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trans,trans-2,4-decadienal induces mitochondrial dysfunction and oxidative stress

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Abstract Lipid peroxidation produces a large number of reactive aldehydes as secondary products. We have previously shown that the reaction of cytochrome c with *trans,trans*-2, 4-decadienal (DDE), an aldehyde generated as a product of lipid peroxidation in cell membranes, results in the formation of adducts. Mass spectrometry analysis indicated that His-33, Lys-39, Lys-72 and Lys-100 in cytochrome c were modified by DDE. In the present work, we investigated the effect of DDE on isolated rat liver mitochondria. DDE (162 μM) treatment increases the rate of mitochondrial oxygen consumption. Extensive mitochondrial swelling upon treatment with DDE (900 nM–162 μM) was observed by light scattering and transmission electron microscopy experiments. DDE-induced loss of inner mitochondrial membrane potentials, monitored by safranin O fluorescence, was also observed. Furthermore, DDE-treated mitochondria showed an increase in lipid peroxidation, as monitored by MDA formation. These results suggest that reactive aldehydes promote mitochondrial dysfunction.

Keywords DDE · Mitochondria · Lipid peroxidation · Mitochondrial swelling

Introduction

Oxidative stress has been implicated in a variety of pathological conditions such as atherosclerosis, cancer, amiotrophic lateral sclerosis and post ischemic reoxygenation (Halliwell and Gutteridge 1999). Lipid peroxidation mediated by a free radical chain mechanism leads to the formation of a large number of reactive aldehydes as secondary products (West and Marnett 2006). Many of these aldehydes are cytotoxic and have been reported to form adducts with biomolecules including glutathione, amino acids, proteins and DNA (Esterbauer et al. 1991; Tsai et al. 1998; Hidalgo and Zamora 2000; Loureiro et al. 2000). Among the different aldehydes that can be formed during the lipid peroxidation process, the most extensively studied are malondialdehyde, acrolein, and 4-hydroxy-2-nonenal (HNE) (Marnett 2000; Uchida 2000; Uchida 2005). Several studies have demonstrated the reactivity of these aldehydes and others such as 2-octenal and 4-oxo-2-nonenal towards amino acids and proteins in vitro (Alaiz and Barragan 1995; Zamora et al. 1999; Liu et al. 2003; Liu and Sayre 2003). HNE has also been shown to modify amino acid residues in LDL (Chen et al. 1992) and a range of proteins including e-FABP (Bennaars-Eiden et al. 2002), apomyoglobin (Liu and Sayre 2003) and cytochrome c oxidase (Musatov et al. 2002). These modifications can lead to protein–protein cross-links and stimulate higher order protein aggregation, promoting loss of function and degradation (Zhang et al. 2004). Regulatory, inhibitory and gene expression effects have recently been ascribed to aldehydes. HNE can regulate glycogen synthase 3-kinase by regulation of P13K/AKT and ERK, two proteins that play a role in the MAPK signaling pathway in human neuroblastoma cells (Laurora et al. 2005). HNE can also inhibit mitochondrial aldehyde dehydrogenase (Doorn et al. 2006) and promote changes in the expression of a range of genes in RKO human colorectal carcinoma cells (West and Marnett 2005). HNE and other lipid peroxidation products have been suggested to promote alterations in mitochon-

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drial parameters such as coupling, respiration and organelle volume by yet undetermined mechanisms (Kristal et al. 1996; Jaburek et al. 2004; Echtay et al. 2005; Shabalina et al. 2006).

One of the most reactive aldehydes produced during the lipid oxidation process is *trans,trans*-2,4-decadienal (DDE). DDE is a widespread environmental α,β-unsaturated aldehyde found, for example, as a contaminant in water and food (Schieberle and Grosch 1991; Brewer and Vega 1995; Konopka et al. 1995; Milo and Grosch 1996; Farkas et al. 1997; Kerler and Grosch 1997; Cha and Cadwallader 1998). DDE is abundant in heated oils and has been associated with lung adenocarcinoma in women due to their exposure to oils during cooking (Chang et al. 2005). Cytotoxic and lethal effects of this aldehyde have been observed in different models such as human fibroblasts, endothelial and erythroleukemia cells. DDE inhibits cell growth, affects cell viability, changes cellular glutathione levels and is involved in DNA fragmentation (Kaneko et al. 1987; Kaneko et al. 1988; Nappez et al. 1996).

Our laboratory and co-workers have reported that DDE epoxides are able to bind to 2′-deoxyadenosine and 2′-deoxyguanosine in DNA, yielding adducts (Carvalho et al. 2000; Loureiro et al. 2000). Moreover, we described recently that DDE promotes modification of cytochrome c leading to the formation of adducts. Mass spectrometry analysis indicated that His-33, Lys-39, Lys-72 and Lys-100 were modified by DDE (Sigolo et al. 2007).

In order to uncover possible functional consequences of DDE in energy metabolism, we investigated alterations in mitochondrial parameters such as organelle volume, membrane potential, oxygen consumption and membrane integrity, using isolated rat liver mitochondria exposed to DDE as an experimental model. Changes in mitochondrial parameters could pinpoint mechanisms in which lipid peroxidation products perform their deleterious effects in vivo.

Materials and methods

Chemicals

trans,trans-2,4-Decadienal was purchased from Aldrich, Milwaukee, WI. Oligomycin, ATP, ADP, rotenone, succinate, dithiotereitol (DTT), ethylene glycol-bis (β-amino-ethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and cyclosporin A were purchased from Sigma Chemical Co., St. Louis, MO. All the other reagents used were of the highest quality commercially available.

Isolation of rat liver mitochondria

Mitochondria were isolated by differential centrifugation from the livers of Sprague-Dawley strain rats fasted overnight as previously described (Kowaltowski et al. 1995). The homogenate was prepared in 250 mM sucrose, 2 mM EGTA and 10 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethane-sulfonic acid (HEPES) buffer, pH 7.2 (KOH). The mitochondrial pellet was suspended in the same buffer at 30 mg protein/ml and kept over ice.

Oxygen consumption assay

Mitochondrial oxygen consumption was measured using a computer-interfaced Clark-type electrode from Hansatech Instruments with magnetic stirring. Oxygen solubility was taken to be 184 nmol/ml. Experiments were performed at 37°C in buffer containing 10 mM HEPES, 2 mM $MgCl_2$, 2 mM KH_2PO_4 and 100 mM KCl, pH 7.2 (KOH).

Mitochondrial membrane potentials

Mitochondrial membrane potentials (Δψ) were estimated by following the fluorescence of safranin O at 495 nm (excitation) and 586 nm (emission) on a Hitachi F4500 spectrofluorometer (Kowaltowski et al. 2002). Experiments were performed at 37°C in a buffer containing 10 mM HEPES, 2 mM $MgCl_2$, 2 mM KH_2PO_4 and 100 mM KCl, pH 7.2 (KOH).

Measurements of mitochondrial volume

Changes in mitochondrial volume were followed over time using a light-scattering technique carried out in a Carry 50 Bio UV-visible spectrophotometer (Varian Inc., Australia) equipped with magnetic stirring at 520 nm. Mitochondria (0.6 mg/ml) were suspended in a buffer containing 10 mM HEPES, 2 mM $MgCl_2$, 2 mM KH_2PO_4 and 100 mM KCl, pH 7.2 (KOH) at 37°C.

Transmission electron microscopy

Mitochondria incubated under the conditions used to study volume changes were centrifuged and the resulting pellet was fixed in 100 mM cacodylate buffer with 2% glutaraldehyde and then in 2% osmium tetroxide. After samples were dehydrated by ethanol, they were embedded in 100% Spurr resin. The resin was polymerized at 72°C and the ultra thin sections were cut and stained with uranyl acetate and lead nitrate. Samples were analyzed with a Jeol Jem-1010 transmission electron microscope at 80 kV.

Malondialdehyde determination

Mitochondria were incubated for 10 min under conditions used for volume measurements at 37°C in presence of DDE. After a 10 min centrifugation period at 2,000 rpm, 500 μl of a 0.4% (*w*/*v*) solution of TBA in HCl 0.2 N/H_2O (2:1) and 60 μl of 0.2% (*w*/*v*) solution of BHT in 95% ethanol were added to 500 μl of the supernatant obtained from the mitochondrial suspension. The mixture was heated to 90°C for 45 min, cooled on ice, and extracted with an equal volume of isobutanol. The isobutanolic phase was injected through a Shimadzu auto injector model SIL-10AD/VP (Shimadzu, Kyoto, Japan) in a Shimadzu HPLC system, consisting of two pumps LC-6AD connected to a Lichrosorb 10 RP-18 (Phenomenex, Torrance, CA) reversed-phase column. The flow rate was 0.8 ml/min (5–25% acetonitrile in H_2O deionized). The MDA-TBA adduct was detected with a RF-10A/XL fluorescence detector set at an excitation wavelength of 515 nm and an emission wavelength of 550 nm, and data were processed using Shimadzu Class-VP 5.03 software. Malonaldehyde-bisdiethylacetal was used as a standard.

Statistical analysis

Comparisons were conducted through two-way analysis of variance (ANOVA) using Prism software (GraphPad). Data represent means ± SEM or representative traces of at least three experimental repetetions conducted with different mitochondrial preparations.

Results

Rat liver mitochondria isolated and incubated under our conditions were fully coupled, as indicated by the difference in respiratory rates observed in the presence of ADP and after the addition of the ATP synthase inhibitor oligomycin (Fig. 1a). Interestingly, the addition of DDE increased oxygen consumption rates in the presence of oligomycin (Fig. 1a). A significant increase in oxygen consumption was observed with the addition of 162 μM DDE (Fig. 1b). At this DDE concentration, RCR values droped from 7.02± 0.48 to 3.64±0.25, (≈ 48% of control, $P< 0.0001$). DDE-promoted increases in respiratory rates were not attributable to O_2 consumption due to lipid oxidation reactions, since they were completely absent when mitochondrial respiration was inhibited by antimycin A (results not shown). Instead, increased respiratory rates promoted by DDE in the presence of oligomycin suggest this compound leads to uncoupling of mitochondrial respiration from ATP synthesis.

In order to confirm that DDE promoted uncoupling, inner mitochondrial membrane potentials were measured using the fluorescent probe safranin O (Fig. 2), which accumulates in the matrix, leading to lower fluorescence, in a manner proportional to inner membrane potentials. A representative trace obtained in these experiments is shown in Fig. 2a, and Fig. 2b shows the variation in fluorescence intensity obtained in a range of DDE concentrations. We found that 162 μM DDE promotes a significant decrease in inner membrane potentials. The proton ionophore CCCP was added at the end of all traces to promote maximal mitochondrial uncoupling.

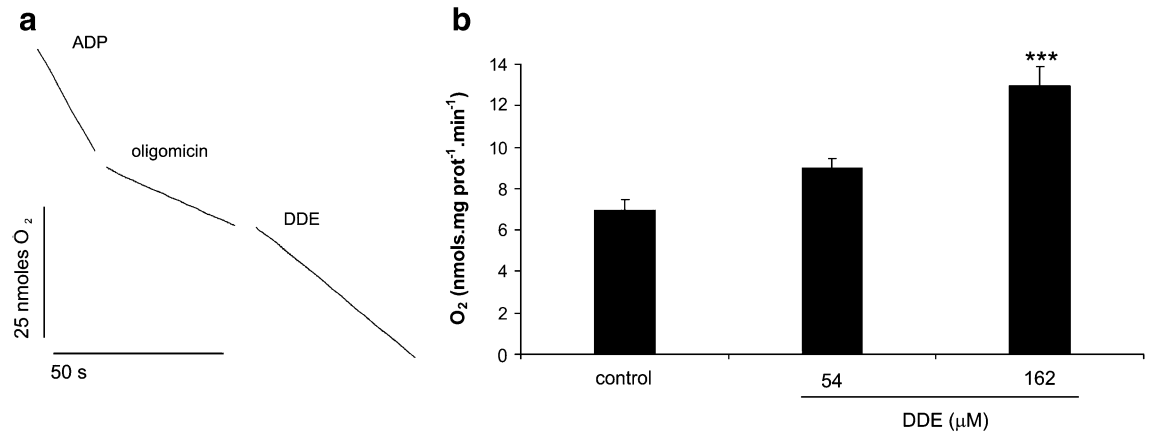

Fig. 1 DDE enhances oxygen consumption rates in isolated mitochondria. Mitochondria (3 mg/ml) incubated in the presence of oligomycin (1 μg/ml) were exposed to increasing DDE concentrations (1.8–180 μM) in experimental buffer containing 10 mM HEPES, 2 mM $MgCl_2$, 2 mM KH_2PO_4 and 100 mM KCl, 2 mM succinate, pH 7.2 (KOH) at 37°C. **a** Representative trace obtained using 162 μM DDE. **b** Oxygen consumption in state 4 mitochondria promoted by DDE. Control denotes state 4 mitochondria in the absence of DDE. ***$P< 0.001$ relative to control, $n=4$

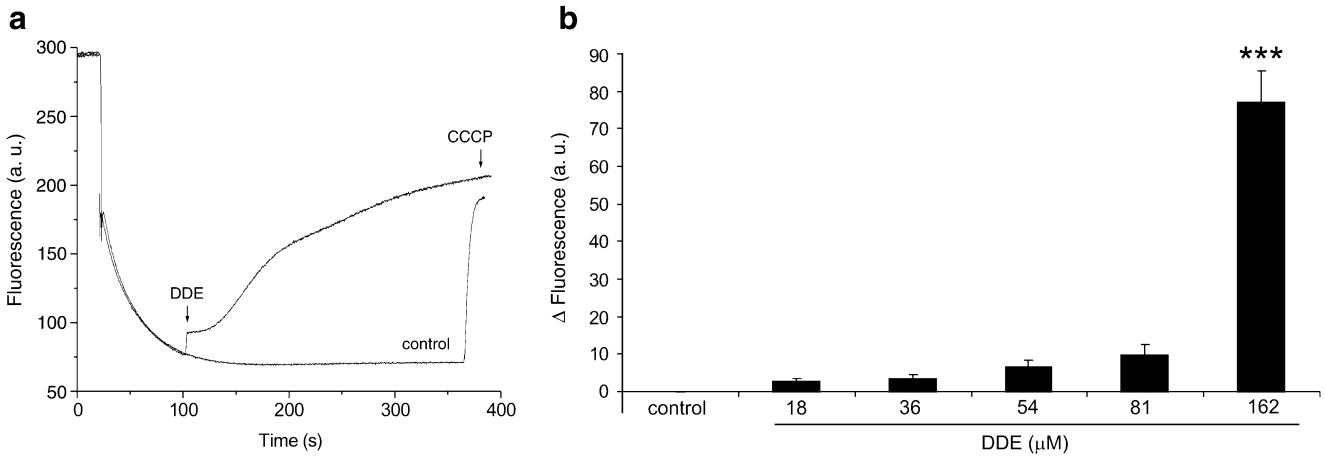

Fig. 2 DDE induces loss in mitochondrial inner membrane potentials. Experiments were performed in buffer containing 10 mM HEPES, 2 mM $MgCl_2$, 2 mM KH_2PO_4, 100 mM KCl, 2 mM succinate, 5 μM safranin O, 2 mM ATP and 1 μg/ml oligomycin, pH 7.2 (KOH) at 37°C. Mitochondria (≈0.2 mg protein/ml) were exposed to 18–162 μM DDE, as indicated. **a** Representative trace obtained using DDE 162 μM. **b** Dose-response graphic. ***$P < 0.001$ versus control

Decreases in mitochondrial inner membrane potentials may occur due to selective permeabilization of this membrane to protons or non-selective inner membrane permeabilization. In order to determine what kind of permeabilization occurred under our conditions, we conducted light scattering assays to measure mitochondrial swelling. Non-selective inner membrane permeabilization promotes the uptake of osmotic support and water into mitochondria, resulting in a decrease in light scattering. No such decrease is observed when only inner membrane permeability to protons is enhanced. We found that exposure to DDE resulted in considerable mitochondrial swelling, in a dose-dependent manner (Fig. 3), and in a concentration range similar to that which promotes increased respiratory rates and loss of inner membrane potentials. The large size of the inner membrane pore promoted by DDE was confirmed by the experiments in Fig. 4. We found that, although the amplitude was smaller, DDE caused mitochondrial swelling in both KCl and sucrose-based media, indicating that it promoted permeabilization to molecules as large as sucrose. The occurrence of large-scale mitochondrial swelling in the presence of DDE was further confirmed by transmission electron microscopy (Fig. 5). We found that DDE causes very significant and dose-dependent mitochondrial swelling, clearly perceptible even at concentrations as low as 900 nM.

Large-amplitude mitochondrial swelling is frequently observed as a result of the mitochondrial permeability transition, a Ca^{2+} dependent form of inner membrane permeabilization that occurs as a result of oxidative stress

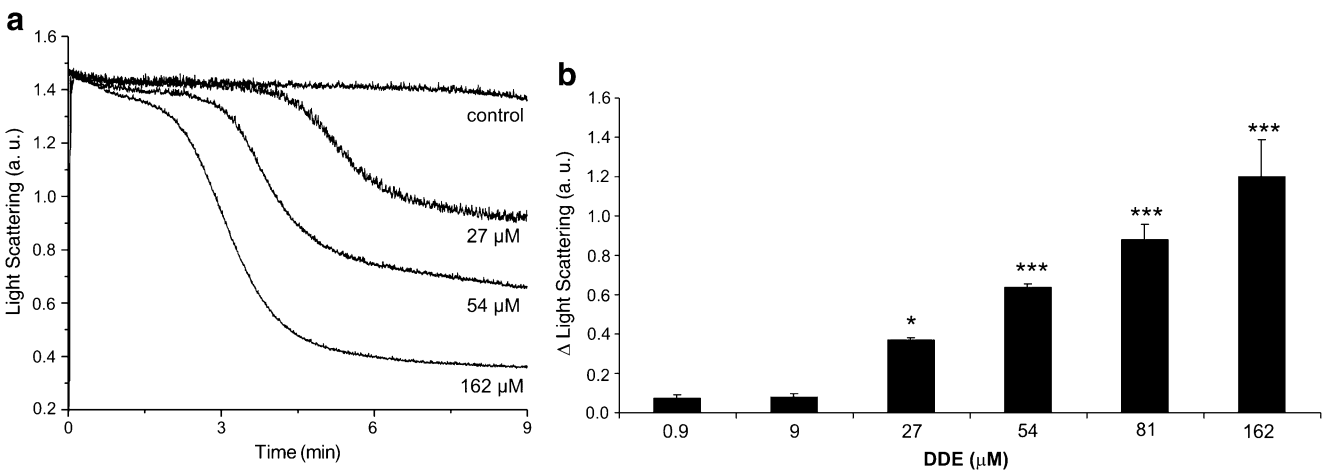

Fig. 3 DDE induces mitochondrial swelling. Mitochondria (≈0.6 mg protein/ml) were incubated at 37°C in experimental buffer 10 mM HEPES, 2 mM $MgCl_2$ and 2 mM KH_2PO_4, 100 mM KCl, pH 7.2, (KOH) plus 2 μM rotenone, 1 mM succinate, 1 μg/ml oligomycin, 2 mM ATP and DDE at indicated concentrations: **a** representative traces, **b** dose-response. *$P < 0.05$ and ***$P < 0.001$ versus control

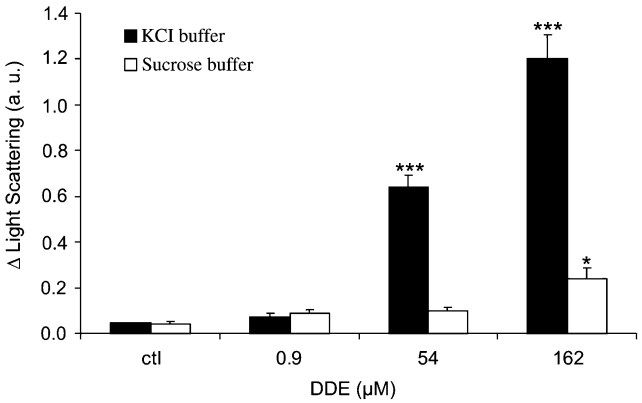

Fig. 4 DDE-promoted mitochondrial swelling in KCl and sucrose-based buffers. Mitochondria were exposed to DDE in buffer containing 10 mM HEPES, 2 mM $MgCl_2$, 2 mM KH_2PO_4 and 100 mM KCl, pH 7.2 (KOH; *black bars*) or in 250 mM sucrose, 2 mM EGTA and 10 mM HEPES, pH 7.2 (KOH; *white bars*). ***$P <$ 0.001 and *$P < 0.05$, versus control

and protein thiol oxidation (Kowaltowski et al. 2001; Armstrong 2006). In order to determine if mitochondrial permeability transition occurred in response to DDE, we followed the effect of its inhibitor, cyclosporin A, on mitochondrial swelling (Fig. 6). Cyclosporin A had no effect on the swelling observed, indicating that swelling was not attributable to the permeability transition. Indeed, inner membrane permeabilization promoted by DDE was enhanced by, but not dependent on Ca^{2+} ions, as verified by the partial protective effect of the Ca^{2+} chelator EGTA. Interestingly, the thiol reductant DTT also partially prevented mitochondrial swelling under these conditions, suggesting it is associated with oxidative mitochondrial damage.

The involvement of oxidative damage in DDE-promoted mitochondrial inner membrane permeabilization was confirmed by conducting lipid oxidation measurements. We found (Fig. 7) that DDE caused substantial lipid oxidation. This oxidation was not prevented by lycopene or vitamin E (20 μM, results not shown).

In summary, the results here indicate that DDE, at micromolar concentrations, promotes mitochondrial oxygen consumption in oligomycin-poisoned organelles as well as a decrease in mitochondrial membrane potentials, associated with massive swelling and lipid peroxidation.

Discussion

Nucleophilic attack to membrane phospholipids by reactive species can lead to breakdown of these molecules produc-

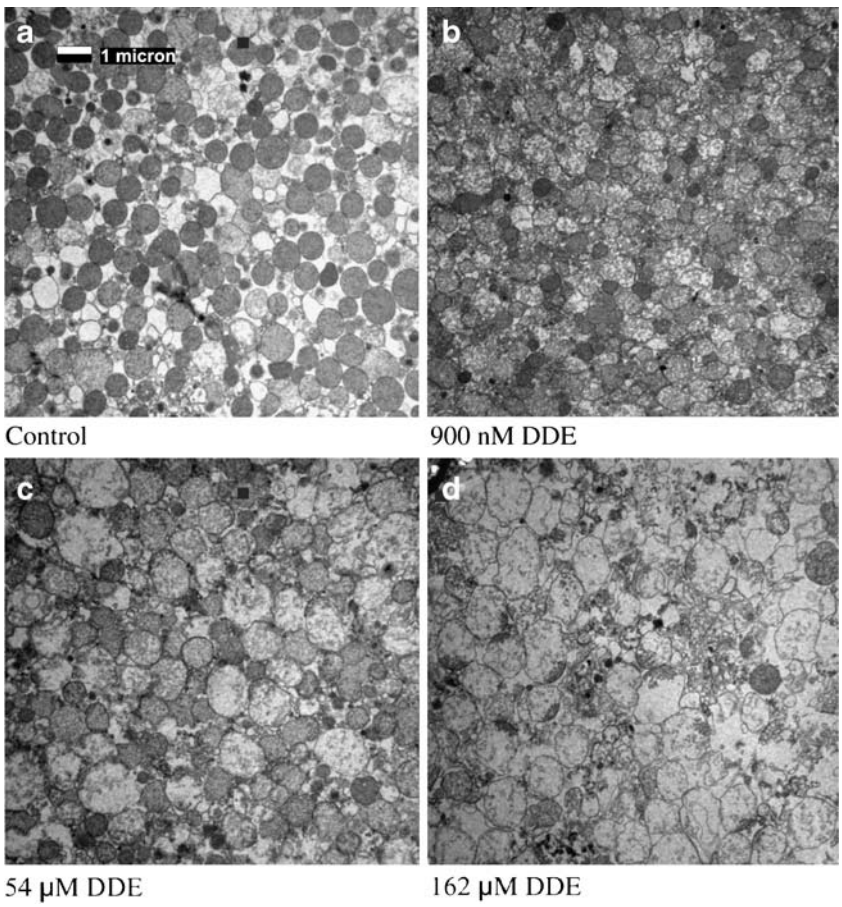

Fig. 5 DDE induces changes in mitochondrial morphology. Electron transmission microscopies of mitochondria (magnification=5000 in **a**–**b** and 6000 in **c**–**d**, scale bar 1 μM) incubated with DDE under the conditions of Fig. 3

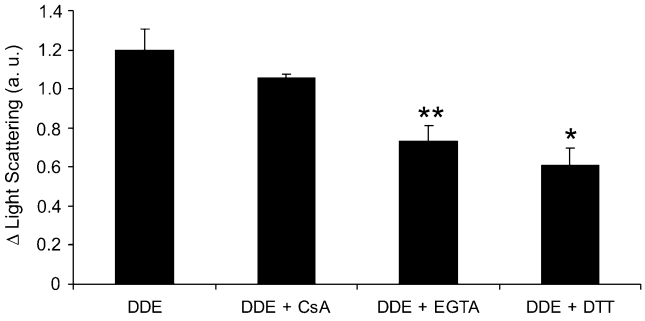

Fig. 6 DDE-promoted mitochondrial permeabilization is partially inhibited by EGTA and DTT. Mitochondria (≈0.6 mg protein/ml) were incubated at 37°C in experimental buffer (100 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 2 mM KH_2PO_4, pH 7.2, KOH) plus 2 μM rotenone, 1 mM succinate, 1 μg/ml oligomycin, 2 mM ATP and 162 μM DDE. Some experiments were carried out in presence of 1 mM EGTA, 1 mM DTT and 1 μM CsA, as indicated. * $P < 0.05$ and ** $P < 0.01$, versus DDE

ing reactive aldehydes as secondary products (Esterbauer et al. 1991). α,β-Unsaturated aldehydes have been shown to promote protein and DNA damage, induce mutagenesis, and to be involved in cell signaling cascades (Loureiro et al. 2000; West and Marnett 2005; West and Marnett 2006). Since mitochondria are the main source of reactive oxygen species, it is likely that this organelle is an important site of oxidative injury. The aim of this study was to evaluate mitochondrial dysfunction promoted by DDE, a well known toxic aldehyde produced in lipid peroxidation processes (Kaneko et al. 1987; Kaneko et al. 1988; Nappez et al. 1996; Chang et al. 2005).

Different aldehydes can promote different changes in mitochondrial parameters. Acrolein, another lipid peroxidation product, stimulates oxygen consumption in a manner related to the tissue investigated and mitochondrial substrate employed. Sun and coworker demonstrated that acrolein can affect both electron transport chain and inner membrane (IMM) integrity in liver mitochondria On the other hand, changes in state 4 respiration induced by acrolein were not observed in isolated brain mitochondria, but increases in ROS generation and inhibition of electron transport were reported (Picklo and Montine 2001; Luo and Shi 2005). 4-Hydroxynonenal (HNE) promotes respiratory inhibition (Chen et al. 2001; Lashin et al. 2006), mitochondrial uncoupling (Jaburek et al. 2004; Echtay et al. 2005; Shabalina et al. 2006) and increases swelling promoted by Ca^{2+} (Kristal et al. 1996). Increased mitochondrial volume was also detected in isolated rat liver mitochondria exposed to 4-hydroxyhexenal (HHE), in a permeability transition-dependent mechanism (Kristal et al. 1996).

We found that micromolar concentrations of DDE increase mitochondrial oxygen consumption under non-phosphorylating conditions (Fig. 1). Our results also indicate decreased inner membrane potentials in mitochondria exposed to DDE (Fig. 2). We suggest that DDE promotes non-selective inner membrane permeabilization, since we found that DDE promotes mitochondrial swelling (Fig. 3–6). Partial inhibition in mitochondrial swelling promoted by DTT indicates oxidative membrane damage, although it is not related to permeability transition, as indicated by the lack of effect of cyclosporin A. In addition to protein oxidation, swelling also involves lipid peroxidation, as verified by MDA measurements (Fig. 7). Taken together, these results indicate that DDE promotes oxidative damage to mitochondria resulting in non-selective membrane permeabilization and loss of function.

Increases in oxygen consumption induced by reactive aldehydes have been demonstrated recently. A mitochondrial uncoupling protein-mediated mechanism has been suggested (Echtay et al. 2003; Murphy et al. 2003; Brand et al. 2004; Echtay et al. 2005). However, there are also reports demonstrating that uncoupling promoted by HNE is not directly related to uncoupling protein activity (Jaburek et al. 2004; Shabalina et al. 2006). This is consistent with the knowledge that uncoupling proteins promote very limited control of inner membrane potentials, while the effects of aldehydes are substantial. Indeed, our results indicate a non-selective inner membrane permeability mediated by DDE.

DDE promotes significant mitochondrial lipid peroxidation. The MDA levels determinated in our system are comparable to these found in mitochondria exposed to Fe^{2+} and Ca^{2+}, a system that produces considerable lipid peroxidation (Gogvadze et al. 2003). DDE is also one of the most cytotoxic alkenals to hepatocytes, leading to lipid peroxidation in these models (Niknahad et al. 2003; Dung et al. 2006). A-549 cells exposed to oil fumes also show high levels of lipid peroxidation, mostly promoted by DDE, the most available alkenal in these fumes (Dung et al. 2006).

Altogether, the results described here demonstrate that DDE can produce important changes in mitochondrial parameters, resulting in membrane oxidative damage and

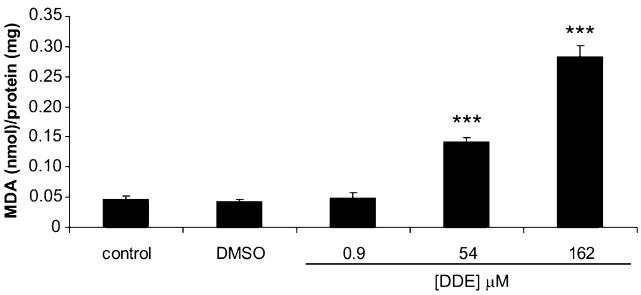

Fig. 7 DDE induces mitochondrial lipid peroxidation. The MDA-TBA adduct formed in mitochondria incubated for 10 min with DDE 900 nM, 54 μM or 162 μM in 10 mM HEPES, 2 mM $MgCl_2$, 2 mM KH_2PO_4, 100 mM KCl, pH 7.2 (KOH) at 37°C was analyzed by HPLC with fluorescence detection. *** $P < 0.001$, versus control

loss of organelle function. Our experimental model suggests that DDE induces formation of non-selective pores in the inner mitochondrial membrane. Mitochondrial swelling and membrane damage promoted by DDE could induce release of cytochrome c and other pro-apoptotic factors from mitochondria, initiating the apoptotic signaling pathway or inducing necrotic cell death (Halestrap et al. 1998; Halestrap et al. 2002; Gogvadze et al. 2003).

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