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Mitochondrial Permeability Transition Induced by Chemically Generated Singlet Oxygen

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Pure singlet molecular oxygen ($^1O_2$) generated by thermal decomposition of the 3,3′-(1,4-naphthylidene) dipropionate endoperoxide ($NDPO_2$), inhibited respiration of isolated rat liver mitochondria supported by NADH-linked substrates or succinate, but not by *N*,*N*,*N*,*N*-tetramehyl-*p*-phenylene-diamine (TMPD)/ascorbate. Under the latter conditions, mitochondria treated with 2.7 mM $NDPO_2$ exhibited a decrease in transmembrane potential ($\Delta\Psi$) in manner dependent on $NDPO_2$ exposure time. This process was sensitive to the mitochondrial permeability transition inhibitors EGTA, dithiothreitol, ADP, and cyclosporin A. The presence of deuterium oxide (D_2O), that increases $^1O_2$ lifetime, significantly enhanced $NDPO_2$-promoted mitochondrial permeabilization. In addition, $NDPO_2$-induced mitochondrial permeabilization was accompanied by DTT or ADP-sensitive membrane protein thiol oxidation. Taken together, these results provide evidence that mitochondrial permeability transition induced by chemically generated singlet oxygen is mediated by the oxidation of membrane protein thiols.

KEY WORDS: Rat liver mitochondria; single oxygen; permeability transition; $NDPO_2$.

INTRODUCTION

Mitochondrial permeability transition (MPT) is characterized by a Ca^{2+}-promoted opening of a nonselective proteinaceous inner membrane pore, the permeability transition pore (PTP), sensitive to the immune suppressor cyclosporin A (CsA) (Gunter *et al.*, 1994; Vercesi *et al.*, 1997). The occurrence of MPT is stimulated by a variety of inducing agents that include inorganic phosphate (P_i), uncouplers, and prooxidants (Gunter *et al.*, 1994; Vercesi *et al.*, 1997). Solid evidence for a pivotal role of reactive oxygen species (ROS) mediated membrane protein thiol oxidation in MPT induced by these compounds has been provided by our group (Fagian *et al.*, 1990; Kowaltowski *et al.*, 2001; Valle *et al.*, 1993). Excessive uptake of Ca^{2+} by mitochondria stimulates the production of ROS (Grijalba *et al.*, 1999) that promotes the opening of the permeability transition pore.

Singlet oxygen ($^1O_2$) is a ROS that exhibits high reactivity with amines, sulfides, and unsaturated lipids because of its high electrophilicity and relatively long lifetime (2–4 μs in H_2O and ~700 μs in CCl_4) (Moroe, 1985). In mammalian cells, $^1O_2$ can be generated during oxidative stress and is able to attack DNA (Di Mascio *et al.*, 1989), protein thiol groups (Devasagayam *et al.*, 1991), and membrane lipids (Cadenas and Sies, 1982). In the presence of P_i, enolyzable carbonyls produced by lipid peroxidation

Key to abbreviations: AA, Antimycin A; CsA, cyclosporin A; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MPT, mitochondrial permeability transition; NDP, 3,3′-(1,4-naphthylidene) dipropionate; $NDPO_2$, 3,3′-(1,4-naphthylidene) dipropionate endoperoxide; $^1O_2$, singlet molecular oxygen; P_i, inorganic phosphate; RLM, rat liver mitochondria; ROS, reactive oxygen species; rot, rotenone; suc, succinic acid; TMPD, *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylene-diamine; TPP^+, tetraphenylphosphonium; $\Delta\Psi$, mitochondrial membrane potential; α-kg, α-ketoglutaric acid.

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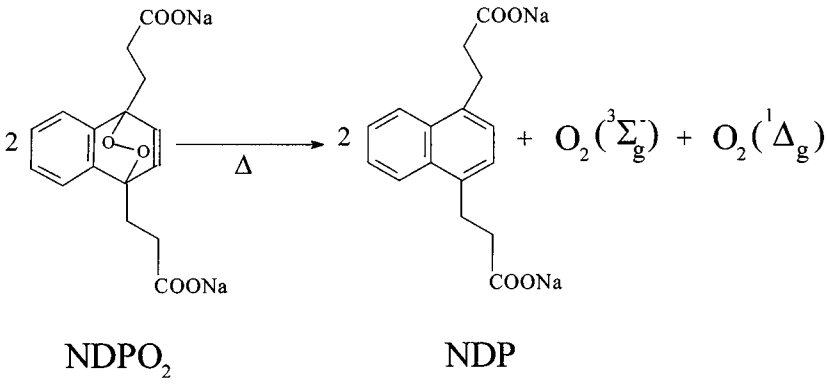

Scheme I. Thermal decomposition of the water-soluble 3,3′-(1,4-naphthylidene) dipropionate endoperoxide disodium salt ($NDPO_2$) producing the parent hydrocarbon (NDP), molecular oxygen in triplet state ($O_2(^3\Sigma_g^-)$) and oxygen in singlet state ($O_2(^1\Delta_g)$).

may be oxidized by cytochromes (Fe^{3+}) producing triplet carbonyls prone to damage mitochondria directly or via $^1O_2$ produced by energy transfer mechanisms (Nantes *et al.*, 1995). Singlet oxygen can also be generated during inflammatory processes (Kanofsky, 1984) by dioxygenases (Duran, 1982), tryptophan pyrrolase (Hayashia and Nozaki, 1969), and lipoxygenases (Chan, 1971). Furthermore, the photodynamic action of some drugs that accumulate in mitochondria from proliferating cells is mediated by $^1O_2$ (Moan and Berg, 1992; Saitow and Nakaoka, 1997). The understanding of the mechanisms underlying $^1O_2$ toxicity is highly relevant once these drugs are used in photodynamic therapy (Dougherty *et al.*, 1998; Moor, 2000; Oseroff *et al.*, 1986). In this regard, we have shown recently (Kowaltowski *et al.*, 2001; Nepomuceno *et al.*, in press) that two distinct groups of compounds named triarylmethane dyes and Fe (III) forms of mesotetrakis(4-methyl piridiniumyl) porphyrins, that may generate $^1O_2$ and have potential applications in antioncotic photodynamic therapy, induce oxidative damage to isolated rat liver mitochondria.

Previously, Salet *et al.* (1997) reported that PTP was inactivated in Ca^{2+}-overloaded rat liver mitochondria, irradiated during 1960s in the presence of hematoporphyrin. The authors concluded that the inhibition of Ca^{2+} release by this compound resulted from a site-selective inactivation of PTP by $^1O_2$ generated during the irradiation. More recently, data from the same group (Moreno *et al.*, 2001) using 4,5′, 8-trimethylpsoralen (TMP), showed that irradiation of this compound induced PTP opening in Ca^{2+} loaded rat liver mitochondria. Since none of these works provided direct evidence that $^1O_2$ was responsible for the observed results, in this paper we investigated the effect of pure singlet oxygen on isolated rat liver mitochondria using the water soluble $^1O_2$ carrier 3,3′-(1,4 naphthalidene) dipropionate endoperoxide ($NDPO_2$). When heated at 30–37°C, this compound releases molecular oxygen half of which is in the singlet excited state (Scheme I) (Martinez *et al.*, 2000; Pierlot *et al.*, 2000). We show that, indeed, $^1O_2$ generated by this compound open PTP via oxidation of membrane protein thiols.

MATERIALS AND METHODS

Reagents

All reagents were of the highest grade of purity available. The endoperoxide of the disodium salt of $NDPO_2$ was prepared according to Di Mascio and Sies (1989), and identified by nuclear magnetic resonance (NMR), IR spectroscopy, and LC mass spectrometry. Absorption measurements of the endoperoxide were performed with a LKB spectrophotometer (model Ultrospec 4050) and its stock solutions were kept in the dark at −80°C until immediately before use.

Isolation of Rat Liver Mitochondria (RLM)

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar rats fasted overnight. The protein concentration was determined by a modified biuret assay (Kaplan and Pedersen, 1983).

Generation of $^1O_2$ by Thermodissociation of $NDPO_2$

Chemical generation of $^1O_2$ was promoted using the thermodissociable endoperoxide $NDPO_2$ (Scheme I).

During the thermal decomposition of the water-soluble $NDPO_2$ to NDP and molecular oxygen, one half of the oxygen is in the excited state (Di Mascio *et al.*, 1989). The maximal rate of $^1O_2$ generation was calculated by measuring NDP formation (Di Mascio *et al.*, 1989). For example, at 6 min after addition of 10 mM endoperoxide, the maximal rate of $^1O_2$ generation was 38 μM per min decreasing to 10 μM after 30 min. Thus, the steady state of $^1O_2$ in the incubation medium is calculated to be 31×10^{-12} M at 6 min and 8×10^{-12} M at 30 min after the addition of $NDPO_2$ (Di Mascio *et al.*, 1989).

Standard Incubation Procedure

With the exception of Fig. 1, RLM (1.0 mg/mL) were preincubated in 125 mM sucrose, 65 mM KCI, 10 mM Hepes (pH = 7.2), 1 mM K_2HPO_4, 1 mM $MgCI_2$, 2 μM rotenone, 1 μM oligomycin, 1 μg/mL antimycin A in the presence of 2.7 mM $NDPO_2$ or 2.7 mM NDP and 14.5 μM free Ca^{2+}. As control experiments, reverse additions of NDP to the mitochondrial suspension were made. They consisted of 2.7 mM $NDPO_2$ additions to the reaction medium 4 h before mitochondria. $NDPO_2$ is fully degraded to NDP and O_2 within 3 h at 30°C (Martinez *et al.*, 2000).

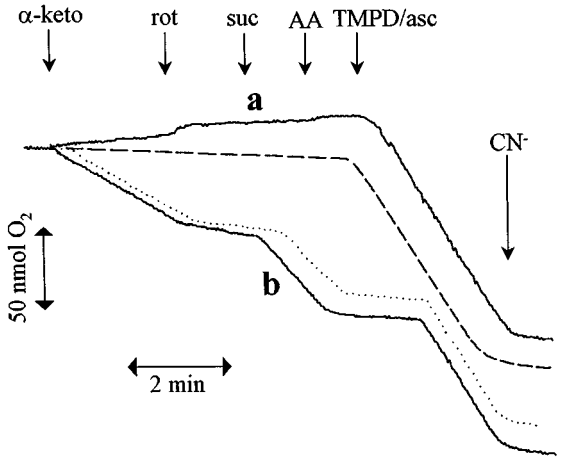

Fig. 1. Effect of $NDPO_2$ on oxygen consumption by rat liver mitochondria. RLM (1.0 mg/mL, 30°C) were preincubated during 3 min in 1.3 mL of reaction medium in the presence of 2.7 mM $NDPO_2$ (*line a*), 2.7 mM NDP (*line b*), or in the absence of both (*dotted line*) plus 1 μM FCCP. Where indicated, TMPD (100 μM) plus 1 mM ascorbate, 2 mM KCN, 10 mM α-ketoglutarate, 2 μM rotenone, 2 mM succinate, or 2 μM antimycin A were added. The dashed line contained mitochondria (1 mg/mL), 10 mM α-ketoglutarate, 2 μM rotenone, 2 mM succinate, and 2 μM antimycin A.

Measurement of Mitochondrial Respiration

Mitochondrial oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments Co.) in a 1.3 mL glass chamber equipped with magnetic stirring.

Mitochondrial Transmembrane Electrical Potential ($\Delta\Psi$)

Mitochondria (1 mg/mL) were incubated in standard reaction medium containing 2 μM tetraphenylphosphonium (TPP^+). The concentration of TPP^+ in the extramitochondrial medium was continuously monitored with a TPP^+-selective electrode prepared in our laboratory according to Kamo *et al.* (1979). The membrane potential was then calculated assuming that the TPP^+ distribution between mitochondria and medium follows the Nerst equation (Muratsugu *et al.*, 1977). Corrections due to the binding of TPP^+ to the mitochondrial membrane were made according to Jensen *et al.* (1986).

Determination of Ca^{2+} Movements

Variations in free Ca^{2+} concentration were followed by measuring the changes in the absorbance spectrum of the metallochromic indicator arsenazo III (Scarpa, 1979), using an SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm at 30°C. The calibrations were performed by the sequential addition of known concentrations of EGTA. The initial Ca^{2+} concentration in the solution was obtained by atomic absorption spectroscopy and the Ca^{2+} concentration after each EGTA addition was calculated as described before (Vercesi and Docampo, 1992).

Determination of Protein Thiol Groups Content

Mitochondrial membrane thiol groups were determined using 5,5′-dithio-bis(2-nitrobenzoic) acid (Ellman's reagent) as described in Castilho *et al.* (1996) and Kowaltowski *et al.* (1997).

RESULTS

Effect of $^1O_2$ on Mitochondrial Respiration Supported by α-ketoglutarate, Succinate, or TMPD/Ascorbate

Figure 1 shows the rates of FCCP-induced uncoupled respiration supported by α-ketoglutarate, succinate, or

N,*N*,*N*,*N*-tetramethyl-*p*-phenylene-diamine (TMPD)/ascorbate, in mitochondria preincubated at 30°C in the presence of 2.7 mM $NDPO_2$ (*line a*), 2.7 mM NDP (*line b*) or in the absence of both (*dotted line*). The rates of O_2 consumption were similar in the presence or absence of NDP (compare solid line b with dotted line). On the other hand, NDPO2 promoted a clear inhibition of respiration supported by either α-ketoglutarate or succinate but not by TMPD/ascorbate. Considering these results, the experiments aimed at studying the effects of $NDPO_2$ on membrane permeability were carried out using TMPD/ascorbate as substrate to maintain equal energization between control and treated samples.

It can also be noted that in the presence of $NDPO_2$ there was a continous increase in oxygen concentration in the medium that is certainly the result of $NDPO_2$ decomposition. When we compare the experiment in the presence of $NDPO_2$ (line a) with a control done with mitochondria incubated in its absence (dashed line) is possible to observe that the net rate of O_2 appearance (about 5 nmol O_2/min) was linear during the period of observation. This value is not far from that reported by Di Mascio *et al.* (1989).

$^1O_2$ Generated From $NDPO_2$ Induces Drop of $\Delta\Psi$ Supported by TMPD/Ascorbate Oxidation

The experiments depicted in Fig. 2 show that $\Delta\Psi$ supported by TMPD/ascorbate oxidation was gradually decreased by increasing the preincubation time with $NDPO_2$. A decreased capacity of mitochondria to sustain $\Delta\Psi$ could be observed after 1 min preincubation with

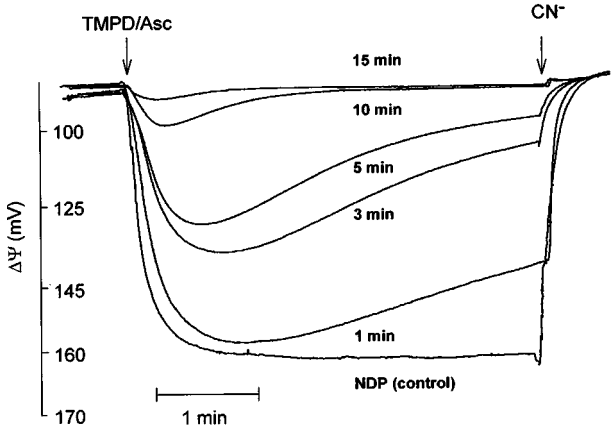

Fig. 2. Mitochondrial transmembrane potential dissipation by $NDPO_2$: effect of preincubation time. RLM (1.0 mg/mL, 30°C) was preincubated in the presence of 2.7 mM $NDPO_2$ in 1 mL of reaction medium during 1, 3, 5, 10, and 15 min. A control experiment was done with the reverse addition of 2.7 mM NDP during 15-min preincubation.

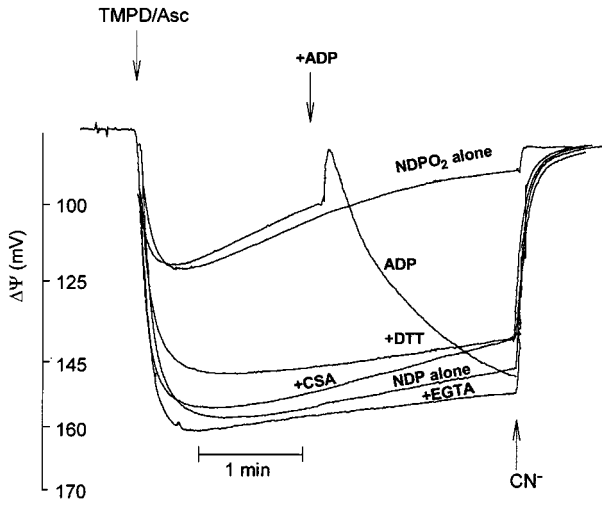

Fig. 3. Protective effects of CSA, DTT, ADP, and EGTA on $\Delta\Psi$ dissipation induced by $NDPO_2$. RLM (1.0 mg/mL, 30°C) were preincubated during 3 min in 1 mL of reaction medium in the presence of 2.7 mM $NDPO_2$ (line $NDPO_2$ alone) or $NDPO_2$ plus: 500 μM EGTA, 300 μM DTT, or 1 μM CSA present from the start. ADP (300 μM) was added where indicated in the experiment containing $NDPO_2$. A control experiment (NDP alone) with reverse addition of 2.7 mM NDP was also done.

2.7 mM $NDPO_2$. With preincubation periods of 10 or 15 min, only a small and transient $\Delta\Psi$ could be observed. The *NDP*-labeled line represents a control experiment in which the preincubation was conducted in the presence of a reverse NDP addition.

Cyclosporin A, DTT, ADP, or EGTA Inhibit $\Delta\Psi$ Dissipation Induced by $^1O_2$

We conducted experiments in the presence of classical MPT inhibitors in order to study the mechanism of $\Delta\Psi$ dissipation by $^1O_2$. Fig. 3 shows that $\Delta\Psi$ drop induced by preincubation of RLM with 2.7 mM $NDPO_2$ during 3 min could be inhibited by cyclosporin A, DTT, and EGTA present in the incubation medium from the beginning of the experiment. ADP could prevent (not shown) or reverse $\Delta\Psi$ drop as is also characteristic of MPT.

D_2O Enhances $^1O_2$ Effect

The experiment depicted in Fig. 4 shows that the $\Delta\Psi$ generated when mitochondria were preincubated in the presence of $NDPO_2$ in D_2O-containing medium was significantly lower than that obtained in the presence of $NDPO_2$ in H_2O. This was expected and confirms that $^1O_2$ was responsible for the observed effects of $NDPO_2$

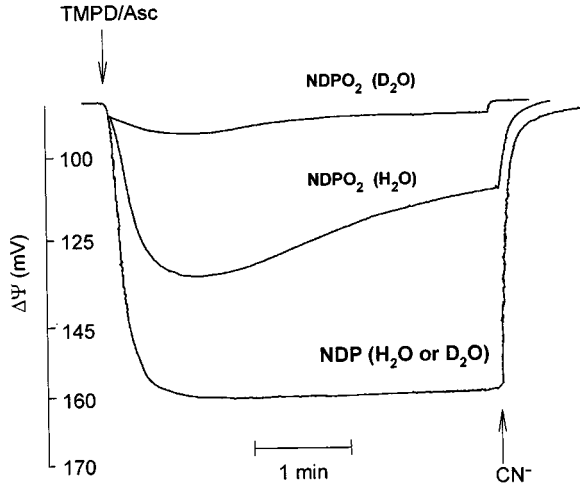

Fig. 4. Mitochondrial transmembrane potential dissipation by $NDPO_2$: effect of D_2O. RLM (1.0 mg/mL. 30°C) were preincubated during 3 min in 1 mL of reaction medium in the presence of 2.7 mM $NDPO_2$ in water or 2.7 mM $NDPO_2$ in 90% D_2O. A control experiment was conducted with reverse addition of 2.7 mM NDP in the presence or absence of D_2O.

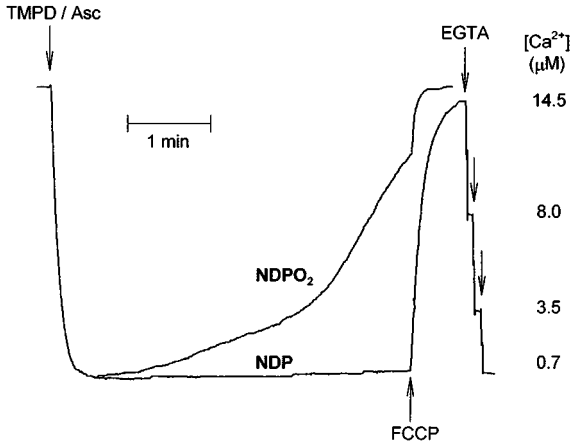

Fig. 5. Mitochondrial Ca^{2+} transport in the presence of $NDPO_2$. RLM (1.0 mg/mL, 30°C) were preincubated during 3 min in 1 mL of reaction medium in the presence of 2.7 mM $NDPO_2$ or 2.7 mM NDP. A control experiment was also conducted with reverse addition of 2.7 mM NDP.

because D_2O is known to increase the lifetime of $^1O_2$ (Kajiwara and Kearns, 1973). The control experiment (NDP) was done in medium containing or not containing D_2O.

Mitochondrial Ca^{2+} Release Induced by $^1O_2$

One of the characteristics of MPT is its dependence on Ca^{2+} accumulation by mitochondria. At the relative low Ca^{2+} loads used in these experiments (14.5 μM Ca^{2+}), mitochondria could sustain both $\Delta\Psi$ (preceding experiments) and accumulated Ca^{2+} (Fig. 5, NDP) during the experimental time. However, when RLM were preincubated during 3 min in the presence of 2.7 mM $NDPO_2$ their capacity to retain accumulated Ca^{2+} significantly decreased (Fig. 5, $NDPO_2$).

Ca^{2+} uptake was supported by TMPD/ascorbate oxidation and release was induced by the protonophore FCCP to allow calibration with EGTA at the end of the experiment.

Oxidation of Mitochondrial Membrane Protein Thiol Groups by $^1O_2$

It is well known that MPT is associated with membrane protein thiol group oxidation (Castilho *et al.*, 1996; Fagian *et al.*, 1990). The inhibition by DTT of $\Delta\Psi$ drop induced by $NDPO_2$ in Fig. 3 suggested that thiol oxidation was involved in this mechanism. Indeed, Table I shows a significant decrease of membrane protein thiols when mitochondria were preincubated in the presence of $NDPO_2$. Both MPT inhibitors, ADP, and DTT, protected against thiol oxidation.

DISCUSSION

Singlet oxygen can be generated in biological systems during photo-oxidation of a variety of biological compounds or by nonphotochemical processes such as enzymatic reactions (Kanofsky, 1984). It can also be generated by energy transfer to triplet molecular oxygen from triplet species produced during oxidative stress conditions (Nantes *et al.*, 1995). In this work, we studied the nature of oxidative damage induced to isolated rat liver mitochondria by pure $^1O_2$ generated from the

Table I. Effect of ADP and DTT on Mitochondrial Membrane Protein Thiol Oxidation Induced by $NDPO_2$

Conditions	Absorbance (412 nm)
2.7 mM NDP (control)	0.598 ± 0.010
2.7 mM $NDPO_2$	0.385 ± 0.009
2.7 mM $NDPO_2$ + 300 μM ADP	0.520 ± 0.016
2.7 mM $NDPO_2$ + 300 μM DTT	0.484 ± 0.009

Note. The experimental conditions were similar to those of Fig. 3. RLM (1.0 mg/mL) were preincubated in the presence of 2.7 mM $NDPO_2$ plus either 300 μM ADP or 300 μM DTT. A control experiment was conducted with reverse addition of 2.7 mM NDP. Mitochondrial membrane protein thiol content was determined as described in Materials and Methods. The values represent average of three experiments ±SD.

water-soluble compound $NDPO_2$ (Martinez *et al.*, 2000; Pierlot *et al.*, 2000).

The capacity of $^1O_2$ to oxidize thiol groups (Devasagayam *et al.*, 1991) prompted us to investigate the possibility that this species could generate MPT. Indeed, this hypothesis was confirmed by the experiments showing that $^1O_2$ induces a decrease in $\Delta\Psi$ sensitive to the MPT inhibitors EGTA, CsA, ADP, and DTT (Fig. 3). The participation of protein thiol group oxidation in the mechanism of $^1O_2$-induced MPT is supported by the experiments demonstrating both protection by DTT and a decreased content of reduced membrane thiol groups. The ADP effect against the decrease in both $\Delta\Psi$ (Fig. 3) and oxidized thiols (Table I) by $^1O_2$ is an additional evidence for the involvement of the ADP/ATP carrier in the structure of the PTP (Halestrap *et al.*, 1997, see also the reviews, Kowaltowski *et al.*, 2001; Vercesi *et al.*, 1997). It might be possible that ADP binding to the ADP/ATP carrier induces changes in the protein conformation protecting its thiol groups against the oxidant action of $^1O_2$.

Regarding the action of $^1O_2$ on mitochondria, Salet *et al.* (1997) reported that this species, when produced by irradiation of hematoporphyrin, led to inactivation of the mitochondrial permeability transition pore. The disagreement between their data and ours may result from differences in the amount or location of $^1O_2$ generation. This possibility was also suggested by experiments using 4,5′, 8-trimethylpsoralen, a photosensitizer that binds to protein sites that differ from those of hematoporphyrin (Moreno *et al.*, 2001).

In the present experiments, the direct participation of $^1O_2$ is supported by at least two specific properties of this species. First, the enhancement of $NDPO_2$-induced mitochondrial permeabilization by D_2O (Fig. 4), a compound known to increase the life time of $^1O_2$ (Kajiwara and Kearns, 1973), and second, the inhibition of mitochondrial respiration supported either by NADH-linked substrates or succinate (Fig. 1) (Salet and Moreno, 1981).

In conclusion, the present data provide evidence that $^1O_2$ promotes oxidative damage to isolated rat liver mitochondria by a mechanism sensitive to the MPT inhibitors EGTA, DTT, ADP, and cyclosporin A. This strongly suggests that MPT may be the mechanism by which photodynamic therapy leads to mitochondrial injury and tumor cell death. Considering that large $\Delta\Psi$ drops favor PTP opening (Bernardi and Petronilli, 1996), we propose that the toxicity of $^1O_2$ to mitochondria in situ can be the result of two independent effects, i.e., thiol oxidation and the decrease in $\Delta\Psi$ that occurs because of the inhibition of respiration supported by NAD-linked substrates, the main respiratory substrates in vivo.

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