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Reactive oxygen species are involved in the stimulation of the mitochondrial permeability transition by dihydrolipoate

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Abstract

Dihydrolipoic acid (DHLA) has been found to stimulate the Ca^{2+}-induced mitochondrial permeability transition (MPT) in rat liver mitochondria (RLM) [Biochem. Mol. Biol. Int. 44 (1998) 127] which could be due to its prooxidant properties. We therefore investigated whether DHLA stimulated superoxide anion ($O_2^{\bullet -}$) generation in RLM and in bovine heart submitochondrial particles (SMP). In RLM DHLA caused a concentration-dependent $O_2^{\bullet -}$ generation assayed by lucigenin chemiluminiscence. The stimulation was seen with the lowest concentrations of DHLA (5 μM) with pyruvate as the respiratory substrate, with 2-oxoglutarate or especially succinate the stimulation was less pronounced. Stimulation of $O_2^{\bullet -}$ production by DHLA was also observed in bovine heart SMP using an electron spin-trapping technique. Radical scavengers (butylhydroxytoluene and TEMPO) decreased $O_2^{\bullet -}$ generation induced by DHLA and inhibited MPT. Slight reduction of the mitochondrial membrane potential by a small amount of a protonophorous uncoupling agent also delayed the DHLA-induced MPT. These data indicate that the stimulation of MPT by DHLA is due to DHLA-derived prooxidants, i.e. stimulated production of $O_2^{\bullet -}$ and possibly other free radicals.

Keywords: Dihydrolipoic acid; Mitochondria; Permeability transition; Pyridine nucleotides; Superoxide anion; Ubisemiquinone

1. Introduction

α-Lipoic acid (LA) and its reduced form DHLA are effective antioxidants that are currently used therapeutically in diabetes, in heart and neural pathologies, and in various infections [1]. LA and DHLA prevent lipid peroxidation [2] and increase the pool of GSH both in the cytosol and in the mitochondrial matrix [3]. By promoting the reduction of oxidized antioxidants they drive the antioxidant network [1].

The MPT plays a central role in several pathological processes and cell death [4,5]. MPT is due to the opening of a large pore that allows components with a molecular mass up to 1.5 kDa to diffuse across the inner membrane and causes uncoupling, swelling, rupture of the outer membrane and release of proapoptotic factors [6,7]. Pore opening is influenced by many factors including prooxidants that stimulate MPT by oxidizing pyridine nucleotides (PN) and thiols in the pore [8], while substances that keep the SH groups reduced are known to inhibit MPT [8,9]. LA as a disulfide could thus be expected to promote MPT while DHLA as a dithiol to suppress it. However, both LA and DHLA were found to induce MPT in RLM, DHLA more potently [10]. We then found that addition of low concentrations of DHLA caused oxidation of PN [11,12], which suggests increased production of reactive oxygen species (ROS). Generation of ROS by xanthine plus

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Abbreviations: $\Delta\psi$, mitochondrial inner membrane transmembrane potential; BHT, 2,6-di-*tert*-butyl-4-methylphenol; DBPMPO, 5-(di-*n*-butoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DHLA, dihydrolipoate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; LA, lipoate; LDCL, lucigenin-derived chemiluminescence; MPT, mitochondrial permeability transition; PN, pyridine nucleotides; RLM, rat liver mitochondria; ROS, reactive oxygen species; SMP, submitochondrial particles; SOD, superoxide dismutase; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

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xanthine oxidase (EC 1.1.3.22), that form the superoxide anion ($O_2^{\bullet -}$), and glucose plus glucose oxidase (EC 1.1.3.4), that generate hydrogen peroxide, also stimulated MPT similar to DHLA [13]. The induction of MPT by DHLA could thus be due to ROS production and exhaustion of the antioxidant capacity of mitochondria. In the present study we have assayed the production of $O_2^{\bullet -}$ in RLM and bovine heart (SMP) and found it to be stimulated by DHLA. Both MPT and $O_2^{\bullet -}$ production were prevented by radical scavengers. These findings support the conclusion that the stimulation of MPT by DHLA is due to increased production of ROS.

2. Materials and methods

2.1. Preparation of rat liver mitochondria and bovine submitochondrial particles

RLM were prepared by differential centrifugation after homogenization in a mannitol–sucrose medium containing EGTA and bovine serum albumin as described previously [13]. Mitochondria were washed two to three times using a medium without EGTA and albumin. Finally they were suspended in a medium containing 220 mM mannitol, 70 mM sucrose, 10 mM Hepes/Tris, pH 7.4.

SMP were prepared from bovine heart mitochondria as described in [14,15]. Briefly, 6 mL of the mitochondrial suspension (10 mg protein/mL) in the medium containing 2 mM EDTA, pH 9.0, was sonicated at 40 W with a Branson Sonifier. Sonication for 1 min was carried out in bursts of 3 s, with cooling to 0° in the intervals. Mitochondria were removed by centrifugation at 7500 *g* for 10 min and SMP separated from the supernatant fraction by centrifugation at 144,000 *g* for 30 min. Mitochondrial protein was assayed by the Biuret method using bovine serum albumin as standard.

2.2. Measurements of MPT

Onset of MPT was recorded by following mitochondrial swelling or the redox state of PN as a parameters indicating inner membrane permeabilisation [8,16]. Swelling was recorded as decrease in absorbance at 540 nm on an Ultrospec 3000 pro spectrophotometer. Fluorescence changes due to redox changes of PN were followed on a Hitachi F4000 spectrofluorometer, using the excitation wavelength 340 nm and the emission wavelength 460 nm. In control experiments the MPT blocker cyclosporin A [9] was used to ascertain that the DHLA-induced changes were due to MPT.

2.3. Assay of $O_2^{\bullet -}$ production in RLM with lucigenin

Owing to its sensitivity, lucigenin-derived chemiluminescence (LDCL) has frequently been used for the detection of $O_2^{\bullet -}$ production by various enzymatic and cellular systems [17–19]. One benefit of using the lucigenin probe is its ability to detect $O_2^{\bullet -}$ generated in hydrophobic areas that are not accessible to superoxide dismutase (SOD) (EC 1.15.1.1).

Generation of LDCL was monitored with a Lucifer-10M luminometer at 37°. The reaction mixture contained 1 mg mitochondrial protein in 1 mL air-saturated medium in the presence of various substrates. The reaction was started by adding 25 μM of lucigenin, followed by 5–40 μM DHLA 1 min later. Chemiluminiscence was continuously monitored for 5–10 min. Control experiments were carried out in the absence of DHLA. The data are expressed as the relative increase in chemiluminiscence over the control (ΔLDCL, %).

2.4. EPR spectroscopy of $O_2^{\bullet -}$ production in SMP

Production of $O_2^{\bullet -}$ in SMP was measured as an EPR signal using 5-(di-*n*-butoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DBPMPO) or 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) as a spin trap [20,21]. EPR spectra of the spin trap radical adducts were recorded at room temperature with a Bruker ESP 300E in a flat quartz cell. SMP suspended in oxygenated medium were respiring on succinate in the presence or absence of DHLA. Spin trap was present in the medium at the concentration of 100 mM. The total incubation time was 23 min. SOD was added to the SMP suspension at the same time as DHLA. General EPR settings were as follows: microwave power, 50 mW; modulation frequency, 100 kHz; field, 3458 G; sweep, 120 G. The intensity of the EPR signal was calculated by summing up 15 scans and expressed in arbitrary units.

2.5. Measurement of ubisemiquinone EPR signal in SMP

EPR spectra were recorded by the same equipment (Bruker ESP 300E) at liquid nitrogen temperature. The SMP suspension was incubated with succinate and DHLA at room temperature. Afterwards the reaction mixture was placed in quartz tube and frozen in liquid nitrogen. EPR spectra were recorded under the following conditions: temperature, 200 K; microwave power, 20 mW; microwave frequency, 9.49 GHz; modulation frequency, 100 kHz; modulation amplitude, 4 G, scans, 3.

2.6. Incubation conditions

RLM were incubated at room temperature in a medium containing 100 mM KCl, 2 mM KH_2PO_4, 10 μM $CaCl_2$, 10 mM Hepes (pH 7.4) and 5 mM of the respiratory substrate. Other experimental details are described in the figure legends. SMP were incubated in 100 mM KCl, 20 mM Tris, using oxygen saturated medium. In order to prevent catalytic interference from iron during oxygen radical measurement with EPR, 1 mM diethyltriaminepen-

taacetic acid (DETAPAC) was used to chelate Fe in redox-inactive complexes.

2.7. Reagents

Reagents were of analytical grade, obtained from Sigma Aldrich. Racemic DHLA was also obtained from Calbiochem. CsA was a kind gift of Novartis.

2.8. Statistical methods

The Student's *t*-test was used for evaluating the statistical significance of differences in means. In Fig. 2, the non-parametric treatment of Wilcoxson [22] was used to ascertain the trend of rise in signal with [DHLA].

3. Results

3.1. Effect of DHLA on $O_2^{\bullet -}$ production

Since DHLA-stimulated ROS formation was implied as a factor in the promotion of MPT by DHLA, we investigated its effect on $O_2^{\bullet -}$ production in RLM. Fig. 1A shows the experimental protocol for measuring $O_2^{\bullet -}$ production in RLM with lucigenin. RLM were suspended in the standard incubation medium in the presence of respiratory substrate. After 1 min preincubation with lucigenin, 30 μM DHLA was added (trace 1). The signal was higher than in the control without DHLA (trace 2), indicating that DHLA stimulated the $O_2^{\bullet -}$ production. There was no increase in the signal on addition of DHLA in the absence of RLM (trace 3), showing that mitochondrial respiration chain is generating $O_2^{\bullet -}$. The difference (expressed in percent) in the integrated signal with and without DHLA is summarised in Fig. 1B. The relative increase in lucigenin-mediated chemiluminescence depended on the concentration of DHLA and on the respiratory substrate used. This increase in the signal in the presence of pyruvate was higher than in the presence of 2-oxoglutarate. With succinate plus rotenone the signal increase was quite small at corresponding DHLA concentrations. In the presence of pyruvate, as low as 5 μM DHLA had an effect and with 10 μM DHLA the signal was increased by 25% over that in the control, with only slightly more at higher DHLA concentrations. The same ΔLDCL value for mitochondria respiring on 2-oxoglutarate was obtained with three to four times higher concentrations of DHLA, in this case 5 μM DHLA had hardly any effect. In the presence of succinate plus rotenone the signal increase was even smaller. Chemiluminescence was increased only by 10–15% when 40 μM DHLA was used.

The stimulation of $O_2^{\bullet -}$ production by DHLA was studied in a simpler system SMP using EPR to record the generation of $O_2^{\bullet -}$. Succinate (20 mM) was used as the respiratory substrate to study $O_2^{\bullet -}$ production in the respiratory chain—the main source of ROS in mitochondria. Owing to the sensitive detection system and absence of antioxidants, some $O_2^{\bullet -}$ production was observed in SMP (0.5 mg/mL) respiring on 20 mM succinate. The addition of 100 μM DHLA increased the $O_2^{\bullet -}$ adduct-derived EPR signal by 47% (Fig. 2). However, if the value obtained in the presence of SOD is substracted, which is close to the reagent blank, the relative increase in signal was 115%. This relatively high concentration of DHLA was used, since an increased $O_2^{\bullet -}$ production in RLM respiring on succinate was observed only at higher DHLA concentrations. The EPR signal was reduced by addition of SOD to SMP respiring on succinate in the presence of DHLA to the blank value (data not shown). This indicates that the signal was due to $O_2^{\bullet -}$. Similar results were obtained using DEPMPO as the spin trap (data not shown). These findings are in line with the data on DHLA-stimulated generation of $O_2^{\bullet -}$ in RLM and support the inter-

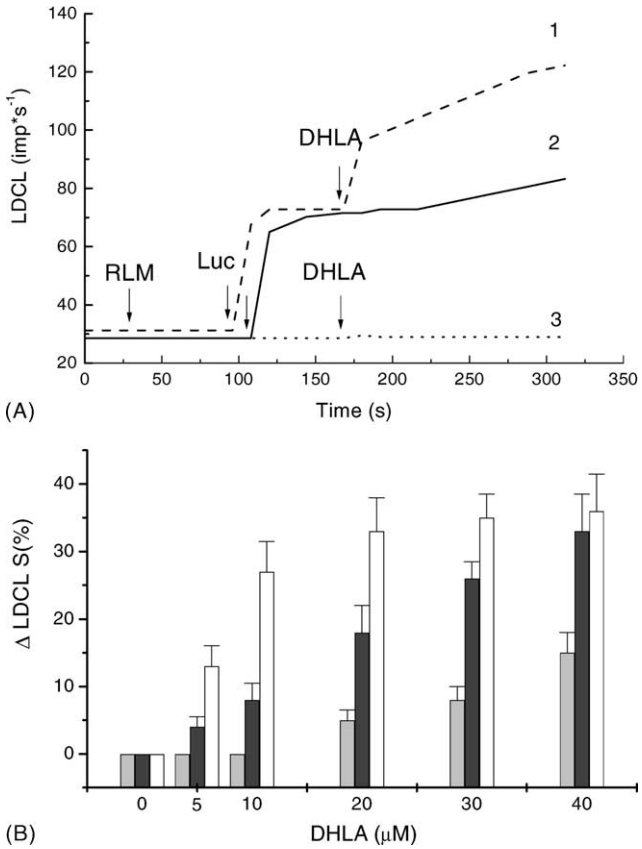

Fig. 1. The stimulation by DHLA of mitochondrial $O_2^{\bullet -}$ generation measured as lucigenin chemiluminescence. RLM (1 mg/mL) were incubated in medium as described in Section 2 in the presence of 5 mM substrate. (A) Original traces using pyruvate as substrate. Trace 1, addition of 30 μM of DHLA; trace 2, control; trace 3, control without RLM. The chemiluminiscence is given as photon impulses/s (IMP s^{-1}). (B) Concentration-dependence of DHLA-increased lucigenin chemiluminiscence RLM respiring on different substrates. Data are expressed as difference in percent from control (0 μM DHLA). Grey columns, succinate + rotenone; black, 2-oxoglutarate; white, pyruvate. The rise in signal was statistically significant ($P < 0.05$) using Wilcoxson's non-parametric method.

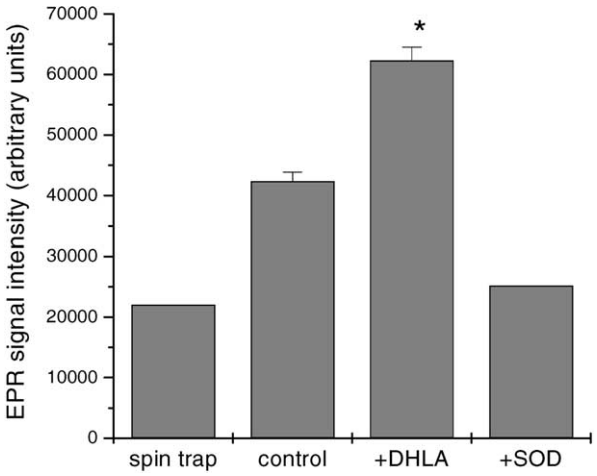

Fig. 2. Effect of DHLA on $O_2^{\bullet -}$ generation in SMP. SMP (0.5 mg/mL) were incubated in oxygenated buffer in the presence of 20 mM succinate and 100 mM DBPMPO as described in Section 2. Spin trap, DBPMPO reagent blank in medium in the absence of SMP; control, no addition of DHLA; +DHLA, 100 μM of DHLA added to the reaction mixture; +DHLA + SOD; SOD added to reaction mixture with 100 μM DHLA. The statistical significance of difference in means between control and +DHLA columns was calculated by *t*-test ($^*P < 0.01$).

pretation that the stimulated $O_2^{\bullet -}$ production occurs in the respiratory chain.

Auto-oxidation of ubisemiquinone is an important source of $O_2^{\bullet -}$ in mitochondria [23]. It is of interest that we found 100 μM DHLA to reduce the EPR signal of ubisemiquinone by 70% in SMP.

3.2. *Effect of radical scavengers on the potency of DHLA to induce MPT and stimulate generation of $O_2^{\bullet -}$*

Since we had found DHLA to increase the $O_2^{\bullet -}$ production, we tested the effect of radical scavengers on MPT and the lucigenin-mediated chemiluminiscence. Fig. 3 shows the effect of the radical scavengers 2,6-di-*tert*-butyl-4-methylphenol (BHT) and 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) on the DHLA-induced MPT with pyruvate as substrate. When 50 μM BHT (Fig. 3, trace 2) or 500 μM TEMPO (trace 3) were added to RLM prior to DHLA, the MPT-induced swelling was largely inhibited compared to DHLA alone (trace 1). Since $O_2^{\bullet -}$ may cause oxidation of reduced PN, we studied the effect of the radical scavengers TEMPO and BHT on the redox state of PN. The effect of varying the concentration of TEMPO and BHT is summarised in Table 1. The effect of the radical scavengers was calculated at the time of maximal oxidation of PN induced by DHLA. Relatively low concentrations of

Table 1
Effect of radical scavengers on redox state of PN

	Reduced PN (%)
BHT (μM)	
5	17.5 ± 2.4
10	33.4 ± 3.4
15	51.0 ± 3.2
20	68.3 ± 4.1
30	82.2 ± 2.9
50	88.0 ± 2.1
TEMPO (μM)	
100	41.0 ± 3.0
250	64.4 ± 2.2
500	79.4 ± 1.3

Mitochondria (1 mg/mL) were incubated in medium as described in Section 2. Oxidation of PN was induced by 20 μM DHLA and prevented by different concentrations of BHT and TEMPO. The PN fluorescence before addition of DHLA taken to be 100% reduced PN, and the fluorescence signal after completion of the DHLA-induced maximal oxidation to represent 0%. The redox state of PN after addition of BHT or TEMPO was measured at the time of maximal oxidation induced by DHLA.

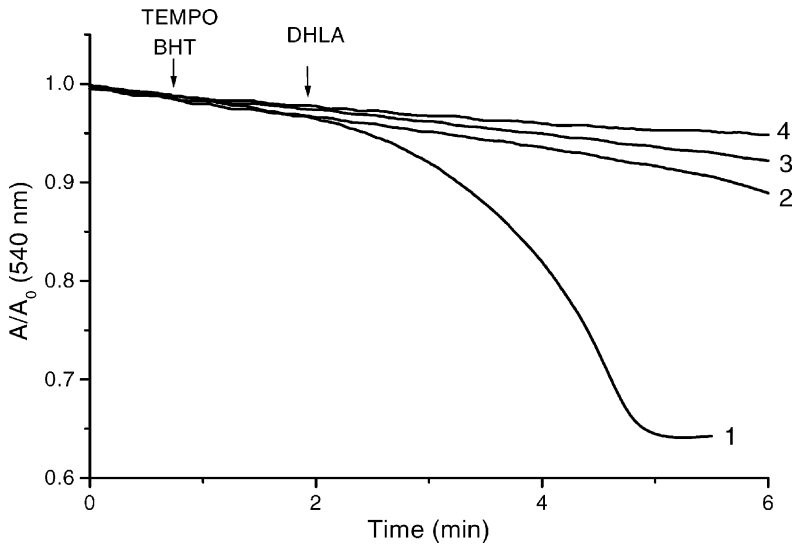

Fig. 3. The effect of radical scavengers on the potency of DHLA to induce swelling. Incubation conditions as described in Section 2 in the presence of 5 mM pyruvate. Trace 1, 20 μM of DHLA; trace 2, additions: 20 μM of DHLA and 50 μM BHT; trace 3, 500 μM TEMPO added instead of BHT; trace 4, control without addition of DHLA.

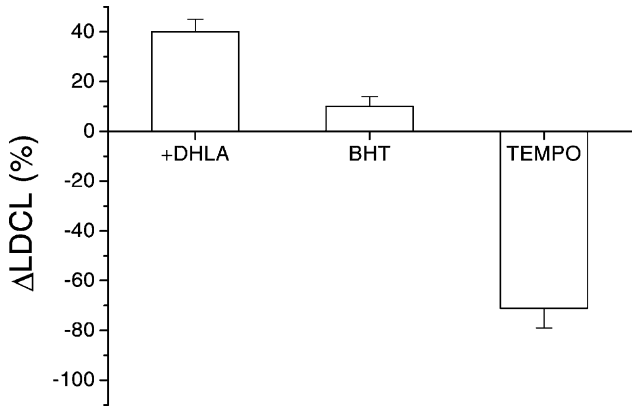

Fig. 4. Effects of BHT and TEMPO on the DHLA-induced $O_2^{\bullet-}$ generation in RLM. Experimental conditions as in Fig. 1. RLM were respiring after 2 min preincubation time 40 μM DHLA was added in column +DHLA. In addition TEMPO 500 μM or BHT 50 μM were present (TEMPO or BHT columns, respectively). The chemiluminiscence signal was recorded for 5 min. The % indicates the difference to the control LDCL level in RLM without additions. The standard errors of the relative differences are shown in the columns. The differences were statistically significant, $P < 0.01$.

BHT, 10–30 μM, had an inhibitory effect on PN oxidation, while TEMPO was inhibitory in the range 100–500 μM.

Fig. 4 shows the effects of radical scavengers on the lucigenin-mediated chemiluminescence signal in mitochondria respiring on 5 mM pyruvate in the presence of 40 μM DHLA. The DHLA-induced increase in the signal was substantially reduced by 50 μM BHT (30%), and even more by 500 μM TEMPO (71%) (Fig. 4).

3.3. Effect of lowering $\Delta\psi$ on DHLA-induced MPT

The $O_2^{\bullet-}$ production is influenced by the mitochondrial inner membrane transmembrane potential ($\Delta\psi$) [24]. We therefore measured the effect of a small decrease in $\Delta\psi$ brought about by low concentrations of the protonophorous uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on the DHLA-promoted MPT. Onset of MPT was followed photometrically as mitochondrial swelling. Prior to addition of the uncoupler, RLM were allowed to respire on pyruvate for approximately 1 min and to accumulate Ca^{2+} from the medium. DHLA was added 1 min after addition of the uncoupler (Fig. 5). Mild uncoupling induced by addition of small amounts of FCCP, up to 25 nM, prolonged the lag time before the onset of the MPT induced by DHLA.

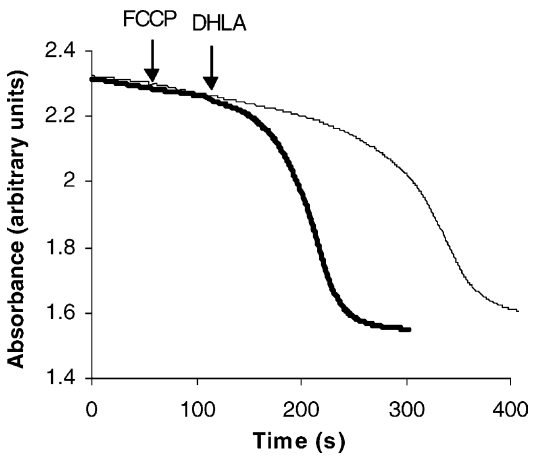

Fig. 5. The effect of lowering $\Delta\psi$ on DHLA-induced MPT. Experimental conditions were as described in Section 2. Additions as indicated by arrows: 20 μM DHLA (thick line) was added after 2 min incubation in both traces, 20 nM FCCP 1 min before DHLA (thin line).

4. Discussion

The main finding in the present study is stimulation of $O_2^{\bullet-}$ generation by DHLA in RLM and SMP (Figs. 1 and 2). The increase in $O_2^{\bullet-}$ generation was greatest with pyruvate as respiratory substrate compared with 2-oxoglutarate and succinate. This corresponds to the potency of DHLA to promote MPT in mitochondria respiring on various substrates [11] and suggests that DHLA induces MPT due to increased ROS formation. This finding contrasts to the strong antioxidant capacity of LA/DHLA in cells and tissues [1–3]. In isolated mitochondria and SMP the ROS formation may be more readily observed, since less antioxidant systems are present with which the LA/DHLA could interact. For a recent review of anti- and prooxidant effects of LA and DHLA, see [25].

There are several methods for the detection of ROS, but the use of many of these is limited by the direct interaction of DHLA with the detection system used: cytochrome *c*, epinephrine or horseradish peroxidase (EC 1.11.1.7). We chose the lucigenin and spin trap methods for measuring $O_2^{\bullet-}$ because DHLA did not give any unspecific signal with lucigenin and the spin traps in the absence of RLM or SMP (Fig. 1A, trace 3). In this study we used a low concentration of lucigenin (25 μM), that should not cause any extra production of $O_2^{\bullet-}$ in mitochondria [18]. SMP were used to test if DHLA stimulates generation of $O_2^{\bullet-}$ in the mitochondrial electron transport chain.

The involvement of free radicals in the mechanism for MPT stimulation is also supported by the inhibition of the DHLA-induced MPT and PN oxidation by the radical scavengers BHT and TEMPO (Fig. 3, Table 1) as both compounds suppressed the stimulation of MPT by DHLA and the DHLA-induced increase in the lucigenin-mediated chemiluminiscence (Fig. 4). The inhibitory effect of BHT on the DHLA-induced MPT was reported before [10]. These observations indicate that the DHLA-promoted induction of MPT involves formation of $O_2^{\bullet-}$ and probably also of lipid radicals, as BHT is a lipid radical scavenger [26,27]. Additional experiments were performed in order to elucidate the mechanism of DHLA-stimulated $O_2^{\bullet-}$ generation.

We found that DHLA substantially (by 70%) reduced the level of ubisemiquinone in SMP. Thus, a one-electron

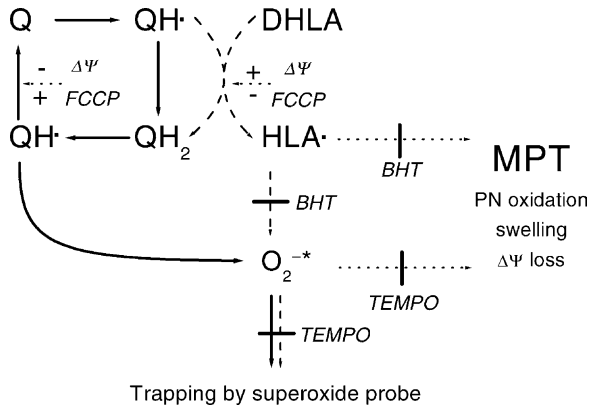

Fig. 6. Proposed scheme for DHLA-induced $O_2^{\bullet -}$ generation and MPT in RLM. HLA·, sulfur-centered radical (thiyl radical); continuous line arrows, processes that take place in RLM in the absence of DHLA; dashed line arrows, DHLA-dependent processes; dot-line arrows, regulation of the processes with '+' indicating stimulation and short transverse lines indicating inhibition by radical scavengers.

reaction in the reduction of ubisemiquinone to ubiquinol by DHLA should be involved. This implies an initial formation of DHLA or/and LA sulfur-centered radicals [28–30], which is responsible for secondary oxygen activation with formation of $O_2^{\bullet -}$ [31,32], and subsequently induction of MPT (see scheme in Fig. 6). These radicals are unstable and therefore difficult to trap. Formation of sulfur-centered radicals from DHLA and subsequent generation of $O_2^{\bullet -}$ has been reported [33].

Mild uncoupling of mitochondria with low concentrations of protonophorous uncoupling agents, which reduce the ubisemiquinone level [34], would thus be expected to reduce the DHLA-dependent $O_2^{\bullet -}$ production. The experiments in Fig. 5 indicate that in the presence of 20 nM of FCCP, the lag phase for MPT after DHLA addition is prolonged due to decreased ROS production. This is observed in the range of 10–25 nM FCCP (data not shown).

The scheme of the proposed mechanism for formation of sulfur-centered radicals, $O_2^{\bullet -}$, stimulation of MPT and the effects of BHT and TEMPO is shown in Fig. 6. Since TEMPO has been shown to be mainly a $O_2^{\bullet -}$ scavenger [18,35] and BHT scavenges lipid radicals effectively [26,27] and both compounds prevent MPT and stimulation of $O_2^{\bullet -}$ production, we propose that in addition to $O_2^{\bullet -}$, also sulfur-centered radicals from DHLA may be formed initially and be involved in MPT stimulation.

The common explanation for DHLA-induced ROS production is reduction of ferric iron followed by iron redox cycling and radical formation by the Fenton reaction [28,36]. In the cell, lysosomes may contain redox-active iron formed after autophagocytosis of iron-containing organelles, and desferrioxamine was found to inhibit lysosomal ROS formation [36]. However, we found no effect of desferrioxamine on the DHLA-promoted MPT (data not shown). Thus, the prooxidant effect of DHLA was not due to contaminating iron in the medium or to lysosomes present in the mitochondrial preparation. This finding does not rule out an iron-mediated ROS production in mitochondria, since desferrioxamine may not reach all redox-active iron sites in the inner membrane.

The proposed mechanism for DHLA-dependent $O_2^{\bullet -}$ generation does not explain why there is a difference in $O_2^{\bullet -}$ generation in mitochondria respiring on different substrates [11] (Fig. 1). It is of interest that pyruvate dehydrogenase is sensitive to inactivation by ROS [37], and both pyruvate and oxoglutarate dehydrogenase (EC 1.2.4.2) to lipid peroxidation products [38]. The mechanism involves reduction of enzyme-bound LA to DHLA, which could occur when DHLA is added. Studies are in progress to decide which mechanisms actually are involved.

Our data thus indicate that DHLA increased $O_2^{\bullet -}$ production in isolated RLM and SMP. The mechanism may be due to ubisemiquinone-dependent oxidation of DHLA to form thiyl radicals that may generate $O_2^{\bullet -}$ by autoxidation. The stimulation of MPT by DHLA is due to increased production of ROS.

Acknowledgments

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