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Oxidative Stress Underlies the Mechanism for Ca^{2+}-induced Permeability Transition of Mitochondria

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Recent studies demonstrated that the generation of intracellular reactive oxygen species (ROS) was enhanced prior to the onset of mitochondrial membrane permeability transition (MPT), a critical step for the induction of DNA fragmentation and apoptosis. Although Ca^{2+} induces typical MPT that involves depolarization and swelling of mitochondria and finally releases cytochrome c into cytosol, the mechanism by which ROS induce MPT remains unclear. In the presence of inorganic phosphate, Ca^{2+} increased the oxygen consumption and ROS production by isolated mitochondria as determined by a chemiluminescence (CHL) method using L-012. Ca^{2+} increased the generation of H_2O_2 by some mechanism that was inhibited by cyclosporin A but not by superoxide dismutase (SOD) and trifluoperazine. Ca^{2+} decreased the content of free thiols in adenine nucleotide translocase (ANT) in mitochondrial membranes with concomitant increase in ROS generation. The presence of cyclosporin A, trifluoperazine, or SOD inhibited the Ca^{2+}-induced increase of L-012 CHL and decrease in the free thiols of ANT. These results indicate that Ca^{2+} increases the generation of ROS which oxidize the free thiol groups in mitochondrial ANT, thereby inducing MPT to release cytochrome c.

Keywords: Adenine nucleotide translocase; Apoptosis; Cyclosporin A; Membrane permeability transition; Protein thiol; Reactive oxygen species

Abbreviations: ANT, adenine nucleotide translocase; CHL, chemiluminescence; diS-C3-(5), 3,3′-dipropyl-2, 2′thiodicarbocyanine iodide; HRP, horse radish peroxidase; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl-hydrazone; L-012, 8-amino-5-chloro-7-phenulpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dion; MPT, membrane permeability transition; SOD, superoxide dismutase

INTRODUCTION

It has been well documented that the decline of the respiratory function decreases mitochondrial ATP generation but enhances the production of reactive oxygen species (ROS).[1] Recent studies demonstrated that the generation of ROS was enhanced prior to the onset of DNA fragmentation leading to apoptosis.[2,3] Several lines of evidence indicate that mitochondria play important roles in the induction of apoptosis through opening membrane permeability transition (MPT) pore.[4] When cytochrome c and related proteins are released from mitochondria through MPT, they activate caspases to induce apoptosis.[5] Thus, mitochondria play critical roles in determining both the survival and death of a cell through energy transduction and release of apoptosis-related proteins, respectively. The classical type of MPT characterized by depolarization, swelling and high sensitivity to cyclosporin A and trifluoperazine is induced by Ca^{2+} in the presence of inorganic phosphate (Pi) and respiratory substrate.[6] Recent studies reported that Pi plus Ca^{2+} enhanced the production of H_2O_2 and induced MPT.[7–9] However, the mechanism for the induction of MPT and its relationship with the ROS generated by mitochondria remains unclear. The present work describes the effect of cyclosporin A, trifluoperazine and superoxide dismutase (SOD) on

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the Ca^{2+}-induced generation of ROS by mitochondria, the thiol status of their adenine nucleotide translocase (ANT) and the induction of MPT. The different effects of cyclosporin A and trifluoperazine on the induction of oxidative stress in mitochondria were also described. The results clearly indicated that the ROS generated in mitochondria oxidized the critical thiol groups of ANT, thereby inducing the release of cytochrome c to initiate the sequence of events leading to apoptosis.

MATERIALS AND METHODS

Chemicals

Antipyrylazo III, cyclosporin A, carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone (FCCP) and horseradish peroxidase (HRP) were obtained from Sigma Company Ltd. (USA). *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) and anti-cytochrome c antibody were purchased from Molecular Probes (USA) and PharMingen (USA), respectively. Anti-ANT antibody and 8-amino-5-chloro-7-phenylpyrido [3,4-*d*] pyridazine-1, 4-(2*H*, 3H) dion (L-012) were donated from Dr Hiroshi Terada (Tokushima University, Japan) and Dr Isuke Imada (Osaka City University, Japan), respectively. A cyanine dye 3,3′-dipropyl-2, 2′-thiodicarbocyanine iodide [diS-C3-(5)] was obtained from Kanko-Shikiso Research Institute (Japan).

Assay for Mitochondrial Functions

Oxygen consumption of mitochondria was measured using an oxygen electrode. For the assay of swelling and membrane potential, mitochondria (0.1 mg protein/ml) were incubated in a standard medium (10 mM Tris–HCl, pH 7.4, containing 150 mM KCl) at 25°C. The change in the absorbance at 540 nm was recorded in a Shimadzu UV-3000 dual-wavelength spectrophotometer equipped with a magnetic stirrer and thermostatic control. Membrane potential of mitochondria was measured by fluorescence intensity of diS-C3-(5) at 670 nm during excitation at 622 nm.[10] Uptake of Ca^{2+} was analyzed by monitoring the fluorescence intensity of antipyrylazo III at 720–790 nm.[11] Uptake and release of Ca^{2+} by mitochondria were analyzed by adding 0.1 μM FCCP at the end of each experiment.

Assay for Mitochondrial Generation of ROS

Because mitochondria contain a large amount of Mn-SOD that effectively dismutates the superoxide radical, a highly sensitive chemiluminescence (CHL) probe, L-012, was used for the analysis of ROS. Isolated rat liver mitochondria (0.1 mg protein/ml) were incubated in the medium containing 2.5 mM succinate and 1 mM phosphate buffer (pH 7.4) in the presence of 100 μM L-012 at 25°C.[12] After incubation for 1 min, the reaction was started by adding 20 μM $CaCl_2$. During the incubation, CHL intensity was recorded continuously for 20 min using an Intracellular Ion Analyzer (Jasco CAF-110).

Generation of H_2O_2 was assayed at 25°C in a fluorescence spectrophotometer (Hitachi 650-10LC) using an Amplex Red/HRP system.[13] Mitochondria (0.1 mg protein/ml) were suspended in the standard medium containing 1 mU/ml of HRP and 20 μM Amplex Red in the presence or absence of 20 μM $CaCl_2$. The change in the fluorescence intensity was measured at 590 nm with excitation at 550 nm.

Western Blotting

After SDS-polyacrylamide gel electrophoresis, proteins were transferred electrophoretically from the gel onto an Immobilon membrane (Millipore, Waltham, MA). The membrane was blocked in TBS (0.15 M NaCl, 10 mM Tris–HCl, pH 7.4) containing 5% skim milk, and then incubated with the primary antibodies diluted with TBS containing 0.05% Tween 20 (TBST) at room temperature for 1 h. After washing three times in TBST, the membrane was incubated at room temperature for 1 h with the HRP-conjugated secondary antibody (DAKO, Glostrup, Denmark) diluted in TBST. Immunoreactive bands were visualized using an ECL system (Amersham Biotech, Uppsala).[14]

Analysis of Cytochrome c release

The release of cytochrome c from mitochondria was analyzed as described previously.[14] Mitochondria (0.1 mg protein/ml) suspended in the standard medium containing 2.5 mM succinate and 1 mM phosphate buffer (pH 7.4) were incubated with 15 μM $CaCl_2$ at 25°C for 10 min in the presence or absence of 1 μM cyclosporin A or 20 μM trifluoperazine. After centrifugation at 7000 × *g* for 10 min, the supernatants were added to a 0.5 volume of SDS-sample buffer containing 125 mM Tris–HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue. After incubation at 100°C for 5 min, the samples were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting analysis using specific antibodies and ECL kit (Amersham).

Analysis of Thiol Status of Mitochondrial Proteins

The thiol status of mitochondrial proteins and low molecular weight compounds were analyzed as

described previously.[15] Briefly, mitochondria (0.5 mg protein) were suspended in ice-cold 10% trichloroacetic acid (w/v) containing 1 mM diethylenetriamine-penta-acetic acid. The suspension was centrifuged for 5 min at 12,000 × *g* and 4°C. The acid-insoluble precipitate and soluble supernatant were suspended in 0.5 M potassium phosphate buffer (pH 7.4) containing 0.2 mM 5, 5′-dithiobis-(2-nitrobenzoic acid) and 5 mM EDTA, and then incubated at 4°C for 30 min. After removing insoluble materials by centrifugation at 12,000 × *g* for 5 min, optical density of the supernatant was measured at 412 nm.

Thiol status of ANT was analyzed using phenylarsine oxide-conjugated agarose (ThioBond resin, Invitrogen, Carlsband, CA) as described previously.[15] Briefly, mitochondria (2 mg protein) treated with 100 μM $CaCl_2$ in the presence or absence of various reagents were centrifuged at 12,000 × *g* and 4°C for 5 min. Proteins in the precipitate were solubilized in 100 μl of 50 mM HEPES buffer (pH 7.4) containing 150 mM Na_2SO_4, 1 mM EDTA, 3% Triton X-100, 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 1 μM leupeptine. After centrifugation of the lysate at 15,000 × *g* and 4°C for 5 min, the supernatant fraction was incubated with 4-aminophenylarsine oxide-conjugated agarose equilibrated with 50 mM HEPES buffer (pH 7.4) containing 150 mM Na_2SO_4, 1 mM EDTA, 0.25% Triton X-100, 1 mM PMSF and 1 μM leupeptine. After incubation at 4°C for 15 min, the agarose was washed three times with 0.5 ml of the buffer by centrifugation at 15,000 × *g* and 4°C for 1 min. Proteins bound to the agarose were eluted by 50 μl of the buffer containing 10 mM dithiothreitol, and analyzed by SDS-PAGE followed by Western blotting using anti-ANT antiserum.

RESULTS

Mitochondrial Ca^{2+} Transport and Respiration

In the presence of respiratory substrates and Pi, mitochondria rapidly accumulated Ca^{2+} and then released it after being uncoupled by high concentrations of Ca^{2+} (Fig. 1A). The release of Ca^{2+} from mitochondria was suppressed either by cyclosporin A or trifluoperazine. Other types of phospholipase A_2 inhibitors, such as chlorpromazine, also inhibited the release of Ca^{2+} from mitochondria (data not shown).

In the presence of Pi and succinate, Ca^{2+} increased the oxygen consumption of mitochondria in a biphasic manner (Fig. 1B). The second phase of the increased oxygen consumption

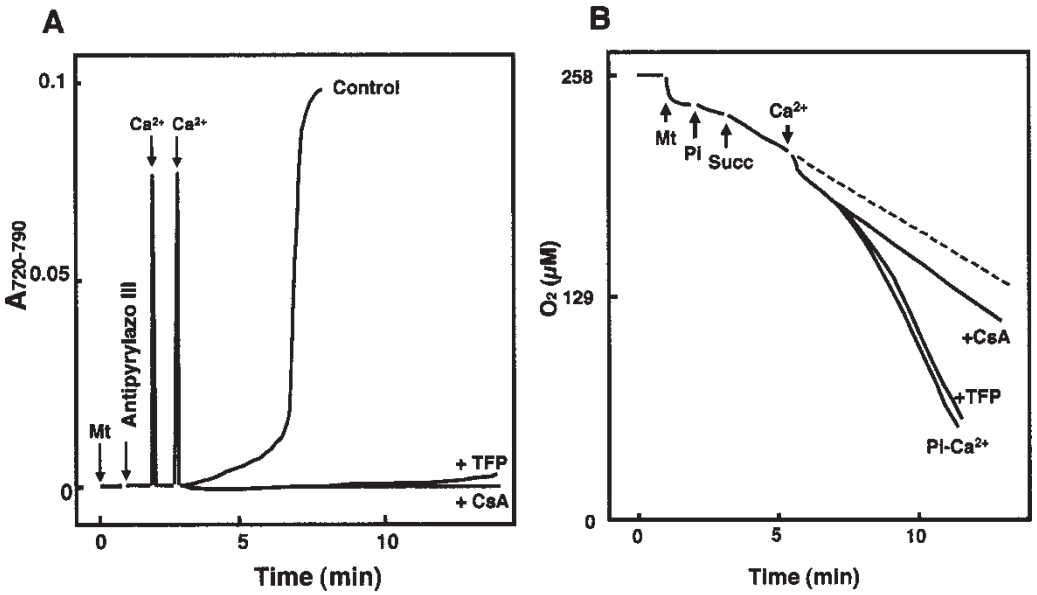

FIGURE 1 *Effect of cyclosporin A and trifluoperazine on Ca^{2+}-induced Ca^{2+} transport and oxygen uptake*. Mitochondria (0.5 mg protein/ml) were incubated in the standard medium (10 mM Tris–HCl, pH 7.4, containing 150 mM KCl) at 25°C in the presence or absence of the reagents. (A) Effect of cyclosporin A (CsA) and trifluoperazine (TFP) on the release of Ca^{2+} from mitochondria. Mitochondria were incubated in the standard medium consisting of 2 mM Pi, 5 mM succinate, and 50 μM antipyrylazo III, and Ca^{2+} transport was monitored by the difference in the absorbance at 720–790 nm. Ca^{2+}-transport in mitochondria was induced by adding 50 μM Ca^{2+} in the presence or absence of 1 μM cyclosporin A and 20 μM TFP. Cyclosporin A and trifluoperazine were added in the incubation medium prior to start the experiment. Similar results were obtained in three separate experiments. (B) Effect of cyclosporin A and trifluoperazine on the Ca^{2+}-induced oxygen uptake by mitochondria was analyzed using an oxygen electrode in the presence of 2 mM Pi and 5 mM succinate. Added concentrations of Ca^{2+}, cyclosporin A and trifluoperazine were 100 μM, 1 μM and 20 μM, respectively. Similar results were obtained with three separate experiments.

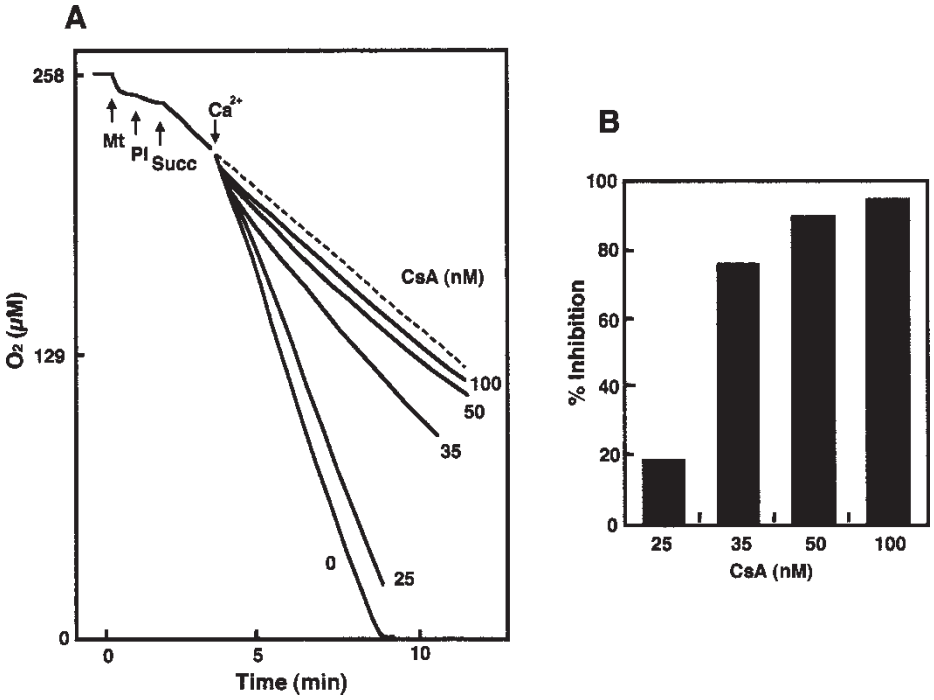

FIGURE 2 *Inhibition of Ca^{2+}-induced oxygen uptake of mitochondria by cyclosporin A.* Experimental conditions were the same as described in Fig. 1. Oxygen uptake was suppressed by cyclosporin A (A) in a concentration dependent manner (B). Similar results were obtained with three separate experiments.

was due to uncoupling of oxidative phosphorylation as described by Shinohara *et al*.[16] The uncoupled respiration was suppressed by the presence of cyclosporin A but not trifluoperazine, an inhibitor of phospholipase A_2, respectively. The suppressing activity of cyclosporin A was concentration dependent, 50 nM of the agent was required for the suppression (Fig. 2).

Effect of Ca^{2+} on the Swelling and Membrane Potential of Mitochondria

It has been reported that the release of Ca^{2+} from mitochondria is associated with their swelling.[17] In fact, Ca^{2+} induced swelling of mitochondria in a biphasic manner (Fig. 3A). Both phases of the swelling were inhibited by the presence of either cyclosporin A or trifluoperazine. Ca^{2+} also induced depolarization of mitochondria by a mechanism that was suppressed by the presence of either cyclosporin A or trifluoperazine (Fig. 3B).

ROS Generation by Ca^{2+}-treated Mitochondria and its Sensitivity to Cyclosporin A and Trifluoperazine

To elucidate the mechanism by which Ca^{2+} induced uncoupling, swelling and depolarization of mitochondria, possible occurrence of ROS was tested using highly sensitive CHL probe L-012; L-012 CHL predominantly reflects the generation of superoxide and hydroxyl radicals.[12] When Ca^{2+} was added to mitochondrial suspension, the CHL intensity increased rapidly and then decreased thereafter (Fig. 4A). The Ca^{2+}-dependent increase in the CHL was inhibited by cyclosporin A in a concentration dependent manner. To obtain complete suppression, 100 nM of cyclosporin A was required (Fig. 4B). Similar inhibition of L-012 CHL was also observed with trifluoperazine, superoxide dismutase (SOD), and 10 μM deferoxamine, a potent chelater for iron (data not shown).

To determine the site(s) and chemical nature of ROS generated by Ca^{2+}-treated mitochondria, generation of H_2O_2 was measured in the presence of rotenone using an Amplex Red/HRP system. Ca^{2+} increased the fluorescence intensity of Amplex Red in the presence but not absence of succinate (Fig. 5). The enhanced generation of H_2O_2 was further increased by the presence of antimycin A, a blocker of ubisemiquinone. The increased generation of H_2O_2 was inhibited by cyclosporin A in a concentration dependent manner; 50 nM of the agent was required for the suppression of H_2O_2 generation (Fig. 6A and B). However, no significant effect was observed with SOD and trifluoperazine (Fig. 6A). These results indicate that the electron leakage from coenzyme Q in mitochondrial inner

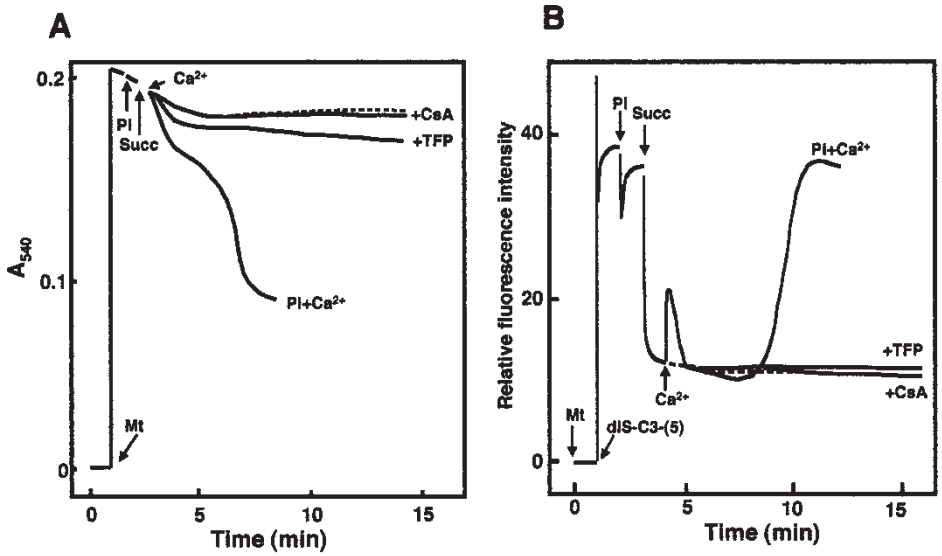

FIGURE 3 *Effect of Ca^{2+} on the swelling and depolarization of mitochondria*. (A) Mitochondria (0.1 mg protein/ml) were incubated in the standard medium, and their swelling was monitored spectroscopically at 540 nm. Mitochondrial swelling was induced by adding 15 μM Ca^{2+} and 1 mM Pi in the presence or absence of 2.5 mM succinate, 1 μM cyclosporin A (CsA) and 20 μM trifluoperazine (TFP). (B) Membrane potential was monitored using diS-C3-(5). Mitochondria were incubated in the standard medium containing 0.2 μg/ml diS-C3-(5) at 25°C. Membrane depolarization was induced by 20 μM Ca^{2+}. Other conditions were the same as described in (A). Similar results were obtained in three separate experiments.

membranes underlies the mechanism for the Ca^{2+}-induced generation of ROS.

Effect of Ca^{2+}, Cyclosporin A and Trifluoperazine on Mitochondrial Thiol Status and Cytochrome c Release

Because Ca^{2+} stimulated the generation of ROS, mitochondrial constituents might be exposed to oxidative stress. Thus, we analyzed the effect of Ca^{2+} on the thiol status of mitochondria. Figure 7 shows the effect of Ca^{2+} on the concentrations of protein-associated and low molecular weight thiols in mitochondria. Levels of free thiol groups of both proteins and low molecular weight compounds in mitochondria remained unaffected during the experiments (~30 min).

ANT is an important component for the ADP/ATP exchange reaction across the inner membranes of mitochondria. ANT has three critical thiol groups; the cross linking between the Cys^{160} and Cys^{257} increases the affinity of ANT for cyclophilin D, thereby inducing MPT through inhibition of the ADP/ATP exchange reaction.[18] To test the possibility that

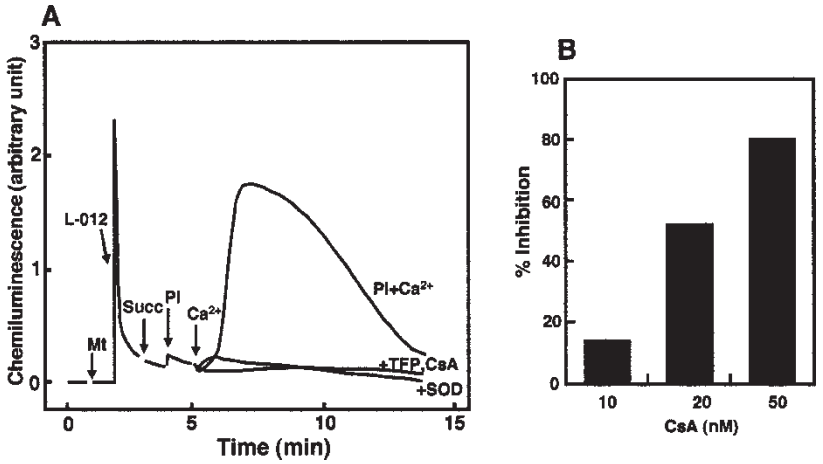

FIGURE 4 *Effect of Ca^{2+} on the ROS generation by mitochondria*. Mitochondria (0.1 mg protein/ml) were incubated in the standard medium containing 100 μM L-012 in the presence of various reagents at 25°C. Concentrations of succinate, Pi, Ca^{2+}, cyclosporin A (CsA), trifluoperazine (TFP), and SOD were 2.5 mM, 1 mM, 20 μM, 1 μM, 20 μM, and 100 mU/ml, respectively. (A) ROS generation was induced by Ca^{2+} after addition of succinate and Pi. L-012 CHL was monitored by an intracellular Ca^{2+} Analyzer (Jasco CAF-110, CHL mode). (B) The ROS generation was suppressed by cyclosporin A in a concentration dependent manner. Similar results were obtained with three separate experiments.

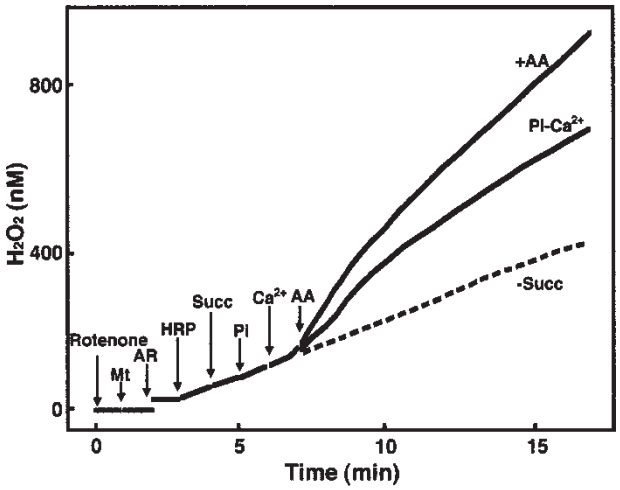

FIGURE 5 *Effect of Ca^{2+} on H_2O_2 generation by mitochondria.* Mitochondria (0.1 mg protein/ml) were incubated in the standard medium containing 1 mU/ml HRP, 20 μM Amplex Red, and 5 μM rotenone. H_2O_2 was generated by adding 20 μM $CaCl_2$ in the presence of 1 mM Pi and 2.5 mM succinate. Generation of H_2O_2 was increased by 0.5 μM antimycin A. The change in the fluorescence intensity was measured at 590 nm with excitation at 550 nm. Similar results were obtained with three separate experiments.

the enhanced generation of ROS might affect the thiol status of ANT, effect of Ca^{2+} was examined using phenylarsine oxide-conjugated agarose which selectively interacts with vicinal thiols in various proteins.[15] Figure 8 shows that ANT from intact mitochondria bound to the phenylarsine oxide-conjugated agarose by a mechanism that was inhibited by pretreating mitochondria with Ca^{2+}. The inhibitory effect of Ca^{2+} on the binding of ANT to the agarose was suppressed by the presence of either cyclosporin A or trifluoperazine during the incubation.

It has been well documented that MPT occurs when thiol groups of inner membrane proteins, including ANT, are oxidized to induce conformational changes that form large non-selective pores.[9] Thus, oxidation of the critical thiols of ANT induces the release of cytochrome c from mitochondria. In fact, treatment of mitochondria with Ca^{2+} released cytochrome c by a mechanism that was inhibited by either cyclosporin A or trifluoperazine (Fig. 9).

DISCUSSION

The present work describes that, in the presence of succinate and Pi, relatively high concentrations of Ca^{2+} transiently accumulated in mitochondria and increased the generation of ROS by releasing free electrons from coenzyme Q with concomitant induction of mitochondrial swelling, depolarization and uncoupling by a mechanism that was inhibited by cyclosporin A and trifluoperazine. The results also show that the ROS generated by Ca^{2+}-treated mitochondria selectively oxidized the critical thiol groups of ANT to induce MPT that release cytochrome c by a mechanism that was inhibited by cyclosporin A and trifluoperazine.

It should be noted that the Ca^{2+} release, swelling and depolarization of mitochondria were inhibited by either cyclosporin A or trifluoperazine while the uncoupled respiration was inhibited only by the former. Thus, the two inhibitors might have

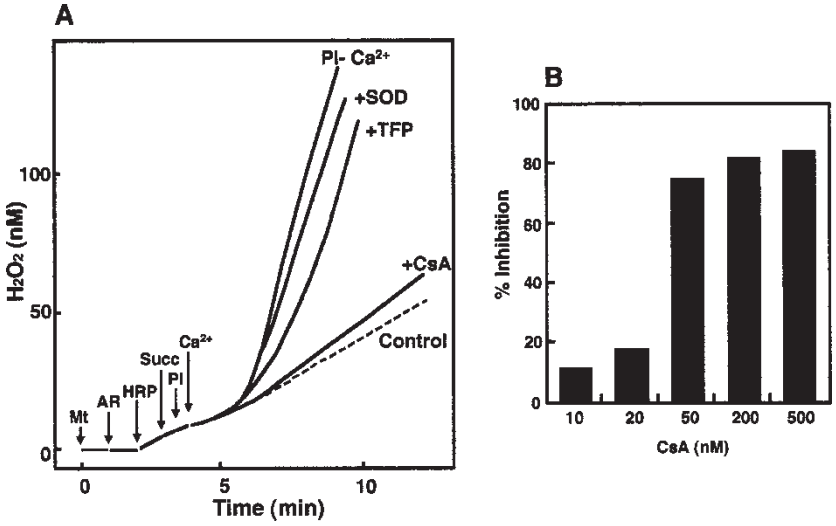

FIGURE 6 *Effect of various compounds on Ca^{2+}-induced generation of H_2O_2.* Experimental conditions were the same as described in Fig. 5. (A) Concentrations of succinate, Pi, Ca^{2+}, cyclosporin A, trifluoperazine (TFP) and SOD were 2.5 mM, 1 mM, 20 μM, 1 μM, 20 μM, and 5 U/ml, respectively. (B) Suppression by cyclosporin A of the H_2O_2 generation occurred in a concentration dependent manner. Similar results were obtained with three separate experiments.

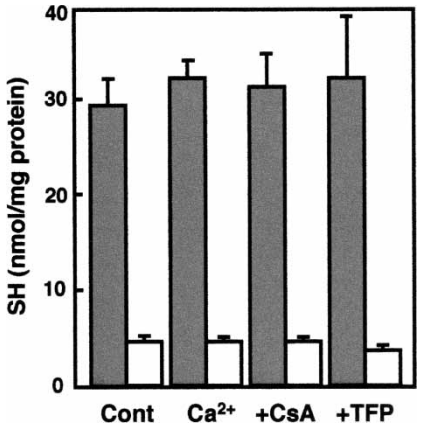

FIGURE 7 *Effect of various reagents on protein-associated and low molecular weight thiols*. Mitochondria (0.5 mg protein/ml) were incubated at 25°C for 5 min in the standard medium containing 2.5 mM succinate, 1 mM Pi, 100 μM Ca^{2+} in the presence or absence of 1 μM cyclosporin A (CsA) and 50 μM trifluoperazine (TFP). The protein-associated (hatched columns) and low molecular weight thiols (open columns) were measured spectrophotometrically as described in the "Materials and Methods Section". Data are expressed by mean ± S.E. from three separate experiments.

interacted with different site(s) in mitochondria to inhibit MPT. Kinetic analysis suggested that cyclosporin A inhibited the initial step of ROS generation while trifluoperazine suppressed the peroxidation process induced by the generated ROS.

Because mitochondria are highly enriched with Mn-SOD, it has been practically difficult to analyze the occurrence of the superoxide in and around intact mitochondria. The present work used a highly sensitive CHL probe L-012,[12] which permitted studies on the generation of superoxide and related

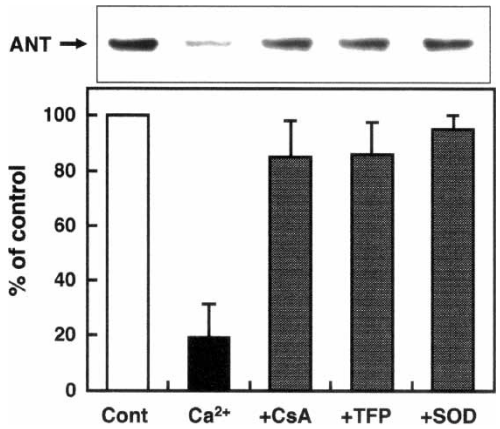

FIGURE 8 *Effect of Ca^{2+} on the thiol groups of ANT*. Mitochondria (2 mg/ml) were incubated at 25°C for 5 min in the standard medium containing 2.5 mM succinate, 1 mM Pi, 100 μM Ca^{2+} in the presence or absence of 1 μM cyclosporin A (CsA), 50 μM trifluoperazine (TFP), and 5 U/ml SOD. Then the samples were lysed with Triton X-100 at 4°C for 15 min in the presence of phenylarsine oxide-agarose (25 μl/mg of protein). Proteins bound to the agarose were eluted with 10 mM dithiothreitol and subjected to a Western blot analysis using anti-ANT antibody. Data are expressed as mean ± S.E. derived from three separate experiments.

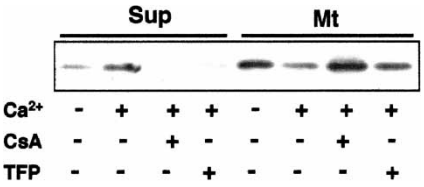

FIGURE 9 *Suppression of Ca^{2+}-induced cytochrome c release from mitochondria*. Mitochondria (0.1 mg protein/ml) were incubated in the standard medium containing 1 mM Pi and 2.5 mM succinate at 25°C. Cytochrome c release was induced by 15 μM Ca^{2+} for 10 min in the presence or absence of 1 μM cyclosporin A (CsA) and 20 μM trifluoperazine (TFP). After incubation, the mixture was fractionated into mitochondria (Mt) and supernatant (Sup). Cytochrome c was detected by Western blot using anti-cytochrome c antibody. Similar results were obtained with three separate experiments.

metabolites. Although ROS generation by Ca^{2+}-treated mitochondria was insensitive to antimycin A in the presence of rotenone, it required the presence of succinate as a respiratory substrate. Thus, the release of free electron from coenzyme Q in mitochondrial inner membranes seems to be responsible for the generation of the superoxide radical. Kinetic analysis using L-012 and Amplex Red revealed that the superoxide generated by Ca^{2+}-treated mitochondria is converted to H_2O_2. Because deferoxamine also inhibited the Ca^{2+}-induced L-012 CHL, the occurrence of the hydroxyl radical seems to be responsible, at least in part, for the increased L-012 CHL. Because mitochondria have been known to contain small amounts of free iron,[19] it is not surprising that H_2O_2 was converted to hydroxyl radical by iron-catalyzed Fenton reaction.

It should be also noted that the Ca^{2+}-induced H_2O_2 generation in mitochondria was inhibited by cyclosporin A but not by trifluoperazine. Kinetic analysis of the inhibitory action of cyclosporin A supports the notion that cyclosporin A inhibited the initial step of ROS generation while trifluoperazine suppressed its the later step.

It is conceivable that generation of ROS by Ca^{2+}-treated mitochondria oxidatively impaired membrane constituents to induce MPT. In this context, Kowaltoswski *et al.*[7–9] reported that H_2O_2 generated by Ca^{2+}-treated mitochondria oxidatively injured lipids and proteins in mitochondria, thereby inducing MPT. Zhang *et al.*[20] also reported that ROS derived from Complex III oxidized ANT to induce MPT, thereby releasing cytochrome c to induce apoptosis.

Accumulated evidences indicate that MPT is prevented by thiol reductancts such as dithiothreitol,[21–23] while thiol oxidants such as diamide and phenylarsine oxide promoted MPT.[24,25] Thus, thiol oxidants and reductants seem to regulate MPT by affecting the redox state of the critical thiols relating to MPT pore formation. MPT occurs when thiol groups of inner membrane proteins are oxidized to

induce conformational changes that form large non-selective pores. Thus, the cross-linkage of thiol groups seems to be essential for the incubation of the conformational change, because only dithiol reagents promoted MPT. In fact, cross-linking of inner membrane proteins was observed with mitochondria after induction of MPT.[21,23,26,27]

To our surprise, no direct evidence showing the oxidation of the critical thiol residues in ANT have been reported with the Ca^{2+}-treated mitochondria. In this context, McStay *et al*.[18] suggested that oxidation of the Cys^{160} and Cys^{257} in ANT markedly increased the affinity of cyclophilin D to the Pro^{62} residue of the protein, thereby inducing MPT. The present work using phenylarsine oxide-conjugated agarose shows that the ROS generated by Ca^{2+}-treated mitochondria oxidatively modified the critical thiol groups in ANT, thereby inducing MPT to release cytochrome c. Both oxidative modification of the thiol groups of ANT and the release of cytochrome c were inhibited either by cyclosporin A or trifluoperazine. Thus, oxidative modification of the critical thiol groups in ANT by Ca^{2+}-treated mitochondria might increase the affinity of this protein for cyclophilin D to induce MPT.

We previously reported that tributyltin-induced cytochrome c release is inhibited selectively by dithiothreitol and 2,3-dimercaptopropane but not by monothiols.[15] Thus, some vicinal dithiols and/or two proximal thiols of ANT and related proteins in mitochondrial membranes might underlie the mechanism for the Ca^{2+}-induced release of cytochrome c.[15] As observed in Fig. 7, treatment of mitochondria with Ca^{2+} decreased the binding of ANT to phenylarsine oxide conjugated agarose. This finding suggests that Ca^{2+} enhanced the cross-linking of the two thiol groups of ANT, such as Cys^{160} and Cys^{257}, thereby inducing MPT to release cytochrome c.

It has been well known that Ca^{2+} has multiple sites of action inside cells. For example, Ca^{2+} interacts with the carboxyatractylate binding site of ANT to decrease membrane potential of mitochondria.[28] Ca^{2+} also activates phospholipase A_2, thereby releasing free fatty acids which induce uncoupling of mitochondria[29] by a mechanism that was inhibited by cyclosporin A.[30] It has been also reported that free fatty acids interact with ANT at its atractyloside-binding site.[31] Because trifluoperazine has been known to inhibit phospholipase A_2, the effect of this inhibitor on the Ca^{2+}-induced swelling and depolarization of mitochondria may reflect the suppression of the release of free fatty acids. However, recent studies suggested that the surface potential effect of trifluoperazine might explain the synergism between this compound and cyclosporin A as MPT inhibitors.[32] It should be noted that trifluoperazine has a potent activity as an antioxidant.[33] Preliminary experiments in this laboratory revealed that, among various inhibitors of phospholipase A_2, only those with antioxidant activity showed strong inhibition of mitochondrial swelling induced by Ca^{2+}. Thus, the antioxidant activity of trifluoperazine might also be responsible for its inhibitory effect on the Ca^{2+}-induced MPT. This notion is consistent with the hypothesis that MPT could be suppressed by cyclosporin A and trifluoperazine inhibition of the initial step of ROS generation and peroxidative injury of mitochondria by ROS, respectively.[33]

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