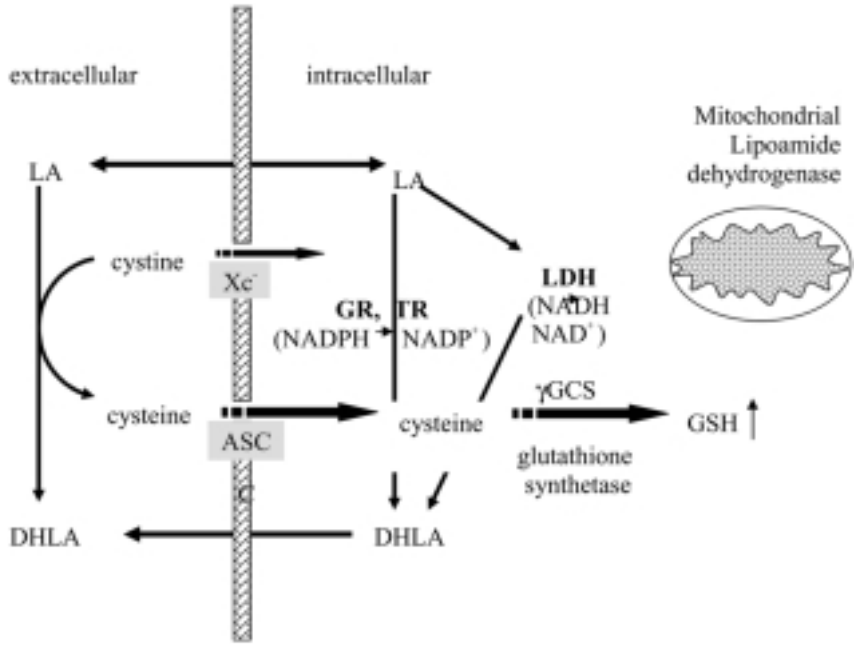


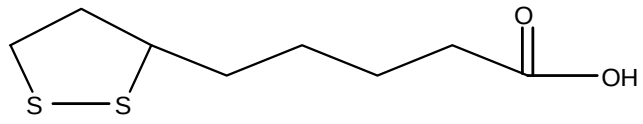
Fig. 2. Cellular pathways for the conversion of alpha-lipoic acid (LA) to dihydrolipoic acid (DHLA) and lipoate mediated upregulation of cellular glutathione (GSH) biosynthesis via increase in cysteine bioavailability. TR, thioredoxin reductase; GR, glutathione reductase; γGCS, gamma glutamyl cysteine synthetase; LDH, lipoamide dehydrogenase.

reduced by dihydrolipoamide dehydrogenase far better than LA. Low amounts of DHLA-plus have been detected in the mitochondrial suspension medium. This suggests that some DHLA-plus is release from the mitochondria. It was clear that LA-plus is more efficiently taken up and reduced in liver mitochondria than LA. LA-plus therefore is better suited than LA to serve as a reductant in mitochondria.

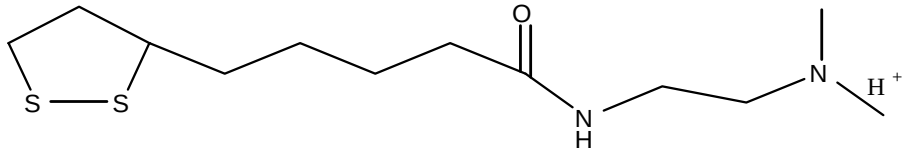
3.2. Mitochondrial membrane potential, swelling and calcium cycling

Mitochondria have been used in flow cytometry and their size has been determined. By diluting the mitochondria 500 times we find that preloaded rhodamine 123 leaves the mitochondria upon loss of membrane potential thus allowing the analysis of mitochondrial membrane potential. Using this approach it was strikingly found that free LA at 150 μM (not the protein bound LA) alone induced a loss of mitochondrial membrane potential, whereas LA-plus at 150 μM actually protected and maintained mitochondrial membrane potential over time (Fig. 5).

The balance of reduced/oxidized forms of the compounds indicate that a high ratio of disulfide (S-S) to dithiols (-SH) can trigger mitochondrial permeability transition while changing the ratio to low S-S and high SH can actually stabilize the mitochondria against the induction of MPT. The fact that LA or LA-plus are exogenous compounds and not part of the total thiol pool of the mitochondria is important. Control of MPT induction by exogenous thiol status can be explained by a quick equilibrium between the thiols that control the formation of the MPT pore and the thiols that are being delivered to the mitochondria. In spectrophotometer monitored swelling of isolated liver and brain mitochondria it was observed that LA alone at 150 μM or in the presence or absence of oxidants, induces swelling of mitochondria, whereas LA-plus at 150 μM maintains the integrity of mitochondria whether or not exposed to oxidants. Brain mitochondria were found to be extremely sensitive to LA (150 μM) induced



Alpha-Lipoic acid



LA-Plus; 5-[1,2] Dithiolan-3-yl-pentanoic acid (2-dimethylamino-ethyl)-amide

Fig. 3. Chemical structures of lipoic acid and LA-plus.

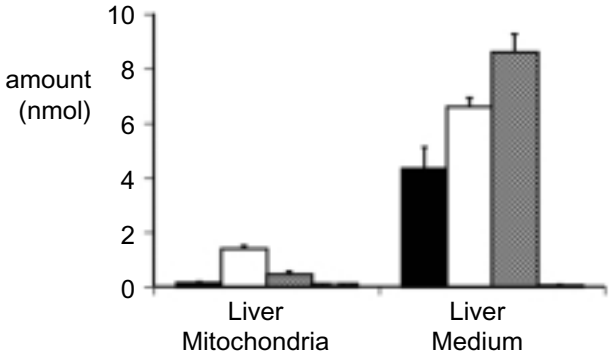


Fig. 4. LA or LA-plus uptake and reduction by rat mitochondria and corresponding extra-mitochondrial content. Mitochondria (0.5 mg) were treated with 150 μM of the compounds for 10 min. Mitochondria were separated from the medium and LA or LA-plus and their corresponding reduced forms were analyzed as described in Tirosh et al. [61]: LA-plus, closed bars; DHLA-plus, open bars; LA, gray cross hatched bars; DHLA, vertical line bars.

swelling but not to TBH treatment. Therefore, the effect of LA was most probably not facilitated by an oxidation-related mechanism, but due to a specific disulfide interaction. Both stereoisomers of LA induced swelling in rat liver mitochondria suggesting that the disulfide bond was the mediator of the MTP promoting effect.

Previously, two independent groups [23,54] have shown Ca^{2+} release induced by LA in isolated liver mitochondria. Our own observations confirm those findings. However, LA-plus does not induce Ca^{2+} release, at concentrations below 220 μM. At 75 to 150 μM, LA-plus did not trigger Ca^{2+} release. Under conditions of oxidant stress induced by TBH, mitochondria released Ca^{2+} in the presence of LA (normal biphasic release) faster than with TBH alone. However, in the presence of LA-plus there is a very long lag phase in the TBH induced Ca^{2+} release. Furthermore when the Ca^{2+} release is initiated it is only a slow linear release. This can be interpreted as an inhibitory affect of LA-plus against TBH induced Ca^{2+}

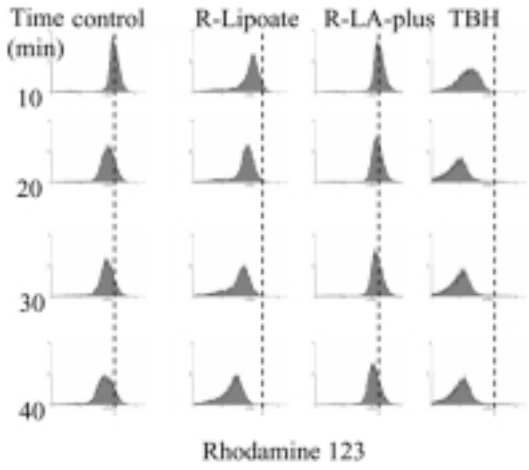


Fig. 5. Changes in mitochondrial membrane potential of succinate energized rat liver mitochondria. Isolated rat liver mitochondria (2 mg/ml) in MSH were treated with rotenone 2 μM, calcium 60 nmol/mg protein, succinate 5 mM and LA, LA-plus (150 μM) or TBH (250 μM). Rh123, 2 μg/ml was added. At the different time intervals indicated, samples were diluted 500 times (2 μl in 1 ml MSH buffer) and analyzed by flow cytometry immediately following dilution. Histograms represent 10,000 events. Histograms are presented as Rh123 fluorescence in log scale (x-axis) Vs' events (Y-axis). Shifts of points in the histogram to the left indicate loss of Rh123 fluorescence and therefore a decline of mitochondrial membrane potential.

release. This is contrary to the effect of LA at 150 μM, which destabilizes mitochondria and potentates the release of calcium. In view of the swelling and membrane potential results its likely that high levels of oxidized form LA is inducing extensive Ca^{2+} cycling and this is eventually causing loss of membrane potential and swelling.

It is clear from the uptake experiments that more LA-plus is taken up and reduced by the mitochondria than LA. The enzyme kinetic experiment supports this observation [61]. Thus it is highly likely that the reduced form of LA-plus could be keeping the vicinal thiol reduced, thus inhibiting Ca^{2+} release. On the other hand when the oxidized form is present in higher amounts (as in the case of LA) there is Ca^{2+} release due to oxidation of the vicinal thiol. Thus, it may be predicted that LA would facilitate cell death and this is exactly what has been observed in Jurkat cells [56]. Such toxic effect of LA is possibly due in part to an interaction and destabilization of cell mitochondria, which may explain the synergistic potentiation in killing tumor cells exposed to doxorubicine [15].

4. Selenium-thiol interaction and mitochondrial permeability transition

Sodium selenite is a common dietary form of selenium, recognized as essential in animal and human nutrition [11,12]. In the amino acid bound form, selenocysteine, it is a component of a number of antioxidant enzymes, e.g. the enzyme glutathione peroxidase and thioredoxin reductase [2,8]. Selenium supplementation induces immune boosting, chemo-protective as well as anticancer activities. Such activities have been associated with selenium intake that corrects for nutritionally deficient status in animals. A higher intake of selenium in mice, 2 ppm, prevented mammary tumorigenesis more effectively, and independently of glutathione peroxidase levels of expression [34]. Therefore, it seems that selenium intake at concentration higher than those associated with maximal expression of the selenocysteine-containing enzymes is beneficial [11,12].

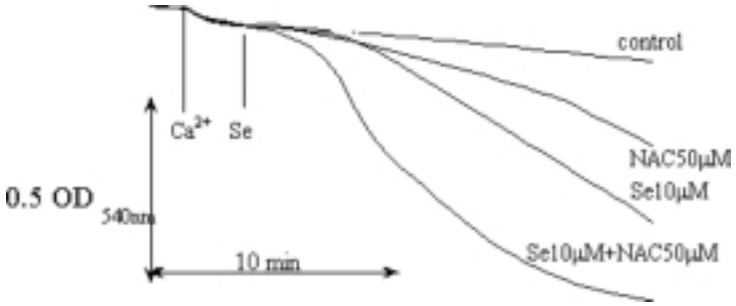


Fig. 6. Effect of selenium and thiols on MPT pore opening. Swelling monitored at OD_{540} in succinate-energized mitochondria in the presence of rotenone: 10 μM sodium selenite and 50 μM NAC were used to induce swelling. Adding thiols to selenium caused intensified high-amplitude swelling.

Little information is available on the biological activity of selenium or on its function in its enzyme-free form; most experiments on the topic have involved its activity while incorporated into selenoproteins [2, 38,53]. Induction of apoptosis of cancer cells is the preferred way of eliminating them. In addition, an efficient and functional apoptotic process in normal cells prevents malignant transformation and helps multicellular organisms with developmental processes. There is a known connection between selenium and apoptosis [16,22,58,64,65]. Selenium may facilitate the reactions of cysteine residues by transient formation of more reactive S-Se intermediates leading to cell death [16]. In recent years, efforts have been made to explain the pro-apoptotic effect of selenium. It was shown in a human hepatic cell line and human hepatoma cell line that SeO_2 prompts apoptosis in correlation with down-regulation of Bcl-2 and up-regulation of p53 levels [22,64]. However, selenium can trigger apoptosis independent of DNA damage in cells having a null p53 phenotype [16]. It has been suggested that the cell-cycle protein kinase cdk2 and protein kinase C are strongly inhibited by various forms of selenium due to the formation of selenium adducts of the selenotrisulfide (S-Se-S) or selenenylsulfide (S-Se) type, or catalysis of disulfide formation [16]. Ebselen, a selenium-containing compound, has been recently found to induce apoptosis via induction of mitochondrial permeability transition (MPT) [67].

Interaction with thiols is a major aspect of selenium biochemistry. The reaction of selenite with the reduced form of glutathione (GSH) leads to the formation of ROS [58]. Such oxidizing activity may regulate the opening of the MPT pore. However, monitoring of ROS production levels in the mitochondrial matrix showed no elevation in mitochondria-derived ROS. Moreover, thiol antioxidants not only failed to prevent the effect of selenium, thiols potentiated Se-induced opening of the MPT pore (Fig. 6).

Therefore, it is possible that the reducing power of mitochondrial thiols facilitated rapid sodium selenite reduction leading to selenium-dependent MPT pore opening, swelling and cytochrome c release. It is therefore suggested that selenium in the form of sodium selenite interacts with thiols. Such interaction facilitates MPT pore opening and supports apoptosis. Thus, interaction between two antioxidants, thiols and selenium, may lead to a ROS-independent pro-apoptotic effect.

In conclusion, works reviewed in this chapter suggest that low mitochondrial membrane potential may serve as a protective barrier against ROS-induced mitochondrial degeneration and that thiol antioxidants such as lipoic acid may support the apoptotic processes via its effects on the mitochondria. Oxidized (disulfide) thiols such as lipoic acid directly opens the MPT pore while reduced thiols need an electron transfer intermediate molecule such as selenium to facilitate MPT pore opening. On the other hand excessive reduced thiol (SH) accumulation in the mitochondrial matrix such as in the case of LA-

plus protect and prevent MPT. LA-plus has outstanding therapeutic potential in disorders related to mitochondriopathy.

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