Mitochondrial swelling and membrane protein thiol oxidation associated with mitochondrial permeability transition induced by Ca\textsuperscript{2+} and inorganic phosphate are inhibited in a dose-dependent manner either by catalase, the thiol-specific antioxidant enzyme (TSA), a protein recently demonstrated to present thiol peroxidase activity, or ebselen, a selenium-containing heterocycle which also possesses thiol peroxidase activity. This inhibition of mitochondrial permeability transition is due to the removal of mitochondrial-generated H\textsubscript{2}O\textsubscript{2} which can easily diffuse to the extramitochondrial space. Whereas ebselen required the presence of reduced glutathione as a reductant to grant its protective effect, TSA was fully reduced by mitochondrial components. Decrease in the oxygen concentration of the reaction medium also inhibits mitochondrial permeabilization and membrane protein thiol oxidation in a concentration-dependent manner. The results presented in this report confirm that mitochondrial permeability transition induced by Ca\textsuperscript{2+} and inorganic phosphate is reactive oxygen species-dependent. The possible importance of TSA as an intracellular antioxidant, avoiding the onset of mitochondrial permeability transition, is discussed in the text.

It is well known that mitochondria may lose their ability to phosphorylate ADP, suffer decrease in membrane potential, and begin to swell when incubated in the presence of Ca\textsuperscript{2+} ions. This Ca\textsuperscript{2+}-dependent, nonspecific inner mitochondrial membrane permeabilization was named mitochondrial permeability transition (MPT)\textsuperscript{1} and was found to be inhibited by the immune suppressor cyclosporin A (for reviews, see Refs. 1 and 2). Ca\textsuperscript{2+}-induced mitochondrial permeability transition (MPT) can be stimulated by a wide variety of compounds with different chemical characteristics, known as inducers, including inorganic phosphate (P\textsubscript{i}), oxidizing agents, dithiol reagents, protonophores, and ligands of the adenine nucleotide translocator (for a list see Ref. 2), although inorganic phosphate is the most probable MPT inducer in vivo. The observation that MPT was stimulated by prooxidants (2, 3) first led us to the hypothesis that MPT could be caused by reactive oxygen species (ROS) produced by the mitochondrial respiratory chain (4–6). This hypothesis was confirmed by studies showing that MPT induced by prooxidants could be prevented by the presence of catalase (5, 7), \(\alpha\)-phenanthroline (5), or the absence of oxygen (5). Further studies demonstrated that MPT induced by P\textsubscript{i} and protonophores was also ROS-dependent (8, 9). Mitochondria-generated ROS promote the oxidation and cross-linkage of mitochondrial membrane protein thiol groups, leading to MPT (5, 6, 10, 11). Despite extensive evidence (for review see Ref. 4), recently the hypothesis that MPT is ROS-dependent has been questioned (12).

The role of MPT is still unknown, but evidence is accumulating that MPT may be an initial event in the process of cell death which occurs in a variety of pathological states, such as ischemia/reperfusion (13, 14). Many studies have also proposed that MPT may be an initial step of apoptosis (15, 16). In this regard, Kroemer and co-authors (15) have recently demonstrated that mitochondrial swelling due to MPT leads to the release of a 50-kDa protein, the “apoptosis inducing factor,” from the intermembrane space into the cytosol. This protein, when isolated, causes apoptotic changes in isolated nuclei (15). Based on these results, Skulachev (16) formulated the hypothesis that ROS-producing mitochondria suffer MPT, leading to release of the apoptosis inducing factor. If the number of ROS-producing mitochondria is large, sufficient quantities of the apoptosis inducing factor accumulate, leading to cell death. Thus, MPT would be a mechanism for eliminating cells which have high ROS generation (16).

The thiol-specific antioxidant enzyme (TSA), also known as thioredoxin peroxidase, is a new antioxidant enzyme originally isolated from yeast and later found in mammalian tissues (17, 18). This protein has the ability to protect biomolecules from oxidative damage if a thiol reductant such as dithiothreitol (DTT) or thioredoxin is present (17, 19). Recently, the antioxidant property of TSA was attributed to its thiol peroxidase activity as follows: 2 RSH + H\textsubscript{2}O\textsubscript{2} \to RSSR + 2 H\textsubscript{2}O (20). Because proteins belonging to the TSA family are usually present in large quantities in protozoan, yeast, and mammalian cells (21–24), we questioned whether this antioxidant protein may play an important role in defense against MPT. The results presented in this paper were designed to investigate both if TSA is capable of inhibiting mitochondrial permeabilization associated with MPT and if the inhibition of MPT by peroxidases is strictly related to their H\textsubscript{2}O\textsubscript{2} removal activity. The effect of ebselen, a selenium-containing heterocycle and analogue of glutathione peroxidase, which presents peroxidase, but not free-radical scavenging activity (25), was also studied.
Fig. 1. Effect of TSA and catalase on mitochondrial swelling induced by Ca\(^{2+}\) and P\(_{i}\). Rat liver mitochondria (0.5 mg/ml) were incubated in standard reaction medium in the presence of 100 \(\mu\)M EGTA or 1 \(\mu\)M cyclopasin A (line a), 16 \(\mu\)M TSA (line b), 2 \(\mu\)M catalase (line c), 16 \(\mu\)M C170S (line d), no further additions (line e), or 16 \(\mu\)M C47S (line f). P\(_{i}\) (0.5 mM) was added where indicated by the arrow.

EXPERIMENTAL PROCEDURES

Standard Incubation Procedure—Mitochondria were isolated as described in Refs. 5 and 6 and incubated at 28 °C in a standard reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer, pH 7.2, 10 \(\mu\)M CaCl\(_2\), 2 mM succinate, and 4 \(\mu\)M rotenone. Other additions are indicated in the figure legends. The results shown are representative of a series of at least three experiments.

Determination of Mitochondrial Swelling—Mitochondrial swelling was estimated from the decrease in the absorbance at 520 nm measured by a SLM Aminco DW2000 spectrophotometer (SLM Instruments, Inc., Urbana, IL).

Determination of Protein Thiol Groups Content—Mitochondrial thiol groups were determined using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent) as described in Refs. 11 and 26. In standard reaction medium, mitochondrial protein thiol content was approximately 45 nmol/mg protein and was reduced to approximately 30 nmol/mg protein after treatment with Ca\(^{2+}\) and P\(_{i}\).

Determination of Mitochondrial \(H_2O_2\) Generation—\(H_2O_2\) production was assessed by the oxidation of scopoletin by horseradish peroxidase in the presence of H\(_2\)O\(_2\) (27). Scopoletin fluorescence was monitored at excitation and emission wavelengths of, respectively, 365 and 450 nm, on a Hitachi F-4010 fluorimeter. Calibration was performed by adding known quantities of \(H_2O_2\). In these determinations, citrate was used as a \(Ca^{2+}\) chelator, because the presence of EGTA interferes with scopoletin fluorescence. Cyclosporin A was present in all determinations in order to prevent artifacts due to mitochondrial swelling.

Oxygen Uptake Measurements—Oxygen concentration was measured using a Clarke-type electrode (Yellow Springs Instruments Co.) in a glass cuvette equipped with magnetic stirring.

Materials—Antimycin A, catalase (C-10), horseradish peroxidase (type IV-A), ebselen, reduced glutathione, cyclosporin A, EGTA, Hepes, rotenone, and succinate were obtained from Sigma. All other reagents were commercial products of the highest purity grade available. Yeast TSA has two highly conserved cysteine residues (Cys-47 and Cys-170) (18). The wild-type and mutant proteins C47S and C170S, where the respective cysteine residues were replaced by serine, were purified as described previously (28).

RESULTS AND DISCUSSION

The hypothesis that MPT is dependent on the presence of mitochondria-generated ROS (for review, see Ref. 4) is further supported by the results presented in Fig. 1, showing that mitochondrial swelling associated with MPT induced by Ca\(^{2+}\) and P\(_{i}\) (line c) was prevented by the presence of 16 \(\mu\)M thiol-specific antioxidant enzyme (TSA, line b). This inhibition was similar to that observed in the presence of 2 \(\mu\)M catalase (line e). Similar results were also obtained using MER-5 (results not shown), a mammalian mitochondrial isoform of TSA that is 58% identical to the yeast protein (28). However, we chose to use yeast TSA in this investigation due to the ready availability of site-specific mutant forms. C47S, a mutant form of TSA devoid of thiol peroxidase activity (see “Experimental Procedures” and Refs. 19 and 20), did not show an inhibitory effect on mitochondrial permeabilization (line f), demonstrating that the protective effect of TSA on mitochondrial swelling is not due to a nonspecific protein effect. The full inhibition of the mitochondrial swelling obtained in the presence of cyclosporin A (line a) confirms that the permeabilization is due to MPT (2).

In cell-free systems, the peroxidase activity of TSA is dependent on the addition of a thiol reductant such as thioredoxin or DTT (20). Since in our experiments TSA was added in its oxidized form, it is safe to assume that it is reduced by components of the mitochondrial suspension, possibly thioredoxin, reduced glutathione, or even outer membrane protein thiol groups. Indeed, thioredoxin, a 12.5-kDa protein containing two vicinal cysteines, has been considered the physiological substrate for TSA (19). C170S is a site-specific mutant of TSA that is active in the presence of low molecular weight thiols such as DTT but is inactive when thioredoxin is used as electron donor (see “Experimental Procedures” and Refs. 19 and 20). C170S promoted partial inhibition of MPT induced by Ca\(^{2+}\) and P\(_{i}\) (line d), indicating that mitochondrial thioredoxin is at least partially responsible for the reduction of TSA. Although TSA seems to have higher affinity for thioredoxin (19), this antioxidant protein is also reduced by other thiol compounds (17, 20). Since mitochondrial suspensions are rich in reduced thiols, such as outer membrane protein thiol groups, it is reasonable to assume that these reductants can also donate electrons to TSA. Indeed, the addition of thioredoxin to the mitochondrial suspension did not stimulate the effect of TSA (results not shown), indicating that TSA is fully reduced by mitochondrial components.

A comparison between the effects of TSA, catalase, and ebselen, a selenium-containing heterocyclic compound that exhibits peroxidase-like activity (for review, see Ref. 25), on mitochondrial swelling and membrane protein thiol oxidation induced by Ca\(^{2+}\) and P\(_{i}\) is shown in Fig. 2. We observed that catalase (■) and TSA (●) inhibit mitochondrial swelling and membrane protein thiol oxidation in a dose-dependent manner, although TSA requires concentrations 6–8 times higher than catalase to present the same effect. This is in agreement with the hydrogen peroxide removal activities of these antioxidant proteins (20). Ebselen (▲) also presented an inhibitory effect on mitochondrial swelling and membrane protein thiol oxidation.

FIG. 2. Effect of catalase (■), TSA (●), and ebselen (▲) on mitochondrial swelling (A) and membrane protein thiol oxidation (B) induced by Ca\(^{2+}\) and P\(_{i}\). Rat liver mitochondria (0.5 mg/ml) were incubated for 10 min in standard reaction medium containing catalase (■), TSA (●), or ebselen, in the presence of 2 \(\mu\)M reduced glutathione (▲), in the concentrations indicated. P\(_{i}\) (0.5 mM) was added after 2 min incubation. Percentages of swelling extent and protein thiol oxidation were calculated as proportions of total mitochondrial swelling extent and protein thiol oxidation, obtained in the absence of catalase, TSA, or ebselen.
induced by Ca\(^{2+}\) and P, in concentrations similar to TSA, but required the concomitant presence of reduced glutathione. Inhibition of mitochondrial swelling and membrane protein thiol oxidation in the presence of 16 \(\mu\)M ebselen or reduced glutathione alone was less than 10% that observed in the presence of both reagents (results not shown).

In Fig. 3, mitochondrial H\(_2\)O\(_2\) generation was monitored by the oxidation of scopoletin catalyzed by horseradish peroxidase in the presence of H\(_2\)O\(_2\) (27). As observed previously by monitoring the formation of horseradish peroxidase compound II (8, 9), the H\(_2\)O\(_2\) generation of Ca\(^{2+}\)-loaded mitochondria is largely increased by the addition of P (line a), an event which precedes mitochondrial swelling observed in Fig. 1. The presence of the Ca\(^{2+}\) chelator citrate completely prevented the burst in mitochondrial H\(_2\)O\(_2\) generation induced by P (line c), demonstrating that this production is Ca\(^{2+}\)-dependent. Ebselen plus reduced glutathione (line b), catalase (line c), and TSA (line d) decreased significantly the detection of mitochondria-generated H\(_2\)O\(_2\). These compounds, which are large molecules not capable of entering intact mitochondria, remove mitochondria-generated H\(_2\)O\(_2\) that diffuses through the membrane and would combine with horseradish peroxidase. We propose that the presence of these peroxidases, by decreasing significantly the concentration of extramitochondrial H\(_2\)O\(_2\), would stimulate H\(_2\)O\(_2\) diffusion through the membrane, resulting in lower intramitochondrial H\(_2\)O\(_2\) concentrations. This would decrease the intramitochondrial generation of the highly reactive hydroxyl radical, probably the main radical species responsible for mitochondrial membrane protein thiol oxidation leading to MPT (4, 5). Further supporting this hypothesis, mitochondrial permeabilization induced by the thiol cross-linking reagent phenylarsine oxide, which directly promotes mitochondrial membrane protein thiol cross-linking (26), leading to MPT independently of the presence of ROS, was not inhibited by TSA or ebselen (results not shown). This indicates that neither TSA nor ebselen are capable of inhibiting MPT by directly reducing mitochondrial membrane protein thiol groups.

Recently, Scorrano et al. (12) attempted to reproduce our results showing that MPT is inhibited by catalase and anoxia, obtaining in both situations only a partial inhibition. Based on these results, the authors concluded that MPT was stimulated but not dependent on ROS. We believe that the partial inhibition of ROS-induced MPT by H\(_2\)O\(_2\) removing compounds is due to the fact that mitochondria-generated H\(_2\)O\(_2\) must diffuse through the membrane in order to be removed by catalase, ebselen, or TSA. Therefore, if mitochondrial ROS generation is too intense, as occurs under experimental conditions in which mitochondrial permeabilization is very fast, part of the generated H\(_2\)O\(_2\) may accumulate, decreasing the inhibitory effect of these peroxidases. Indeed, the authors (12) observed that catalase presented a larger inhibitory effect on MPT promoted under low mitochondrial Ca\(^{2+}\) loads. The observation that MPT induced by uncouplers was not completely inhibited by the lack of molecular oxygen, as detected by a Clarke electrode (12), is not so readily explained, since mitochondria in this situation are expected not to generate ROS. However, it is important to remember that even under very low oxygen tensions, many organic molecules remain tightly bound to molecular oxygen. The experiments shown in Fig. 4 were designed to verify the efficiency of oxygen removal from a mitochondrial suspension. Here, respiring mitochondria were incubated in a closed glass chamber in the presence of the proton ionophore carbonyl cyanide p-trifluoromethoxyphenylnitrazone and allowed to gradually consume the oxygen present in the reaction medium. After different time periods, mitochondrial respiration was inhibited with antimycin, resulting in different final oxygen tensions, as measured by a Clarke electrode. After total inhibition of mitochondrial respiration occurred, mitochondrial swelling (●) and membrane protein thiol oxidation (■) induced by Ca\(^{2+}\) and P added at different oxygen tensions (see numbers in parentheses) were measured. Under these experimental conditions, in which mitochondria were uncoupled and Ca\(^{2+}\) uptake was driven through a high Ca\(^{2+}\) gradient (300 \(\mu\)M Ca\(^{2+}\), see Refs. 5 and 8), mitochondrial swelling and membrane protein thiol oxidation decreased after oxygen concentrations were progressively lowered by mitochondrial respiration. Interestingly, we observed that when mitochondria were allowed to respire during 12 min, and oxygen concentration measured by a Clarke electrode indicated an apparent total anoxia (see numbers in parentheses in Fig. 4), a small but significant mi-
tochondrial swelling and membrane protein thiol oxidation could still be observed. This swelling did not occur when mitochondrial swelling were allowed to respire for an additional period of 3 min, suggesting that even when the electrode indicates complete anoxia in the reaction medium, a small quantity of oxygen may still be present in the mitochondrial suspension, allowing for the generation of ROS and occurrence of MPT.

In conclusion, the present results show that MPT is fully inhibited by the lack of molecular oxygen or the presence of one of three compounds capable of removing extramitochondrial H₂O₂, namely catalase, ebselen, and TSA. TSA is known to protect biomolecules from oxidative damage due to its thiol peroxidase activity (20), and the results obtained suggest that it may play an important role in vivo preventing the occurrence of MPT. MPT inhibition by TSA can be attributed to its hydrogen peroxide removal activity, since no inhibition was observed in the presence of C47S (Fig. 1, line f), a site-specific mutant of TSA which lacks thiol peroxidase activity and does not protect biomolecules from oxidative damage (18–20). Yeast TSA possesses mammalian isoforms, located in both mitochondria (23, 29) and the cytosol (18), although the intramitochondrial forms are probably more important for inhibition of MPT. SP-22 is one of the mammalian mitochondrial isoforms of TSA and seems to account for about 5% of mitochondrial matrix proteins in the bovine adrenal cortex, where steroid hydroxylation generates large amounts of ROS (23). Also, the TSA isoform thioredoxin peroxidase was shown to inhibit endogenous H₂O₂ accumulation which precedes apoptosis induced by a variety of compounds (30), strongly suggesting that these enzymes are important in the defense of mammalian mitochondria against oxidative stress.

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