British Journal of Pharmacology (2005) 145, 469–476
www.nature.com/bjp

Ciclopirox protects mitochondria from hydrogen peroxide toxicity

[1]Sun J. Lee, [1]Youngnam Jin, [1]Hye Young Yoon, [2]Byung-Ok Choi, [3]Hyoung Chun Kim, [4]Yu-Kyoung Oh, [5]Hee-Sun Kim & *,[1,5]Won-Ki Kim

[1]Department of Pharmacology, College of Medicine, Ewha Institute of Neuroscience, Tongdaemoon Hospital, Ewha Womans University, 70 Chongro-6-ga, Chongroku, Seoul 110-783, Republic of Korea; [2]Department of Neurology, College of Medicine, Ewha Institute of Neuroscience, Tongdaemoon Hospital, Ewha Womans University, 70 Chongro-6-ga, Chongroku, Seoul 110-783, Republic of Korea; [3]College of Pharmacy, Kang Won National University, Chunchon, Kwangwon-do, Republic of Korea; [4]Department of Microbiology, College of Medicine, Pochon CHA University, Pochon, Kyoungki-do, Republic of Korea and [5]Laboratory of Neurodegenerative Diseases, Ewha Institute of Neuroscience, Tongdaemoon Hospital, Ewha Womans University, 70 Chongro-6-ga, Chongroku, Seoul 110-783, Republic of Korea

1 The mitochondrial respiratory chain produces reactive oxygen species (ROS) during normal electron transport. Despite producing ROS, mitochondria are vulnerable to oxidative stress. Mitochondrial dysfunction has been associated with many degenerative diseases, making it important to identify compounds that protect mitochondria from ROS-mediated toxicity. Here we report that ciclopirox (CPX) blocks H_2O_2-induced mitochondrial injury by maintaining mitochondrial transmembrane potential ($\Delta\psi m$).

2 CPX completely blocked H_2O_2-stimulated release of lactate dehydrogenase (a marker of cell death) and decrease in MTT reduction (a marker of mitochondrial function) in adenocarcinoma SK-HEP-1 cells.

3 H_2O_2 rapidly depolarized the $\Delta\psi m$, and CPX blocked this H_2O_2-stimulated $\Delta\psi m$ decrease. Similar data were obtained in experiments using mitochondria isolated from rat liver.

4 Furthermore, CPX effectively inhibited H_2O_2-induced mitochondrial permeability transition pore (MPTP) opening. In de-energized mitochondria, however, CPX did not inhibit Ca^{2+}-evoked MPTP opening, indicating that CPX is not a direct inhibitor of the MPTP.

5 Oxygen consumption studies showed that in the presence of pyruvate and malate CPX restored the rate of state 3 to state 4 respiration decreased by H_2O_2. Consistent with this, CPX replenished ATP levels lowered by H_2O_2.

6 The present results indicate that CPX protects SK-HEP-1 cells from H_2O_2 cytotoxicity by inhibiting $\Delta\psi m$ decrease and indirectly preventing MPTP opening.
British Journal of Pharmacology (2005) **145,** 469–476. doi:10.1038/sj.bjp.0706206
Published online 4 April 2005

Keywords: Ciclopirox; hydrogen peroxide; mitochondrial transmembrane potential; mitochondrial permeability transition pore; cell death

Abbreviations: CPX, ciclopirox; CsA, cyclosporin A; $\Delta\psi m$, mitochondrial transmembrane potential; MPTP, mitochondrial permeability transition pore; NEM, *N*-ethyl maleimide; ROS, reactive oxygen species

Introduction

The mitochondrial respiratory chain is a rich source of reactive oxygen species (ROS) (Boveris *et al*., 1972; Loschen *et al*., 1974). While passing through the mitochondrial electron transport chain, some electrons leak out and interact with oxygen molecules to form O_2^- that is quickly dismutated to H_2O_2 by mitochondrial manganese superoxide dismutase (Fridovich, 1986). Under normal physiological conditions, the concentration of H_2O_2 in cells remains low through the actions of catalase and glutathione peroxidase. However, H_2O_2 can accumulate in some pathological states despite the presence of these enzymes (Hyslop *et al*., 1995; Auerbach and Segal, 1997; Cadenas & Davies, 2000) and is associated with cell damage. The Fenton reaction converts H_2O_2 to the highly toxic hydroxyl radical.

*Author for correspondence; E-mail: wonki@mm.ewha.ac.kr
Published online 4 April 2005

Although mitochondria produce ROS, they are also vulnerable to oxidative stress (Hyslop *et al*., 1988). Mitochondrial dysfunction is associated with many human diseases and aging (Shigenaga *et al*., 1994). When complex I of the respiratory chain is suppressed, oxidative stress induced by H_2O_2 can lower and destroy the mitochondrial transmembrane potential ($\Delta\psi m$) (Chinopoulos *et al*., 1999; Zhuang *et al*., 2000). This $\Delta\psi m$ collapse may be a critical event in cell injury during ischemia and Parkinson's disease (Murakami *et al*., 1998; Schapira, 1998). Among enzymes involved in the Krebs cycle, aconitase may be the most sensitive to H_2O_2 (Tretter & Adam-Vizi, 2000), and inhibition of α-ketoglutarate dehydrogenase (KGDH) and succinate dehydrogenase (SDH) by the oxidant also limits the amount of NADH available to the respiratory chain (Nulton-Persson & Szweda, 2001). H_2O_2 opens mitochondrial permeability transition pores (MPTP) in the inner membrane (Madesh & Hajnoczky, 2001; Takeyama

et al., 2002), and this is strongly associated with release of apoptogenic factors (Hirsch *et al*., 1998; Crompton, 1999). $\Delta\psi m$ depolarization facilitates MPTP opening Petronilli *et al*. (1993); Bernardi (1992). Upon consumption of ROS, intact mitochondria are required for reactivation of KGDH and SDH that have been inactivated by H_2O_2 (Nulton-Persson & Szweda, 2001). Therefore, mitochondria must remain intact for cells to overcome cytotoxicity caused by H_2O_2 and other oxidants.

Recently, we reported that ciclopirox (CPX), a well-established synthetic antifungal agent (Bohn & Kraemer, 2000), protected astrocytes from peroxynitrite toxicity by inhibiting peroxynitrite-induced mitochondrial dysfunction (Choi *et al*., 2002). Our preliminary experiments showed that CPX effectively blocked H_2O_2-induced injury/death in the adenocarcinoma cell line SK-HEP-1. Using SK-HEP-1 cells and isolated rat liver mitochondria preparations, in the present study we demonstrate that CPX does not directly inhibit the MPTP but prevents H_2O_2-induced MPTP opening and cell death by inhibiting $\Delta\psi m$ decrease. Further delineation of the mechanisms underlying CPX action would provide a new clinical approach for inhibiting cytotoxicity caused by H_2O_2 or other oxidants *via* protection of mitochondrial function.

Methods

Cell culture and measurement of lactate dehydrogenase (LDH) release and MTT reduction

SK-HEP-1 cells were grown on 100-mm dishes in DMEM supplemented with 10% heat-inactivated fetal bovine serum (GIBCO/BRL), at 37°C in a humidified atmosphere containing 5% CO_2. Cell death was assessed by morphological examination of cells using phase-contrast microscopy and quantified by measuring the activity of LDH released into the medium. Cell viability was also determined by MTT assay. In brief, cells were added to MTT (5 mg ml^{-1} in PBS) and incubated at 37°C for 2–3 h. The resulting dark blue crystals were dissolved with an equal volume of isopropanol containing 40 mM HCl. Absorbance was determined at a test wavelength of 570 nm and a reference wavelength of 630 nm using a Microplate reader (SpectraMax 340pc, Molecular Devices, CA, U.S.A.).

Measurement of ATP

The level of intracellular ATP was measured using a method previously described (Tombaugh & Sapolsky, 1992) with slight modification. Briefly, cells were lysed with 10% trichloroacetic acid, sonicated for 1 min on ice, and added with 2 mM EDTA and 2 mg ml^{-1} bovine serum albumin (BSA). After centrifugation, the supernatant was collected, neutralized with 4 N KOH and the ATP content determined using a luminescence detection kit (Molecular Probes, Eugene, OR, U.S.A.).

Measurement of $\Delta\psi$m in cells

Cells in culture medium were loaded with JC-1 (1.0 μg ml^{-1}; Molecular Probes) for 20 min at 37°C. Depolarization of $\Delta\psi m$ was assessed by measuring fluorescence intensities at 530 and 590 nm emission wavelengths using a fluorescence microplate reader (SpectraMax GeminiXS, Molecular Devices, CA, U.S.A.). During measurements, cells were maintained at 37°C and protected from light. Fluorescence intensity was measured every 15 min or 30 min. All fluorescent measurements were corrected for autofluorescence, which was determined in cells not loaded with JC-1. Autofluorescence was constant throughout the experiment. In control experiments, no photobleaching was observed during fluorescence monitoring.

Isolation of liver mitochondria

Mitochondria were isolated from livers of male (220–280 g) Sprague–Dawley rats (Charles River Breeding Lab, Seoul, Korea). Rats were starved overnight before killing. All steps of the mitochondria isolation were carried out on ice and in a cold medium containing 0.25 M sucrose, 2 mM K^+-EDTA, and 3 mM HEPES, adjusted to pH 7.4 with KOH. The tissue was washed, minced and homogenized on ice using a ground-glass homogenizer. The homogenate was centrifuged at $600 \times g$ for 10 min and the supernatant collected and centrifuged for 20 min at $8000 \times g$. The subsequent mitochondrial pellet washed in EDTA-free medium and used in experiments. Mitochondrial protein concentration was determined by the Bradford (1976) method using BSA as the standard.

All experimental procedures using animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Committee of Ewha Women's University College of Medicine.

Measurement of $\Delta\psi$m in isolated mitochondria

$\Delta\psi m$ in isolated mitochondria was measured according to the method described previously (Emaus *et al*., 1986). Mitochondria (0.5 mg protein) were incubated in 2 ml buffer containing 150 mM sucrose, 5 mM $MgCl_2$, 5 mM succinate, 2.7 μM rotenone, 0.2 μM rhodamine 123 (Molecular Probes), 2 μM Ca^{2+}, 50 mM potassium phosphate, and 20 mM HEPES (pH 7.4). The fluorescence intensity was measured at 37°C for 10 min using a Shimadzu spectrofluorophotometer RF-5301PC (Shimadzu, Kyoto, Japan). Cyclosporin A (CsA, Sigma, St Louis, MO, U.S.A.) and other inhibitors were added to the buffer prior to addition of mitochondria.

Measurement of MPTP opening in isolated mitochondria

MPTP opening was assessed by measuring mitochondrial swelling under energized and de-energized conditions, as described previously (Halestrap *et al*., 1997). Mitochondrial swelling was determined spectrophotometrically (Shimadzu UV-2401PC, Kyoto, Japan) by measuring absorbance changes at 540 nm. Mitochondria (0.5–1 mg protein) were incubated at 25°C under energized conditions in 2 ml medium containing 213 mM mannitol, 70 mM sucrose, 3 mM HEPES (pH 7.4), 10 mM succinate, and 1 μM rotenone. When required, de-energization was achieved by incubating in KSCN buffer (150 mM KSCN, 20 mM MOPS, 10 mM Tris, 0.5 μM rotenone, 0.5 μM antimycin, pH 7.4). CsA and other inhibitors were added to the buffer prior to addition of mitochondria.

Determination of sucrose entry into the mitochondria

MPT after H_2O_2 treatment was also investigated by [U-^{14}C] sucrose accumulation in mitochondria, as described before (Al-Nasser and Crompton, 1986). A mitochondrial suspension (0.5 ml) was transferred to test tubes containing H_2O_2 (final concentration = 200 μM), [U-^{14}C]sucrose (0.6 μCi ml^{-1}; Amersham Biosciences, Seoul, Korea) and CPX (10 μM) in a final volume of 1 ml of buffer A (130 mM KCl, 10 mM HEPES, 5 mM $MgCl_2$, 2 mM potassium phosphate, 5 mM potassium succinate, 10 mM glutamate, 5 mM L-malate, 2 mg ml^{-1} essentially fatty-acid free BSA, pH 7.2). Aliquots (200 μl) were withdrawn at the times indicated in the figures, incubated for 30 s with ice-cold 0.25 M sucrose, 5 mM Mops, 5 mM EGTA and 1.5 μM CyA, filtered through Millipore filters (0.45 μm pore size) under a vacuum, and rinsed three times with the same buffer. The radioactivity remaining on the filters was counted by liquid-scintillation spectrometry.

Measurement of mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured polarographically using a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). Mitochondria (1 mg ml^{-1}) were added to 1.5 ml medium containing 0.25 M sucrose, 10 mM potassium phosphate, 5 mM $MgCl_2$, 20 mM KCl, 20 mM Tris-Cl (pH 7.4), and 1 mM EDTA. All assays were performed at 25°C. A measure of 10 mM glutamate plus 5 mM malate, or 5 mM pyruvate plus 5 mM malate, were used to quantify complex I-dependent respiration, while 3.3 mM succinate was used to quantify complex II-dependent respiration. The respiratory control ratio (RCR) was the ratio of the rates of state 3 (with 300 μM ADP and substrate) to state 4 (with substrate only) respiration. The state 4 respiration was measured after the full consumption of ADP.

Measurement of H_2O_2

The concentration of H_2O_2 was measured spectrophotometrically at 546 nm by the peroxidase-catalysed conversion of 4-aminophenazone to a pink-colored substance (Ioannidis & de Groot, 1993). Cells were incubated with 1 mM H_2O_2 for indicated times at 37°C. After 15 min, the concentration of H_2O_2 in supernatants (20 μl) was measured by adding 1 ml reaction mixture composed of 10 parts solution A (4.2 mM 3,5-dichloro-2-hydroxybenzenesulphonic acid, dissolved in MOPS buffer (50 mM MOPS, 50 mM KCl, pH 7.4)) and 1 part solution B (33 mM 4-aminophenazone and 2.3 U ml^{-1} peroxidase (final activity)).

Assay of aconitase activity

Aconitase activity was determined after exposure of liver mitochondria to various experimental conditions using a previously described method (Hausladen & Fridovich, 1996) with slight modification. Mitochondria (0.05 mg ml^{-1} in 50 ml Tris–HCl, pH 7.4) were sonicated for 30 s. Aconitase activity was determined as the rate of NADP reduction (340 nm, $\varepsilon = 6200\ M^{-1}\,cm^{-1}$) by isocitrate dehydrogenase following addition of 2 mM sodium citrate, 0.4 mM $NADP^+$, and 1.25 U/ml isocitrate dehydrogenase (Sigma) to sonicated mitochondria.

Statistical analysis

Data are expressed as the mean ± standard error of mean (s.e.m.) and analysed for statistical significance using ANOVA followed by Scheffe's test for multiple comparison. A P-value <0.05 was considered significant.

Results

CPX inhibits H_2O_2 cytotoxicity

H_2O_2 induced release of LDH from SK-HEP-1 cells in a time- and concentration-dependent manner (Figure 1a), and this release was completely blocked by CPX (Figure 1b). H_2O_2 significantly decreased MTT reduction (a marker of mitochondrial function) prior to inducing LDH release (Figure 2a). This decreased MTT reduction was completely prevented by CPX (Figure 2a and b).

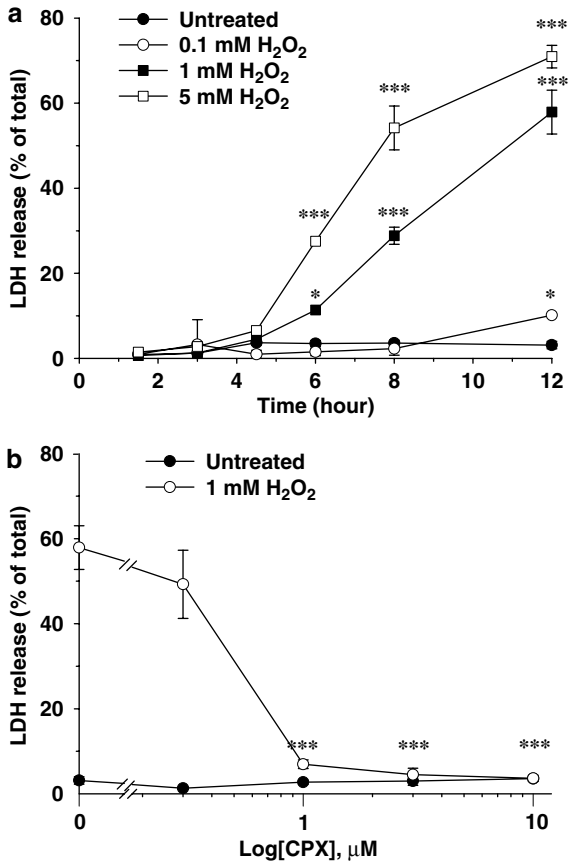

Figure 1 LDH release in SK-HEP-1 cells. (a) H_2O_2 stimulates LDH release in a time- and concentration-dependent manner. (b) CPX inhibits H_2O_2 (1 mM)-induced LDH release. LDH levels were determined after 12 h exposure to H_2O_2. All data are expressed as a percent of total LDH and represent the mean ± s.e.m. $N = 4$. $^*P<0.05$, $^{***}P<0.001$: (Scheffe's test) significantly different from the levels of LDH release in the cells treated with 1 mM H_2O_2 in the absence of CPX.

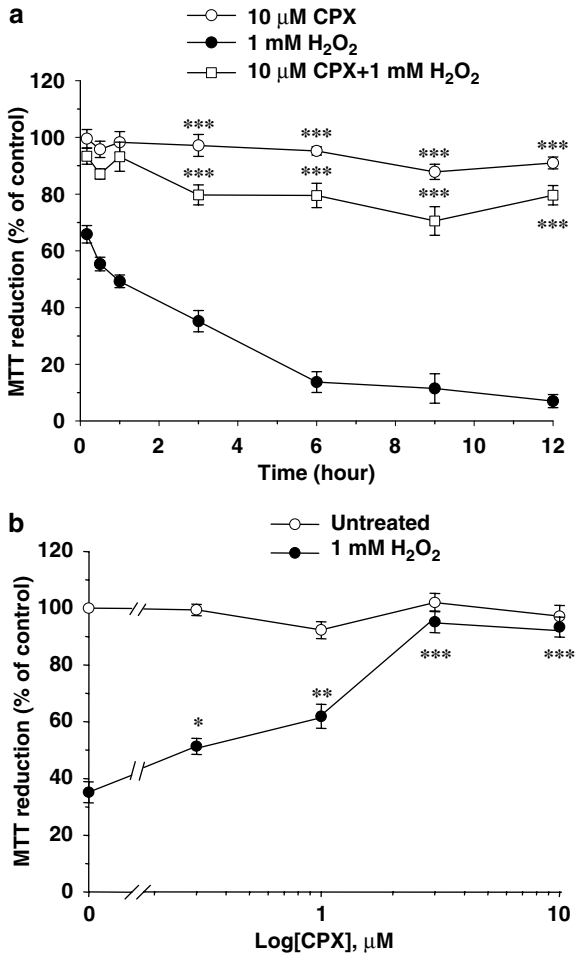

Figure 2 MTT reduction in SK-HEP-1 cells. (a and b) CPX prevents H_2O_2-induced inhibition of MTT reduction in time (a)- and concentration (b)-dependent manners. MTT reduction in (b) was measured after 3 h exposure to H_2O_2. Data represent the mean ± s.e.m. $N=4$. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$: (Scheffe's test) significantly different from the levels of MTT reduction in the cells treated with 1 mM H_2O_2 in the absence of CPX.

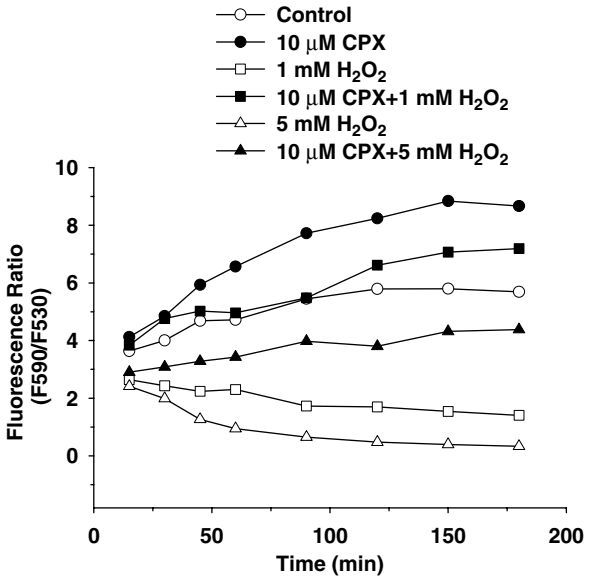

Figure 3 CPX prevents H_2O_2-stimulated $\Delta\psi m$ depolarization in cells. Fluorescence intensities were measured every 15 or 30 min for 3 h at 530 nm (J-monomer) and 590 nm (J-aggregate). Time 'zero' indicates the time of adding H_2O_2 and/or CPX after loading and washing out JC-1. Data are representative of four separate experiments and expressed as the ratios of aggregate fluorescence to monomer fluorescence.

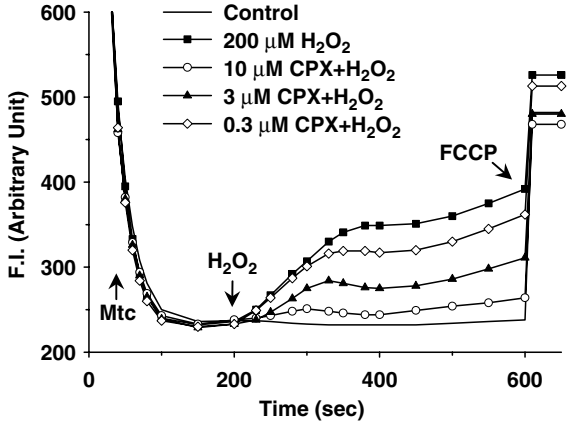

Figure 4 CPX blocks H_2O_2-induced $\Delta\psi m$ decrease in isolated mitochondria. H_2O_2-induced $\Delta\psi m$ was determined using rhodamine 123, as described in the Methods section. Mitochondria were added at 30 s, followed by addition of 200 μM H_2O_2 at 200 s. FCCP (1 μM) was added at the end of each experiment to generate a signal showing the total collapse of $\Delta\psi m$. Traces are representative of four independent experiments.

CPX blocks H_2O_2-stimulated $\Delta\psi$m decrease and MPTP opening

As MTT is reduced by mitochondrial reductases, MTT reduction is likely to be related to mitochondrial function (Mosmann, 1983). We further examined the effect of H_2O_2 on mitochondrial function by studying the $\Delta\psi m$ using the $\Delta\psi m$-sensitive fluorescent dye JC-1. H_2O_2 concentration-dependently decreased the $\Delta\psi m$ prior to release of LDH (Figure 3). The H_2O_2-induced $\Delta\psi m$ depolarization was completely blocked by CPX (Figure 3). This effect of CPX might not be due to interference with JC-1 fluorescence, because the spectral properties of JC-1 were not changed by CPX (data not shown).

Using mitochondria isolated from rat liver, we further examined whether CPX acted directly on mitochondria. Under energized conditions, H_2O_2 rapidly depolarized $\Delta\psi m$, and CPX concentration-dependently blocked this depolarization (Figure 4). Mitochondrial depolarization was previously reported to facilitate MPTP opening (Bernardi, 1992; Petronilli *et al*., 1993). In the present study, trapping of [U-^{14}C]sucrose within the mitochondrial matrix was employed to assess pore formation. The mitochondrial membrane pore formation was induced by adding H_2O_2 and then resealed using EGTA and CyA. We found that CPX concentration-dependently blocked the entry of [U-^{14}C]sucrose into mitochondria (Figure 5).

CPX is not a direct inhibitor of the MPTP

Our preliminary mitochondrial swelling experiments showed that at a concentration (1 mM) used here H_2O_2 alone did not induce the MPTP opening but promotes the MPTP opening

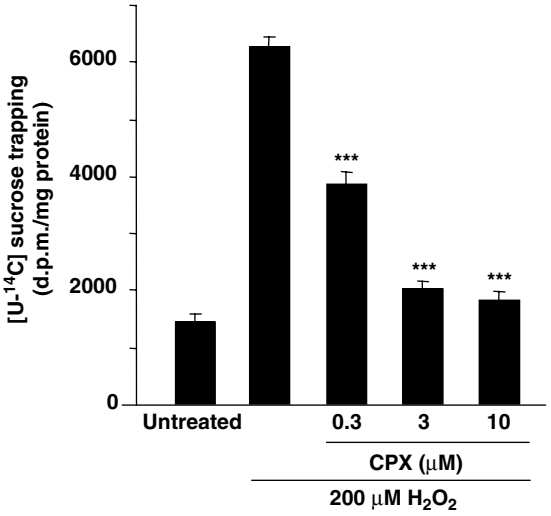

Figure 5 Mitochondrial [U-^{14}C]sucrose trapping. As described in the Methods, a mitochondrial suspension was transferred to test tubes containing H_2O_2 (final concentration = 200 μM), [U-^{14}C]sucrose (0.6 μCi ml^{-1}), and CPX (10 μM). After 30 min, mitochondrial pore was closed by adding EGTA and CyA on ice. Mitochondrial preparation was filtered and the radioactivity remaining on the filters was counted by liquid-scintillation spectrometry. Data are expressed as d.p.m. and the mean ± s.e.m. of five separate experiments. ***$P<0.001$: (Scheffe's test) significantly different from the group treated with H_2O_2 alone.

caused by 20 μM Ca^{2+}. CPX effectively inhibited Ca^{2+}-induced MPTP opening facilitated by H_2O_2 (Figure 6a). CsA (1 μM) and NEM (10 μM) completely blocked H_2O_2-facilitated MPTP opening (Figure 6b). We further confirmed the inhibitory effect of CPX or CsA on the MPTP opening using [^{14}C]sucrose: thus, mitochondrial entrapment of [^{14}C]sucrose caused by 200 μM or 1 mM H_2O_2 and 20 μM Ca^{2+} was largely blocked by CPX (3 μM) and CsA (1 μM) (data not shown). Under de-energized conditions, however, MPTP opening caused by 60 μM Ca^{2+} alone was not altered by CPX, but completely inhibited by CsA (Figure 7). This finding indicates that CPX is not a direct inhibitor of the MPTP.

CPX restores the rate of state 3 to state 4 respiration decreased by H_2O_2

Previously, H_2O_2 was shown to alter mitochondrial respiration (Sims *et al*., 2000). Mitochondrial respiration is normally divided into several states, with state 3 respiration coupled to ATP synthesis and state 4 respiration not coupled to ATP synthesis. Thus, the rate of state 3 to state 4 respiration (RCR) can be used to evaluate the functional health of mitochondria. When the complex II substrate succinate was used, H_2O_2 did not change the RCR (data not shown). However, when complex I substrates glutamate or pyruvate plus malate were used, H_2O_2 reduced both RCR values. CPX partially restored the RCR diminished by H_2O_2 in the presence of pyruvate and malate, but not in the presence of glutamate plus malate (Table 1). To avoid influence of cytochrome *c* and NAD^+ leakage through H_2O_2-induced pore opening, we further performed the RCR experiments in the presence of 50 μM cytochrome *c* and 1 mM NAD^+. Inclusion of cytochrome *c* and NAD^+, however, did not recover RCR decreased by H_2O_2 (data not shown).

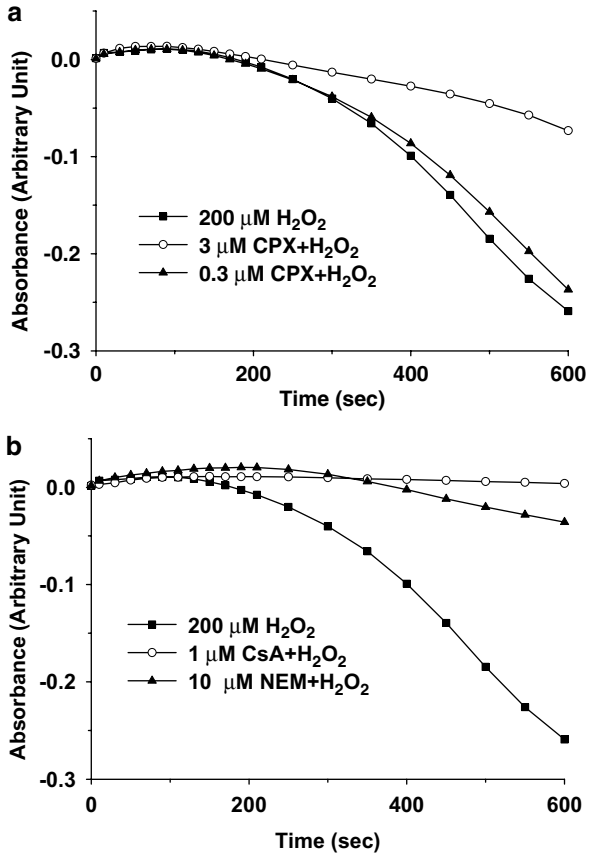

Figure 6 CPX inhibits H_2O_2-induced mitochondrial swelling in isolated mitochondria. (a, b) After addition of mitochondria and inhibitors, 200 μM H_2O_2 and 20 μM Ca^{2+} were added to the incubation mixture. Traces are representative of five independent experiments.

CPX inhibits aconitase inactivation by H_2O_2

Previously, assessment of electron transport chain complexes and Krebs cycle enzymes revealed that aconitase, KGDH and SDH were susceptible to H_2O_2 inactivation (Janero & Hreniuk, 1996; Tretter & Adam-Vizi, 2000; Nulton-Persson & Szweda, 2001). As we found that CPX inhibited the RCR change by H_2O_2 only in the presence of pyruvate and malate, we investigated the inhibitory effect of CPX on aconitase, not KGDH and SDH, inactivated by H_2O_2. Consistent with our previous data, we found that CPX largely protected aconitase from inactivation by H_2O_2 (Figure 8).

CPX restores the level of ATP decreased after H_2O_2 treatment

We measured time-dependent changes in H_2O_2 concentration following its addition to cell cultures. H_2O_2 was very stable in cell-free culture medium, whereas the concentration of H_2O_2 rapidly decreased in SK-HEP-1 cell cultures (Figure 9a). Initially, H_2O_2 rapidly reduced intracellular ATP levels, with levels decreasing 85% after 10-min exposure to 1 mM H_2O_2 (Figure 9b). Cotreatment of CPX could not inhibit the initial decrease of ATP levels but gradually restored ATP levels. Pretreatment with CPX for 1 h before H_2O_2 treatment also did

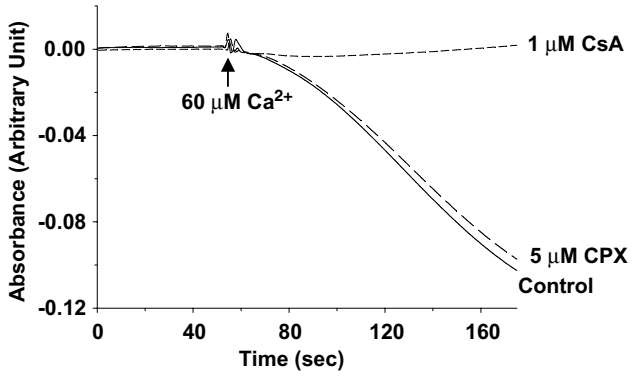

Figure 7 CPX does not block the Ca^{2+}-evoked MPTP opening in de-energized mitochondria. Liver mitochondria were incubated at 25°C under de-energized conditions (see 'Methods') with or without 5 μM CPX or 1 μM CsA. A_{540} was continuously monitored and after 1 min $CaCl_2$ was added to give the final buffered Ca^{2+} level of 60 μM.

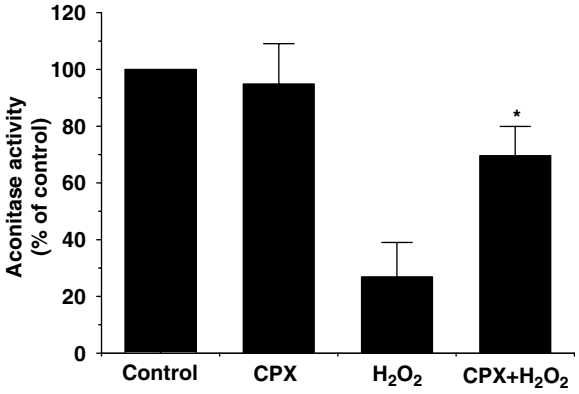

Figure 8 CPX inhibits H_2O_2-induced inactivation of aconitase. Intact mitochondria were incubated in the absence or presence of 200 μM H_2O_2 or 3 μM CPX. Aconitase activity was measured after incubation with H_2O_2 and CPX for 5 min. In the control samples the activity of aconitase was 15.3 ± 1.76 nmol min^{-1} mg^{-1} protein, and this value was deemed 100% activity. Enzyme activities are expressed as percent of control. Data represent mean $\pm$ s.e.m. of five separate experiments, and were analyzed for statistical significance using analysis of variance (ANOVA), followed by Scheffe's test for multiple comparison. *$P<0.05$: compared with the group treated with H_2O_2 alone.

not prevent the initial depletion of ATP, indicating that the lack of effect of CPX on the initial ATP depletion in the previous experiments was not due to slow permeation of CPX.

Discussion

In pathophysiological conditions, H_2O_2 will be continuously generated and its level remains high above normal conditions. Hyslop *et al*. (1995) reported that a significant rise in striatal H_2O_2 levels was observed for about 1 h during reperfusion after middle cerebral artery occlusion, amounting to an increase of approximately 100 μM at the peak. As shown in Figure 8, in the present study the level of H_2O_2 added exogenously was rapidly decreased below 100 μM within an hour in cultured cells possibly by the activity of cellular catalase. Although speculative, therefore, the concentrations of H_2O_2 used in the present study may be within the range of concentrations that occur in some pathological states.

Mitochondria dysfunction is one of the prominent features of ROS-mediated cell death. Oxidative stress including H_2O_2 depolarizes the $\Delta\psi m$ by inhibiting the activities of Krebs cycle enzymes (Chinopoulos *et al*., 1999; Zhuang *et al*., 2000). Oxidative stress can also facilitate Ca^{2+}-evoked MPTP opening, possibly by oxidizing thiols (Madesh & Hajnoczky, 2001; Takeyama *et al*., 2002). In order to protect mitochondria from oxidant-mediated injury, these two distinct, but interactive, events $\Delta\psi m$ depolarization and MPTP opening should be inhibited. In the present study, CPX protected SK-HEP-1 cells from H_2O_2 cytotoxicity mainly by inhibiting the $\Delta\psi m$ depolarization and indirectly the MPTP opening.

In general, maintaining intact mitochondria is very important for survival of cells exposed to oxidative stress. Our present study shows that in energized mitochondria exposed to H_2O_2 CPX inhibits the MPTP opening, as assessed by measuring mitochondrial [U-^{14}C]sucrose trapping (Figure 5) and swelling (Figure 6). Our preliminary electromicroscopy studies also showed that CPX completely blocked mitochondrial enlargement caused by in energized mitochondria

Table 1 Effect of ciclopirox on H_2O_2-treated mitochondrial respiratory activity

Treatment	*Respiratory activity*					
	Pyruvate plus malate			*Glutamate plus malate*		
	State 3	*State 4* (ng O_2/min/mg protein)	*RCR*	*State 3*	*State 4* (ng O_2/min/mg protein)	*RCR*
No addition	508 ± 31.0	160 ± 14.3	3.26 ± 0.24	1239 ± 219.0	168 ± 24.7	7.29 ± 0.63
200 μM H_2O_2	404 ± 31.0	237 ± 18.9*	1.74 ± 0.16*	1446 ± 292.8	284 ± 33.3*	4.94 ± 0.44*
10 μM CPX + H_2O_2	456 ± 28.0	198 ± 15.9	2.33 ± 0.11**	1738 ± 372.7	344 ± 62.1	4.99 ± 0.43
3 μM CPX + H_2O_2	465 ± 35.3	198 ± 20.7	2.40 ± 0.14**	1484 ± 225.4	284 ± 53.6	5.20 ± 0.13
0.3 μM CPX + H_2O_2	376 ± 51.1	194 ± 5.1	1.92 ± 0.25	1446 ± 225.0	310 ± 66.7	5.10 ± 0.44
10 μM CPX	467 ± 30.6	157 ± 10.2	2.97 ± 0.31	1310 ± 221.7	201 ± 17.4	6.52 ± 0.54
3 μM CPX	423 ± 33.7	161 ± 11.0	2.64 ± 0.19	1276 ± 198.2	196 ± 20.3	6.51 ± 0.75
0.3 μM CPX	416 ± 40.1	156 ± 13.7	2.71 ± 0.28	1309 ± 201.9	167 ± 20.6	7.84 ± 0.63

*$P<0.05$; significantly different compared with the corresponding 'No addition' control. **$P<0.05$; significantly different compared with the corresponding '200 μM H_2O_2' control.
States 3 and 4 respiration rates were measured using 10 mM glutamate plus 5 mM malate or 5 mM pyruvate plus 5 mM malate as substrates. H_2O_2 was added to the incubation mix immediately after addition of the mitochondria and CPX was added to the incubation mix before addition of the mitochondria. Values are shown as mean $\pm$ s.e.m. for four separate experiments.

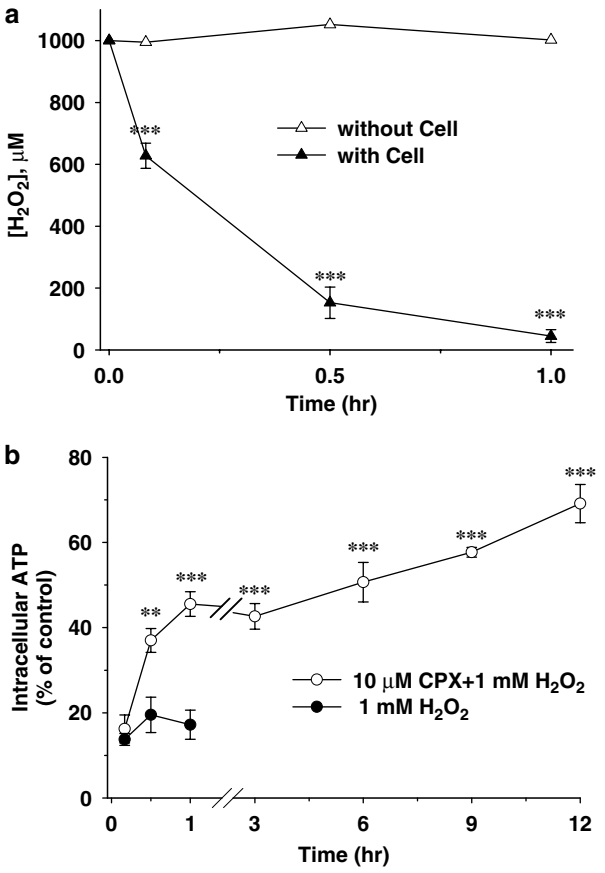

Figure 9 CPX restores ATP levels depleted by H_2O_2. (a) H_2O_2 levels were determined in cell-free culture media (open triangle) and cell cultures (closed triangle). (b) ATP concentrations were determined in cells treated with 1 mM H_2O_2 in the absence and presence of CPX (10 μM). In cells treated with H_2O_2 in the absence of CPX, ATP levels were measured only up to 1 h after addition of H_2O_2 due to cell injury or death. Data are expressed as a percent of the ATP level in control cells (not treated with H_2O_2). Sister cultures were used for both experiments. Data represent mean $\pm$ s.e.m. of five independent determinations. $^{**}P<0.01$, $^{***}P<0.001$: (Scheffe's test) significantly different from the H_2O_2 levels obtained in test tubes (without cells) (a) or the ATP levels at 30 min after treatment wit H_2O_2 alone (b).

exposed to H_2O_2 (J.-J. Choi & W.-K. Kim, unpublished observations). H_2O_2 was reported to inactivate Krebs cycle enzymes such as aconitase, KGDH and SDH (Janero & Hreniuk, 1996; Tretter & Adam-Vizi, 2000; Nulton-Persson & Szweda, 2001). After removal of H_2O_2 by cellular catalase and other antioxidant enzymes, normal mitochondrial function requires Krebs cycle enzymes to recover their activity. Therefore, aconitase regaining its activity in the presence of CPX may contribute to the normal mitochondrial function.

In this study CPX initially and rapidly increased the basal MTP level (Figure 3). Previously, we found that the MPTP blocker CsA also similarly increased the basal MTP level (Ju *et al*., 2000). Results obtained both in intact cells (Ichas *et al*., 1997) and in isolated mitochondria (Hueser *et al*., 1998) also suggested basal MPT activity under resting conditions that the pore opens transiently and reversibly (Petronilli *et al*., 1999), contributing to propagation of electrical and calcium signals (Ichas *et al*., 1997). Transient and reversible openings of the MPT might thus result in some change in MTP, which would not have any detrimental effect on cell viability (Petronilli *et al*., 1999). Like CsA, therefore, CPX is thought to block the basal openings of the MPTP. We are currently investigating which exact molecular targets in mitochondria are modified by CPX. Understanding the exact mechanism for the regulation of MPTP opening by CPX should provide the basis for therapeutic strategies in the hypoxia/ischemia-related diseases.

Mitochondria exposed to abnormally high levels of Ca^{2+} become nonselectively permeable to small molecules (<1500 Da). The sensitivity of mitochondrial permeability to Ca^{2+} level is markedly enhanced by oxidative stress, low $\Delta\psi m$ and adenine nucleotide depletion (Bernardi, 1992; Petronilli *et al*., 1993; Halestrap *et al*., 1997). In contrast, MPTP opening is prevented by high $\Delta\psi m$ and low pH (Bernardi, 1992; Petronilli *et al*., 1993; Bernardi *et al*., 1994). In the present study, CPX did not inhibit Ca^{2+}-evoked mitochondrial swelling in de-energized mitochondria, indicating that CPX is not a direct inhibitor of the MPTP. Thus, the inhibitory effect of CPX on the MPTP opening by H_2O_2 in energized mitochondria is thought due to its maintaining or even hyperpolarizing the $\Delta\psi m$.

CPX has been clinically used as an antifungal agent, and its action may involve disruption of fungus membrane function (Jue *et al*., 1985). In addition to its antifungal activity, CPX also has significant antibacterial activity *via* targeting different metabolic (respiratory) and energy-producing processes (Bohn & Kraemer, 2000). CPX was shown to impair microbial metabolism by affecting mitochondrial electron transport processes in the course of energy production. However, our present and previous (Choi *et al*., 2002) studies demonstrate that CPX prevents H_2O_2- and peroxynitrite-induced impairment of mitochondria in SK-HEP-1 cells and murine astrocytes. More studies are needed to determine how CPX affects microbial and mammalian mitochondria differently.

Recently, we reported that CPX did not scavenge H_2O_2, hydroxyl radicals (HO), NO, peroxynitrite ($ONOO^-$) or $O_2^{\bullet -}$ (Choi *et al*., 2002). Thus, the inhibitory effect of CPX on H_2O_2-induced $\Delta\psi m$ depolarization is not simply caused by scavenging oxidants. This may also explain why CPX was not able to inhibit the initial depletion of ATP (see Figure 7) and GSH (data not shown) in H_2O_2-treated cells.

In summary, CPX protects mitochondria in H_2O_2-treated cells mainly by inhibiting $\Delta\psi m$ depolarization. CPX inhibits inactivation of aconitase by H_2O_2 and, although speculative, indirectly reactivates H_2O_2-inactivated KGDH and SDH by keeping mitochondria intact. Unlike the well-known mitochondria protectors CsA and bongkrekic acid that do not readily permeate the cell membrane, CPX readily enters cells to prevent H_2O_2-stimulated mitochondrial injury. Defining the exact mechanism (including target molecules) underlying CPX inhibition of $\Delta\psi m$ depolarization and Krebs cycle enzymes inactivation may provide the basis for treatment of hypoxia/ischemia-related diseases.

We thank Dr A.P. Halestrap and S.J. Clarke at University of Bristol, United Kingdom, for their technical help for MPTP opening experiments and valuable comment in preparation of this manuscript. This work was supported by the Korea Health 21 R&D Project of the Ministry of Health & Welfare, Republic of Korea.

References

AL-NASSER, I. & CROMPTON, M. (1986). The reversible Ca^{2+}-induced permeabilization of rat liver mitochondria. *Biochem. J.,* **239,** 19–29.

AUERBACH, J.M. & SEGAL, M. (1997). Peroxide modulation of slow onset potentiation in rat hippocampus. *J. Neurosci.,* **17,** 8695–8701.

BERNARDI, P. (1992). Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *J. Biol. Chem.,* **267,** 8834–8839.

BERNARDI, P., BROEKEMEIER, K.M. & PFEIFFER, D.R. (1994). Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. *J. Bioenerg. Biomembr.,* **26,** 509–517.

BOHN, M. & KRAEMER, K.T. (2000). Dermatopharmacology of ciclopirox nail lacquer topical solution 8% in the treatment of onychomycosis. *J. Am. Acad. Dermatol.,* **43,** S57–S69.

BOVERIS, A., OSHINO, N. & CHANCE, B. (1972). The cellular production of hydrogen peroxide. *Biochem. J.,* **128,** 617–630.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–254.

CADENAS, E. & DAVIES, K.J. (2000). Mitochondrial free radical generation, oxidative stress, and aging. *Free Radical Biol. Med.,* **29,** 222–230.

CHINOPOULOS, C., TRETTER, L. & ADAM-VIZI, V. (1999). Depolarization of *in situ* mitochondria due to hydrogen peroxide-induced oxidative stress in nerve terminals: inhibition of alpha-ketoglutarate dehydrogenase. *J. Neurochem.,* **73,** 220–228.

CHOI, J.J., KONG, M.Y., LEE, S.J., KIM, H.C., KO, K.H. & KIM, W.K. (2002). Ciclopirox prevents peroxynitrite toxicity in astrocytes by maintaining their mitochondrial function: a novel mechanism for cytoprotection by ciclopirox. *Neuropharmacology,* **43,** 408–417.

CROMPTON, M. (1999). The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.,* **341** (Part 2), 233–249.

EMAUS, R.K., GRUNWALD, R. & LEMASTERS, J.J. (1986). Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim. Biophys. Acta,* **850,** 436–448.

FRIDOVICH, I. (1986). Superoxide dismutases. *Adv. Enzymol. Relat. Areas Mol. Biol.,* **58,** 61–97.

HALESTRAP, A.P., WOODFIELD, K.Y. & CONNERN, C.P. (1997). Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. *J. Biol. Chem.,* **272,** 3346–3354.

HAUSLADEN, A. & FRIDOVICH, I. (1996). Measuring nitric oxide and superoxide: rate constants for aconitase reactivity. *Methods Enzymol.,* **269,** 37–41.

HUESER, J., RECHENMACHER, C.E. & BLATTER, L.A. (1998). Imaging the permeability pore transition in single mitochondria. *Biophys. J.,* **74,** 2129–2137.

HIRSCH, T., SUSIN, S.A., MARZO, I., MARCHETTI, P., ZAMZAMI, N. & KROEMER, G. (1998). Mitochondrial permeability transition in apoptosis and necrosis. *Cell Biol. Toxicol.,* **14,** 141–145.

HYSLOP, P.A., HINSHAW, D.B., HALSEY Jr, W.A., SCHRAUFSTATTER, I.U., SAUERHEBER, R.D., SPRAGG, R.G., JACKSON, J.H. & COCHRANE, C.G. (1988). Mechanisms of oxidant-mediated cell injury. The glycolytic and mitochondrial pathways of ADP phosphorylation are major intracellular targets inactivated by hydrogen peroxide. *J. Biol. Chem.,* **263,** 1665–1675.

HYSLOP, P.A., ZHANG, Z., PEARSON, D.V. & PHEBUS, L.A. (1995). Measurement of striatal H_2O_2 by microdialysis following global forebrain ischemia and reperfusion in the rat: correlation with the cytotoxic potential of H_2O_2 *in vitro*. *Brain Res.,* **671,** 181–186.

ICHAS, F., JOUAVILLE, L.S. & MAZAT, J.P. (1997). Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signaling. *Cell,* **89,** 1145–1153.

IOANNIDIS, I. & DE GROOT, H. (1993). Cytotoxicity of nitric oxide in Fu5 rat hepatoma cells: evidence for co-operative action with hydrogen peroxide. *Biochem. J.,* **296** (Part 2), 341–345.

JANERO, D.R. & HRENIUK, D. (1996). Suppression of TCA cycle activity in the cardiac muscle cell by hydroperoxide-induced oxidant stress. *Am. J. Physiol.,* **270,** C1735–C1742.

JU, C., YOON, K-N., OH, Y-K., KIM, H-C., SHIN, C.Y., RYU, J.R., KO, K.H. & KIM, W-K. (2000). Synergistic depletion of astrocytic glutathione by glucose deprivation and peroxynitrite: correlation with mitochondrial dysfunction and subsequent cell death. *J. Neurochem.,* **74,** 1989–1998.

JUE, S.G., DAWSON, G.W. & BROGDEN, R.N. (1985). Ciclopirox olamine 1% cream. A preliminary review of its antimicrobial activity and therapeutic use. *Drugs,* **29,** 330–341.

LOSCHEN, G., AZZI, A., RICHTER, C. & FLOHE, L. (1974). Superoxide radicals as precursors of mitochondrial hydrogen peroxide. *FEBS Lett.,* **42,** 68–72.

MADESH, M. & HAJNOCZKY, G. (2001). VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome *c* release. *J. Cell Biol.,* **155,** 1003–1015.

MOSMANN, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods,* **65,** 55–63.

MURAKAMI, K., KONDO, T., KAWASE, M., LI, Y., SATO, S., CHEN, S.F. & CHAN, P.H. (1998). Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. *J. Neurosci.,* **18,** 205–213.

NULTON-PERSSON, A.C. & SZWEDA, L.I. (2001). Modulation of mitochondrial function by hydrogen peroxide. *J. Biol. Chem.,* **276,** 23357–23361.

PETRONILLI, V., COLA, C. & BERNARDI, P. (1993). Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore. II. The minimal requirements for pore induction underscore a key role for transmembrane electrical potential, matrix pH, and matrix Ca^{2+}. *J. Biol. Chem.,* **268,** 1011–1016.

PETRONILLI, V., MIOTTO, G., CANTON, M., BRINI, M., COLONNA, R., BERNARDI, P. & DI LISA, F. (1999). Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophys. J.,* **76,** 725–734.

SCHAPIRA, A.H. (1998). Mitochondrial dysfunction in neurodegenerative disorders. *Biochim. Biophys. Acta,* **1366,** 225–233.

SHIGENAGA, M.K., HAGEN, T.M. & AMES, B.N. (1994). Oxidative damage and mitochondrial decay in aging. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 10771–10778.

SIMS, N.R., ANDERSON, M.F., HOBBS, L.M., KONG, J.Y., PHILLIPS, S., POWELL, J.A. & ZAIDAN, E. (2000). Impairment of brain mitochondrial function by hydrogen peroxide. *Brain Res. Mol. Brain Res.,* **77,** 176–184.

TAKEYAMA, N., MIKI, S., HIRAKAWA, A. & TANAKA, T. (2002). Role of the mitochondrial permeability transition and cytochrome *C* release in hydrogen peroxide-induced apoptosis. *Exp. Cell Res.,* **274,** 16–24.

TOMBAUGH, G.C. & SAPOLSKY, R.M. (1992). Corticosterone accelerates hypoxia- and cyanide-induced ATP loss in cultured hippocampal astrocytes. *Brain Res.,* **588,** 154–158.

TRETTER, L. & ADAM-VIZI, V. (2000). Inhibition of Krebs cycle enzymes by hydrogen peroxide: a key role of [alpha]ketoglutarate dehydrogenase in limiting NADH production under oxidative stress. *J. Neurosci.,* **20,** 8972–8979.

ZHUANG, S., DEMIRS, J.T. & KOCHEVAR, I.E. (2000). p38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *J. Biol. Chem.,* **275,** 25939–25948.

(Received January 24, 2005
Accepted February 17, 2005)