Role of the Mitochondrial Membrane Permeability Transition (MPT) in Rotenone-Induced Apoptosis in Liver Cells

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Rotenone inhibits spontaneously and chemically induced hepatic tumorigenesis in rodents through the induction of apoptosis. However, the mechanism for the induction of apoptosis by rotenone has not been defined. Mitochondrial dysfunction, in particular the induction of the mitochondrial membrane permeability transition (MPT), has been implicated in the cascade of events involved in the induction of apoptosis. Inhibition of the mitochondrial electron-transport chain reduces the mitochondrial transmembrane potential (ΔΨm), which may induce the formation of the mitochondrial permeability transition pore and the subsequent MPT. Fluorescent microscopy of Hoechst 33258-stained WB-F344 cells, a rat-liver cell line, was utilized to examine the effect of the mitochondrial respiratory chain inhibitor, rotenone (0.5–5 μM), atracyloside (5–10 μM), and cyclosporin A (2.5–10 μM) on apoptosis. A time- and concentration-dependent increase in liver cell apoptosis was observed following treatment with rotenone and atracyloside (11.7- and 7.7-fold, respectively, over solvent control). Cotreatment with 7.5- and 10 μM-cyclosporin A for 12 h inhibited the apoptogenicity of 5-μM rotenone treatment. A similar effect was observed following cyclosporin A cotreatment with atracyloside. Rotenone induced a rapid increase in apoptosis (within 20 min of treatment). By 2 h of treatment, the morphological appearance of apoptosis was similar to that observed in cultures treated continuously with rotenone for 12 h. Inhibition studies demonstrated that cyclosporin A prevented apoptosis if the exposure to it occurred prior to the 20-min threshold necessary to induce apoptosis by rotenone. Mitochondrial function was examined by staining with the mitochondrial membrane potential (ΔΨm)-sensitive fluorochrome, MitoTracker Red (CMXRos) and confirmed utilizing cytofluorometric analysis of DiOC6(3)-stained cells. Rotenone (5.0-μM) and atracyloside (5.0-μM) reduced the percent of CMXRos or DiOC6(3)-positive (ΔΨm-positive) liver cells within 15 min and throughout the duration of the study (6 h) to approximately 65–80% and 50–80% of control. However, cotreatment with concentrations of cyclosporin A that inhibited the apoptogenicity of rotenone and atracyloside prevented the rotenone- and atracyloside-induced reduction of the ΔΨm. Therefore, the apoptogenic effect of rotenone and atracyloside appears to occur rapidly (within 20 min) and is irreversible once mitochondrial damage occurs. The inhibition of the rotenone- and atracyloside-induced apoptosis and mitochondrial dysfunction by cyclosporin A suggests the MPT may be involved in the induction of apoptosis by rotenone.

Key Words: rotenone; apoptosis; mitochondrial permeability transition; liver cells.

Activated cell death, or apoptosis, is involved in the maintenance of homeostasis in multicellular organisms, embryogenesis, metamorphosis, and organ involution, and is a controlling factor in counteracting cell proliferation (Ellis et al., 1991; Raff, 1995; Steller, 1995; Wyllie et al., 1980). Several non-genotoxic carcinogens have been shown to inhibit or suppress apoptosis, thus implicating dysfunctional apoptosis in the chemical carcinogenesis process (Bayly et al., 1995; Bursch et al., 1984; Roberts et al., 1995; Wyllie et al., 1980). Cells engaged in apoptosis activate endogenous cellular enzymes involved in the degradation of cytosolic components prior to the loss of plasma membrane integrity. This sequential metabolic suicide produces a distinct cellular morphology characterized by a reduction in cell size, condensation of chromatin, nuclear fragmentation, randomly assorted organelles in the cytoplasm, and ultimately, in the late stages, a loss of plasma membrane integrity and blebbing (Mesner, Jr. et al., 1997).

The consistent characteristics of apoptosis, produced by a variety of stimuli and in a number of different cell types, appear to indicate a common effector pathway(s). The consistent observation of mitochondrial dysfunction prior to the nuclear changes associated with apoptotic cell death implies that it may be a critical regulator of the metabolic events involved in the apoptotic cascade (Deckwerth and Johnson, 1993; Jacobson et al., 1994; Newmeyer et al., 1994; Petit et al., 1995; Schulze-Osthoff et al., 1994; Vayssiere et al., 1995; Zamzami et al., 1995a,b, 1996a). Furthermore, in cell-free systems, mitochondria are a necessary component of the cytosolic fraction to produce apoptotic features in isolated nuclei (Newmeyer et al., 1994). Subsequent evidence revealed only mitochondria undergoing the mitochondrial membrane permeability transition (MPT) are pro-apoptotic in this system (Zamzami et al., 1996a).

The MPT involves the formation of a non-specific pore...
across the inner mitochondrial membrane permitting the free
distribution of ions, solutes, and small-molecular-weight mol-
ecules (<1500 Da) across the membrane (Bernardi et al., 1994;
Zoratti and Szabo, 1995). The collapse of the mitochondrial
membrane potential (ΔΨm) and uncoupling of the electron
transport chain from ATP production have been shown to
promote MPT (Bernardi et al., 1994; Kroemer et al., 1995).
Additionally, consequential to the disruption or collapse of the
ΔΨm and the induction of the MPT, is the loss of matrix Ca2+
and glutathione, increased oxidation of thiols, and further de-
polarization of the inner mitochondrial membrane, which in-
crease the gating potential for the MPT pore. As the conse-
quences of the MPT are also involved in the induction of the
MPT, the MPT may function as a self-amplifying “switch”
that, once activated, irreversibly commits the cell to apoptosis
(Bernardi et al., 1994; Kroemer et al., 1995; Zoratti and Szabo,
1994). Additionally, a variety of known apoptogens, including
the oxidizing agents menadione and hydrogen peroxide, thiol
agents such as diamide, adenine nucleotide-translocating li-
gands such as atractyloside, adenine nucleotide-depleting agents,
and mitochondrial transmembrane-potential (ΔΨm) re-
ducing agents such as rotenone and mCICCP induce the MPT
(Zoratti and Szabo, 1995).

Several agents, including cyclosporin A, prevent opening of
the MPT pore. Cyclosporin A binds to cyclophilin, a peptidyl
prolyl cis-trans-isomerase located in the inner mitochondrial
matrix (Broekemeier et al., 1989; Halestrap and Davidson,
1990). It has been proposed that when bound by cyclosporin A,
cyclophilin remains inactive, thereby maintaining the MPT
pore in a closed state (Broekemeier et al., 1989; Halestrap and
Davidson, 1990). Treatment with inhibitors of the permeability
transition pore and subsequently inhibition of the MPT prevent
apoptosis in a variety of cell types, following treatment with a
variety of apoptogenic stimuli (Kroemer et al., 1995; Zamzami
et al., 1996b). Therefore, inhibition of the MPT by cyclosporin
A appears to prevent the cascade of events leading to apoptotic
cell death.

The naturally occurring pesticide, rotenone, is derived from
the Derris and Lonchorcarpus species root and bark. The
pesticidal activity of rotenone is attributed to irreversible bind-
ing and inactivation of complex I of the mitochondrial electron
transport chain, thereby inhibiting oxidative phosphorylation
(Lindahl and Oberg, 1961). Rotenoids have demonstrated an-
ticancer activity in chemically induced preneoplastic lesions in
mammary organ culture and inhibition of papillomas in the
2-stage mouse-skin model (Gerhauser et al., 1995). Further-
more, the most abundant rotenoid, rotenone, demonstrated
anticancer activity against hepatic tumor formation in B6C3F1
mice following chronic treatment (Abdo et al., 1988) and in an
initiation-promotion protocol (Isenberg et al., 1997). Previous
studies by our group attributed the anticancer activity of rote-
none to the induction of apoptosis (Isenberg et al., 1997).

Mitochondrial respiratory chain inhibitors induce apoptosis
in a variety of cell types including primary cultured hepato-
cytes (Pastorino et al., 1995b, 1993; Wolventang et al., 1994;
Zamzami et al., 1996a). Wolventang et al (1994) examined the
effect of several inhibitors of mitochondrial energy metabolism
on apoptosis in several cell lines. Following treatment with
inhibitors of mitochondrial respiration and uncoupling agents,
apoptosis was induced in these cell lines (Wolventang et al.,
1994). Inhibition of the electron transport chain following
 treatment with rotenone, anoxic conditions, cyaniode, or
1-methyl-4-phenylpyridinium (MPP+) demonstrated that MPT
is critical in the killing of cultured hepatocytes (Pastorino et al.,
1993; Seaton et al., 1998; Synder et al., 1993). Cyclosporin A
has been shown to prevent the MPT (Pastorino et al., 1995b)
and apoptosis induced by anoxia, rotenone, MPP+ or cyaniode
(Pastorino et al., 1995b, 1993; Seaton et al., 1998). These
studies indicate inhibitors of mitochondrial energy metabolism
alter mitochondrial function (reduce the ΔΨm) and this alter-
ation appears to be an important event in the induction of
apoptosis in these cells.

Reduced energy metabolism has been linked to a decrease in
the ΔΨm (Lindahl and Oberg, 1961; Pastorino et al., 1993,
1995b). Furthermore, inhibition of the electron transport chain
by mitochondrial poisons, such as rotenone, has been shown to
reduce the ΔΨm (Zamzami et al., 1995b). Maintenance of the
ΔΨm is necessary for cell survival and the disruption or col-
lapse of the ΔΨm is consistently observed in preapoptotic cells
following treatment with mitochondrial poisons (Deckwerth
and Johnson, 1993; Kroemer et al., 1995; Petit et al., 1995;
Zamzami et al, 1995a,b). In addition to mitochondrial poison-
duced apoptosis, reduction of the ΔΨm has been observed
prior to the characteristic nuclear morphology associated with
apoptosis in nerve growth factor (NGF)-deprived sympathetic
neurons, tumor necrosis factor-alpha (TNF-α)-stimulated U937
cells, Fas ligation, ceramide treatment and gamma irra-
diation (Deckwerth and Johnson, 1993; Garcia-Ruiz et al.,
MATERIALS AND METHODS

Materials

Rotenone, cyclosporin A, atractyloside, carbonyl cyanide m-chlorophenyl-
hydrazone (mCICCP), and DMEM/F12 media were purchased from Sigma
Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from
Hyclone Labs (Logan, UT). Hoechst 33258, MitoTracker Red (CMXRos), and
3,3’-dihexyloxacarbocyanine iodide (DiOC,3) were obtained from Molecu-
lar Probes, Inc. (Junction City, OR).
Cell Culture

WB-F344 cells, a rat-liver cell line, were plated at 1 x 10^6/60 mm^2 culture dish, with a glass coverslip for apoptosis and MitoTracker Red studies and without coverslips in cytofluorometric studies. Cells were allowed to attach and were grown in asynchronous culture for 24 h in DMEM/F12 media containing 5% FBS, dexamethasone, insulin, and antibiotics. The media were then replaced with fresh serum-free media containing the test compounds, for the selected treatment duration. In all experiments involving cyclosporin-A treatment, the media were removed one h prior to treatment and were replaced with fresh serum-free media with or without cyclosporin A. Following one-h pretreatment with cyclosporin A, the media were removed and replaced with fresh serum-free media containing the test compounds, cyclosporin A or cyclosporin A and the test compounds, for the selected treatment duration. All test compounds were dissolved in DMSO and DMSO was examined as a solvent control. Cultures were maintained at 37°C in a humidified atmosphere at 95% O_2 and 5% CO_2.

Measurement of Apoptosis

Apoptosis was quantified by the detection of condensed chromatin with the fluorochrome, Hoechst 33258, as previously described (Bayly et al., 1995; James and Roberts, 1997) with modifications. To assess the effect of rotenone, atractyloside, and cyclosporin A on liver-cell death by apoptosis, fresh serum-free media with or without the test compounds were added to culture dishes containing glass coverslips. Following the selected treatment duration, liver cells were fixed with 4% paraformaldehyde (pH 7.4) for 10 min. Cells were then stained with Hoechst 33258 (5 ng/ml physiological saline) for 5 min and washed twice in distilled water. The coverslips were removed and mounted on glass microscope slides with Supermount™ (Biogenex, San Ramon, CA) to achieve optimal fluorescence. The slides were examined on a Nikon Diaphot inverted microscope utilizing a 350-nm excitation and a 460-nm emission-fluorescent filter. Apoptotic cells were identified by brightly staining condensed chromatin and morphological appearance under phase-contrast conditions. The number of apoptotic cells or apoptotic bodies (cells that generated multiple apoptotic bodies were scored as one) was divided by the total number of cells observed and multiplied by 100 to achieve an apoptotic index. At least 3000–5000 cells per treatment group were counted.

Measurement of Mitochondrial Membrane Potential (\(\Delta \Psi_m\))

MitoTracker Red measurement of the mitochondrial membrane potential (\(\Delta \Psi_m\)). The \(\Delta \Psi_m\) was quantified by microscopic examination of cells stained with the fluorochrome MitoTracker Red (CMXRsos) and confirmed by cytofluorometric analysis of cells stained with DiOC_6(3). Briefly, CMXRsos is an aldehyde fixable cationic lipophilic fluorochrome that passively diffuses through the plasma membrane of viable cells and is selectively sequestered in mitochondria with an active \(\Delta \Psi_m\) and permits the examination of the \(\Delta \Psi_m\) in adherent cells (Haugland, 1996). To assess the effect of rotenone, atractyloside, and cyclosporin A on mitochondrial function in liver cells, fresh serum-free media, with or without the test compounds, was added to culture dishes containing glass coverslips. Fifteen min prior to the duration of each treatment, CMXRos (500 nm) was added to each medium and incubated at 37°C in a humidified atmosphere at 95% O_2 and 5% CO_2. At the conclusion of each treatment duration, the media containing the test compound(s) and CMXRos were removed and the liver cells were fixed with 4% paraformaldehyde (pH 7.4) for 10 min. Following fixation, the cells were washed twice in distilled water and the coverslips were removed and mounted on glass microscope slides with Supermount™ (Biogenex, San Ramon, CA) to achieve optimal fluorescence. A Nikon Diaphot inverted microscope connected to a cooled CCD camera was utilized in each treatment to examine the area of CMXRos staining in liver cells. Fluorescent micrographic images were obtained (ex. 579 nm, em. 599 nm) and BDS Imaging software (Oncor, Inc., Gaithersberg, MD) was utilized to quantify the area of CMXRos staining in each cell. A total of 500–1000 cells per treatment were examined for the determination of the area of CMXRos staining as a measure of mitochondrial function. A reduction in the area of CMXRos staining is indicative of a cell that has mitochondria with reduced membrane potential.

Cytofluorometric measurement of the \(\Delta \Psi_m\). To confirm the CMXRos studies, cytofluorometric analysis of the \(\Delta \Psi_m\) in liver cells was performed. Liver cells were treated with various concentrations of the test compounds for the selected treatment duration. Fifteen min prior to the conclusion of each treatment duration, in vitro labeling of liver cells was performed with 40 nm DiOC_6(3) and incubated at 37°C in a humidified atmosphere at 95% O_2 and 5% CO_2 as previously described (Zamzami et al., 1995b), with minor modifications. The cells were then trypsinized and centrifuged at 1500 x g for 5 min. As a control in some experiments, cells were labeled in the presence of the uncoupling agent mCICCP (50-μM). The supernatant was removed and the pellet was resuspended in physiological saline (Darzynkiewicz et al., 1997; Deitch et al., 1982). For analysis, a FACScan™ cytofluorometer (Becton Dickinson, San Jose, CA) with argon laser excitation at 501 nm was used to analyze 10,000 cells from each sample. Comparison of the forward- and side-light scatters was utilized to gate the major population of normal-sized liver cells. Additionally, as exclusion of vital dyes provides an assessment of cell viability (Darzynkiewicz et al., 1997), propidium iodide (5 μg/ml) staining was utilized to differentiate the live cell population from the dead cell population (data not shown) and to ensure that cells excluded from the gate were either cellular debris or non-viable cells.

Measurement of Cytolethality

To examine the effect of rotenone on non-apoptotic cell death, liver cells were exposed to increasing concentrations of rotenone (0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5, and 10.0 μM) in serum-free medium for 4, 6, 12, and 24 h. Following each treatment duration, 500 μl of medium was removed (sample LDH) from each culture dish and was evaluated as previously described (Ruch et al., 1989). The total LDH from each culture dish from each treatment was determined by solubilizing the plasma membrane with 1% Triton-X for 3 h followed by evaluation of LDH in the medium. To obtain the percent LDH release from each treatment, the sample LDH was divided by the LDH release following solubilization with Triton-X and multiplied by 100.

Statistics

Statistical difference (p < 0.05) from control values was determined by ANOVA followed by a Student-Newman-Keuls test (Gad and Weil, 1986). For analysis of apoptosis, 3 to 4 slides per treatment group were evaluated and the results represent the mean ± the standard deviation. For evaluation of the \(\Delta \Psi_m\) with CMXRos, a total of two slides, and a total of 500–1000 individual cells per treatment were evaluated by ANOVA, followed by a Student-Newman-Keuls test. Analysis of cytofluorimetry data was based on 2 to 3 individual measurements of 10,000 cells each and statistical differences (Student’s t test, p < 0.05) in the percent of DiOC_6(3) positive cells were determined as previously described by (Zamzami et al., 1995b). To determine the correlation between apoptosis and serum concentration in the media, a linear regression analysis was performed (Gad and Weil, 1986).

RESULTS

Effect of Serum on Apoptosis in Liver Cells

Serum starvation has been shown to induce apoptosis in several cell types (Aoki et al., 1997; Gottlieb et al., 1994; Haviv et al., 1998, Xu and Reed, 1998). The effect of serum starvation on liver cells was analyzed in the present study to determine if serum starvation amplifies rotenone-induced apoptosis. In the present study, fluorescence microscopy of Hoechst 33258-stained cells was used to quantitate the per-
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percentage of cells with condensed chromatin (typical of apoptosis) (apoptotic index) following culture in medium containing various concentrations of serum (0–10%). DMSO and rotenone treatment had an inverse relationship between the concentration of serum in the medium and percent of cells undergoing apoptosis ($r^2 = 0.8956$, $p = 0.015$ and $r^2 = 0.8715$, $p = 0.02$, respectively) (data not shown). Based on these results, subsequent studies were performed in serum-free medium.

**Time Course Evaluation of Rotenone-Induced Apoptosis**

The induction of apoptosis following treatment with rotenone and other mitochondrial respiratory chain inhibitors has been demonstrated in several cell types (Jacobson et al., 1994; Newmeyer et al., 1994; Pastorino et al., 1993; Schulze-Osthoff et al., 1994; Wollentang et al., 1994; Seaton et al., 1998). The LDH release assay was utilized to determine the concentration of rotenone to be used in subsequent studies for the evaluation of apoptotic cell death (did not produce LDH release (cytolethality) (Fig. 1). These studies demonstrate that following 12 h of treatment, concentrations of rotenone less than 7.5 mM did not produce LDH release. Furthermore, as the duration of rotenone exposure increased, the cytolethality of rotenone increased, such that following 24 h of continuous exposure, all concentrations of rotenone examined (except 1.0 mM) produced LDH release. Therefore, subsequent treatments were terminated following 12 h of treatment.

Figure 2 shows the effect of increasing concentrations of rotenone and duration of exposure on apoptosis in liver cells. Treatment with 2.0, 2.5, and 5.0 mM rotenone increased the apoptotic index over DMSO-treated controls at all time points examined. Furthermore, a concentration-dependent increase in apoptosis was observed at all time points examined. Treatment with 5.0 mM rotenone for 12 h produced the largest increase in the apoptotic index observed in this study (19.2% or 10.2-fold increase over control).

**Effect of Duration of Rotenone Exposure on Apoptosis**

The effect of the duration of exposure to rotenone on apoptosis is shown in Figure 3. In these studies, liver cells were treated with 2.5 and 5.0 mM rotenone for 1, 2, 4, 6, or 12 h at

FIG. 1. Concentration and time effect of rotenone on LDH release from liver cells cultured in serum-free medium. Liver cells were treated with 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5, and 10.0 mM rotenone for 4, 6, 12, or 24 h. Data represent the mean LDH release ± SD obtained from 4 separate determinations. If no error bar is present, the standard deviation from those data is smaller than the size of the symbol. Statistically significant ($p < 0.05$) from

DMSO control (*).

FIG. 2. Effect of rotenone and time on apoptosis in liver cells cultured in serum-free medium. Liver cells were treated with 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, or 5.0 mM rotenone for 4, 6, or 12 h. Data represent the mean apoptotic index ± SD obtained from 4 separate determinations. If no error bar is present, the standard deviation from those data is smaller than the size of the symbol. Statistically significant ($p < 0.05$) from DMSO control (*).

FIG. 3. Effect of the duration of rotenone treatment on apoptosis in liver cells cultured in serum-free medium. Cells were cultured in medium containing 2.5 or 5.0 mM rotenone for 1, 2, 4, 6, or 12 h, or DMSO-treated liver cells for 12 h. In cultures treated with rotenone for less than 12 h, upon removal of rotenone from the medium, DMSO was added such that the total duration of treatment was 12 h. Data represent the mean apoptotic index ± SD obtained from 4 separate determinations. Statistically significant ($p < 0.05$) from DMSO control (*)
Treatment with all concentrations of cyclosporin A (2.5-, 5.0-, and 10.0-μM) is necessary to prevent the MPT. Therefore, our laboratory (data not shown) demonstrated pretreatment with cyclosporin A, cells were pretreated for 60 min in the appropriate concentration of cyclosporin A. Liver cells were then treated with DMSO, rotenone, rotenone + 2.5 μM cyclosporin A, rotenone + 5.0 μM cyclosporin A, and rotenone + 10.0 μM cyclosporin A continuously for 12 h. Data represent the mean apoptotic index ± SD obtained from 4 separate determinations. If no error bar is present, the standard deviation from those data is smaller than the size of the symbol. Statistically significant (p < 0.05) from DMSO control (*).

The conclusion of each time point, the medium containing rotenone was removed, and the cells were washed and then cultured in medium containing solvent (DMSO) for the appropriate duration of time, such that apoptosis was assessed 12 h after initiation of the study. Treatment with 2.5 and 5.0 μM rotenone increased in the apoptotic index over DMSO-treated controls (1.63%) at all time points examined. Furthermore, treatment with 2.5 and 5.0 μM rotenone for 2 h followed by 10 h of DMSO treatment produced an apoptotic index equivalent to that observed following continuous rotenone treatment for 12 h (6.50% and 10.5% versus 7.42% and 10.6%, respectively). Similar results were obtained for the 4- and 6-h time points. These data indicate that induction of apoptosis in liver cells by rotenone is rapid and irreversible upon withdrawal of rotenone from the medium (Fig. 3).

**Effect of Cyclosporin A on Rotenone-Induced Apoptosis**

To evaluate the role of the mitochondrial membrane permeability transition (MPT) in rotenone-induced apoptosis, the effect of the MPT inhibitor, cyclosporin A, was examined. Previous studies (Malorini et al., 1998; Pastorino et al., 1995b; Yang and Cortopassi, 1998) and preliminary experiments in our laboratory (data not shown) demonstrated pretreatment with cyclosporin A is necessary to prevent the MPT. Therefore, all experiments involved one-h pretreatment with cyclosporin A. Treatment with all concentrations of cyclosporin A (2.5-, 5.0-, 7.5-, and 10.0-μM) at all time points examined in the present study had no effect on apoptosis when compared to DMSO-treated controls (2.2%) (Fig. 4). Increases of A 5.1- and 7.6-fold in the morphological appearance of apoptosis over DMSO-treated control (2.20%) were observed following treatment with 2.5- and 5.0-μM rotenone for 12 h (Fig. 4). Cotreatment with all concentrations of cyclosporin A reduced rotenone-induced apoptosis (Fig. 4). Cotreatment with 5.0-, 7.5-, and 10.0-μM cyclosporin A attenuated the induction of apoptosis by 2.5-μM rotenone (Fig. 4). However, upon cotreatment with 5.0-μM rotenone, 7.5- and 10.0-μM cyclosporin A attenuated the rotenone-induced apoptosis (Fig. 4). Since inhibition of the MPT by cyclosporin A prevents rotenone-induced apoptosis, these results implicate the MPT in rotenone-induced apoptosis in liver cells.

To further evaluate the role of the MPT in apoptosis in liver cells, the effect of atractyloside on apoptosis was examined. Binding of atractyloside to the adenine nucleotide translocase, a putative component of the MPT pore, promotes formation of the MPT pore and the subsequent MPT. Treatment with 5.0, 7.5, or 10.0-μM atractyloside for 12 h had no effect on cytotoxicity (data not shown) in the present study; however the apoptotic index increased 4.7-, 5.4-, or 7.7-fold over DMSO-treated controls (3.56%) (Fig. 5). All concentrations of cyclosporin A examined reduced the atractyloside-induced apoptosis when compared to treatment with atractyloside only (Fig. 5). Cotreatment with 5.0-, 7.5-, or 10.0-μM cyclosporin A attenuated the induction of apoptosis by 5.0-μM atractyloside (Fig. 5). Following cotreatment with 7.5-μM atractyloside, 10.0-μM cyclosporin A was the only concentration of cyclosporin A

**FIG. 4.** Effect of cyclosporin A on rotenone-induced apoptosis in liver cells cultured in serum-free medium. In all treatments containing cyclosporin A, cells were pretreated for 60 min in the appropriate concentration of cyclosporin A. Liver cells were then treated with DMSO, rotenone, rotenone + 2.5 μM cyclosporin A, rotenone + 5.0 μM cyclosporin A, rotenone + 7.5 μM cyclosporin A, and rotenone + 10.0 μM cyclosporin A continuously for 12 h. Data represent the mean apoptotic index ± SD obtained from 4 separate determinations. If no error bar is present, the standard deviation from those data is smaller than the size of the symbol. Statistically significant (p < 0.05) from DMSO control (*).

**FIG. 5.** Effect of cyclosporin A on atractyloside-induced apoptosis in liver cells cultured in serum-free medium. In all treatments containing cyclosporin A, the cells were pretreated for 60 min in the appropriate concentration of cyclosporin A. Liver cells were then treated with either DMSO, atractyloside, atractyloside + 2.5 μM cyclosporin A, atractyloside + 5.0 μM cyclosporin A, atractyloside + 7.5 μM cyclosporin A, or atractyloside + 10.0 μM cyclosporin A continuously for 12 h. Data represent the mean apoptotic index ± SD obtained from 4 separate determinations. If no error bar is present, the standard deviation from those data is smaller than the size of the symbol. Statistically significant (p < 0.05) from DMSO control (*).
rottenone treatment occurs rapidly (within 10–20 min of treatment start) and upon induction of apoptosis by rottenone, cyclosporin A does not have an inhibitory effect on apoptosis. Taken together, these studies further suggest the MPT appears to be involved in rottenone-induced apoptosis and that once the mitochondrial dysfunction (MPT) occurs, the apoptotic process may be irreversibly activated.

**Effect of Rotenone and Atractyloside on Mitochondrial Function in Liver Cells**

Microscopic evaluation of the ΔΨm (Mitotrack Red measurement of the ΔΨm). Liver cells were treated with rottenone, atractyloside, or cyclosporin A for various time points up to 360 min, to examine the effect of these compounds on mitochondrial function. At the conclusion of the treatment duration, mitochondrial function was determined by fluorescent microscopic examination of the ΔΨm-dependent uptake and retention of Mitotracker Red (CMXRos) into mitochondria. Figures 8 and 9 demonstrate the effect of rottenone, atractyloside, and cyclosporin A on the mitochondrial membrane potential (ΔΨm) in liver cells. Cyclosporin A treatments (2.5, 5.0, 7.5, or 10.0 μM) for up to 360 min had no effect on CMXRos staining (data not shown). Treatment with 2.5- and 5.0-μM rottenone reduced the ΔΨm following 15, 30, 60, 120, 240, and 360 min of treatment (Fig. 8). However, cotreatment with 10.0-μM cyclosporin A for 15, 30, 60, 120, 240, and 360 min prevented the reduction of the ΔΨm following treatment with 2.5- and 5.0-μM rottenone (Fig. 8). Similar results were obtained following treatment with 5.0-μM atractyloside for 15, 30, 60, 120, 240, and 360 min. Cotreatment with 10.0-μM rottenone
Cyclosporin A prevented the atractyloside-induced reduction of the ΔΨₘ at all time points examined (Fig. 9).

**Cytofluorometric evaluation of the mitochondrial function (ΔΨₘ) in liver cells.** Cytofluorometric analysis of DiOC₆(3)-stained liver cells was performed to confirm the microscopic analysis of mitochondrial function. Figure 10 illustrates the effect of rotenone, atractyloside, mClCCP, and cyclosporin A on the ΔΨₘ in liver cells. Propidium-iodide staining was utilized to gate the necrotic cell population and debris from the viable cell population (Darzynkiewicz et al., 1997) (data not shown). Treatment with the mitochondrial uncoupler and known reducer of the ΔΨₘ, mClCCP (Zamzami et al., 1995b), disrupted the ΔΨₘ in approximately 50% of the liver cells following 15, 30, 60, 120, and 180 min of treatment (Figs. 11 and 12).

Treatment with 5.0-µM rotenone produced 41.4%, 31.4%, 19.7%, 19.9%, and 29.9% reductions in the population of DiOC₆(3)-positive (ΔΨₘ-positive) liver cells when compared to DMSO-treated controls [96.8% DiOC₆(3)-positive liver cells] following 15, 30, 60, 120, and 180 min of treatment, respectively. When compared to DMSO-treated control, cyclosporin A alone had no effect on the ΔΨₘ (Figs. 11 and 12). Cotreatment with 10.0 µM cyclosporin A prevented the rotenone-induced reduction of the DiOC₆(3)-positive (ΔΨₘ-positive) liver cell population at all time points examined (Figs. 11 and 12).
Apoptosis, or activated cell death, involves the sequential activation of endogenous death-inducing molecules within single cells, which produces the characteristic cellular degradation associated with apoptotic cell death. Apoptosis is involved in the maintenance of homeostasis (Ellis et al., 1991; Raff, 1995; Steller, 1995), in particular, as a counteracting factor for cell proliferation (Wyllie et al., 1980). Nongenotoxic carcinogens appear to alter the balance between cell proliferation and apoptosis through modification of the normal rate of apoptosis in rat and mouse liver (Bursch et al., 1984; Roberts et al., 1995), and they suppress apoptosis in vitro (Bayly et al., 1995; James and Roberts, 1996). Therefore, a role for the dysregulation of apoptosis has been established in the cancer process (Bayly et al., 1995; Bursch et al., 1984; Roberts et al., 1995). In a recent study conducted by Abdo et al. (1988), they observed a reduction in “spontaneous” liver tumor formation in mice following chronic administration of rotenone. A subsequent study by our group suggests the anticancer activity of rotenone may be due in part to the induction of apoptosis in preneoplastic focal lesions (Isenberg et al., 1997).

The toxicity of rotenone has been attributed to the inhibition of cell respiration by blocking the oxidation of nicotinamide adenine dinucleotide (NAD) at complex I of the electron transport chain, thus maintaining a high NADH/NAD⁺ ratio (Lindahl and Oberg, 1961; Pastirino et al., 1995a). This may result in the collapse of the mitochondrial proton motive force necessary to generate the ΔΨm and in turn produce ATP (Lindahl and Oberg, 1961; Pastirino et al., 1995a). Disruption of the ΔΨm has been observed prior to the demonstration of nuclear apoptosis in a variety of cell types (Deckwerth and Johnson, 1993; Jacobson et al., 1994; Newmeyer et al., 1994; Schulze-Osthoff et al., 1994; Petit et al., 1995; Vayssiere et al., 1995; Zamzami et al., 1995a; 1995b; 1996a).

Additionally, the induction of cell death by rotenone in several cell types has been shown to be related to the inhibition of the electron transport chain (Pastirino et al., 1995b; Wolfetang et al., 1994; Zamzami et al., 1995a). This inhibition produces a de-energized mitochondria with a depolarized membrane (Deckwerth and Johnson, 1993; Kroemer et al., 1993; Pastirino et al., 1993; Petit et al., 1995; Zamzami et al., 1995a,b). The reduction of the ΔΨm and decreased ATP are necessary for the induction of the MPT (Deckwerth and Johnson, 1993; Kroemer et al., 1995; Petit et al., 1995; Zamzami et al., 1995a; 1995b). Furthermore, induction of the MPT pro-
duces a further disruption of the $\Delta \Psi_m$ and uncoupling of the respiratory chain which promotes the opening of additional MPT pores (Bernardi et al., 1994; Kroemer et al., 1995; Zoratti and Szabo, 1994).

In the present study, rotenone treatment for greater than 20 min induced apoptosis. Upon induction of apoptosis by rotenone, removal of the compound or treatment with cyclosporin A did not prevent rotenone-induced apoptosis. Therefore, the mitochondrial dysfunction induced rotenone treatment (within 20 min) may be sufficient for the activation of a self-amplification pathway and the induction of further damage in the absence of rotenone in the medium. Alternatively, it is possible that rotenone persists intracellularly upon washing. Therefore, irreversible binding of rotenone to complex I (Gutman et al., 1969) may be responsible for the prolonged effect of rotenone on mitochondrial function observed even in the absence of rotenone in the culture medium. Further support for the early and irreversible nature of rotenone-induced mitochondrial dysfunction in the apoptotic process was apparent in the MitoTracker Red and cytofluorometric study results. Rotenone and atractyloside (a known inducer of the MPT) disrupted the $\Delta \Psi_m$ within 15 min of treatment (Figs. 8, 9, and 10). This disruption occurred prior to a measurable increase in the apoptotic index (20 min, Figs. 6 and 7) and support the proposal that the induction of the MPT is an early, irreversible step in rotenone-induced apoptosis.

In this study, rotenone and other mitochondrial respiratory inhibitors induced apoptosis in cultured rat hepatocytes (Pastorino, 1993, 1995b), in other cultured mammalian cells (Wolventang et al., 1994; Zamzami et al., 1996a,c), and in rat liver cells. Our observation of necrotic cell death following 24 h of treatment may be consequential to ATP depletion in liver cells. Rotenone, as an electron transport chain inhibitor, decreases the generation of ATP through oxidative phosphorylation (Simbula et al., 1997). Several studies have demonstrated the necessity of ATP to activate the metabolic machinery involved in apoptosis, as well as the role of a reduction in ATP concentrations in the induction of apoptosis (Bossy-Wetzel et al., 1998; Leist et al., 1997). Although reduction of ATP concentrations have been observed during apoptosis, it appears to occur relatively late in the process (Bossy-Wetzel et al., 1998) and is required for the downstream events in apoptosis (Eguchi et al., 1997). Treatment with staurosporine or stimulation of the CD95 receptor in Jurkat cells resulted in a switch from apoptosis to necrosis when cells were depleted of ATP (Leist et al., 1997). Therefore, in a state of reduced ATP generation, as observed following rotenone treatment, apoptosis may proceed until cellular ATP stores are depleted or reduced to a point where cell death becomes necrotic rather than apoptotic. Alternatively, the observed increased presence of LDH in the medium following 24-h rotenone treatment may be secondary to the ongoing apoptosis. The presence of LDH in the medium may be a result of the absence of phagocytes that would normally engulf late stage apoptotic cells with leaky plasma membranes (Cejna et al., 1994; Raffray and Cohen, 1997).

The present study also evaluated the role of the MPT in rotenone-induced apoptosis. The MPT is mediated by the opening of a non-specific pore across the inner mitochondrial membrane. The opening of the permeability pore and the subsequent MPT are inhibited by several agents including cyclosporin A (Bernardi et al., 1994; Crompton et al., 1987; Pastorino et al., 1993; Zamzami et al., 1995a, 1996b). Cyclosporin A binds to cyclophilin D, a peptidyl prolyl cis-trans-isomerase located in the inner mitochondrial membrane and a proposed component of the MPT pore, which maintains the permeability pore in a closed state (Broekemeier et al., 1989; Halsestrap and Davidson, 1990). MPT pore inhibitors, including bongkrekic acid, monochlorodimane, and cyclosporin A, prevent apoptosis induced by agents that induce the MPT (Crompton et al., 1987; Pastorino et al., 1993; Zamzami et al., 1996b) as well as several other apoptogens (Bernardi, 1992; Crompton et al., 1988; Hirsch et al., 1998; Pastorino et al., 1993; Yang and Cortopassi, 1998; Zamzami et al., 1996a,c). Since disruption of the $\Delta \Psi_m$ has been shown to induce and be a consequence of the MPT (Deckwerth and Johnson, 1993; Petit et al., 1995; Zamzami et al., 1995a,b), reduction of the $\Delta \Psi_m$, and the subsequent induction of the MPT may be critical events in the apoptotic pathway following treatment with mitochondrial respiratory chain inhibitors and uncouplers (Pastorino et al., 1995b; Raffray and Cohen, 1997; Wolventang et al., 1994; Zamzami et al., 1996c).

Atractyloside, an inducer of the MPT, competitively binds and inhibits adenine nucleoside translocase, thereby preventing translocation of adenosine diphosphate (ADP) across the mitochondrial membrane and also oxidative phosphorylation (Obatomi and Bach, 1998). Previous studies have shown that atractyloside treatment promotes the MPT in isolated liver mitochondria and promotes the subsequent release of cytochrome c from the mitochondrial matrix (Kantrow and Piantadosi, 1997). In the present study, atractyloside induced apoptosis in liver cells (Fig. 5). However, upon cotreatment with cyclosporin A, the atractyloside-induced apoptosis was inhibited or attenuated (Fig. 5). Similarly, following 12 h of treatment with rotenone, an increase in apoptosis was observed that was prevented by cotreatment with cyclosporin A (Fig. 4). The similarity of these results suggests that MPT appears to be involved in the execution of apoptosis in rat liver cells following treatment with rotenone.

The consistent observation of a reduced or disrupted $\Delta \Psi_m$ in preapoptosis indicates maintenance of the $\Delta \Psi_m$ is necessary for cell survival (Deckwerth and Johnson, 1993; Petit et al., 1995; Vayssiere et al., 1995; Zamzami et al., 1995a, 1996c). Isolation and subsequent culture of a population of cells with a subnormal (low) $\Delta \Psi_m$ indicated that when compared to cells with a normal $\Delta \Psi_m$, cells with a reduced $\Delta \Psi_m$ proceed to an apoptotic morphology rapidly (Zamzami et al., 1995a). Disruption of the $\Delta \Psi_m$ has also been shown to be involved in
apoptosis mediated by a variety of apoptogens, including etoposide, doxorubicin, cytosine arabinose, and ceramide (Decaudin et al., 1997; Zamzami et al., 1996b). Furthermore, previous studies demonstrated the reduction or collapse of the $\Delta \Psi_m$ is involved in the induction and is a consequence of the MPT (Bernardi et al., 1994; Kroemer et al., 1995; Zoratti and Szabo, 1994). Therefore, observation of mitochondria with a reduced or diminished $\Delta \Psi_m$ appears to be associated with dysfunctional mitochondria in preapoptotic cells. In the present study, treatment with an apoptogenic concentration of rotenone or atracyloside reduced the $\Delta \Psi_m$ in rat liver cells within 15 min of treatment. However, in the presence of cyclosporin A, the rotenone- and atracyloside-induced reductions of the $\Delta \Psi_m$ are attenuated. Similar to these results, attenuation of the dexamethasone-, irradiation- and etoposide-induced disruption of the $\Delta \Psi_m$ in splenocytes and thymocytes following treatment with cyclosporin A (Zamzami et al., 1995a) and bongkrekic acid (Zamzami et al., 1996b) was detected by cytofluorometric analysis of DiOC$_3$(3)-stained cells. However, Pastorino et al. (1993) reported that cyclosporin A protected cultured hepatocytes from anoxia- and rotenone-induced cell death without attenuating the release of the $\Delta \Psi_m$, as determined by the release of preloaded $[^{3}H]$TPP$^+$ from the mitochondria. Therefore, the discrepancy between these results may be related to the utilization of different methods of detection.

Following treatment with a mitochondrial uncoupler, mClCCP, a rapid induction of the MPT as determined by the loss of the $\Delta \Psi_m$ was observed in the majority of mitochondria (Bernardi et al., 1993). However, when the concentration of mClCCP was decreased, the disruption of the $\Delta \Psi_m$ was observed in only a fraction of the mitochondria (Bernardi et al., 1993). Similar results were obtained in the present study with rotenone. Although a reduction of the $\Delta \Psi_m$ or induction of the MPT was not evident in the majority of cells following treatment with either rotenone or atracyloside, a concentration response was observed (Fig. 12). Inhibition of the mitochondrial dysfunction and apoptosis by cyclosporin A also suggests the induction of the MPT is an important event in the preapoptotic mitochondrial dysfunction induced by rotenone (Figs. 4 and 12).

Several tumor promoters have been shown to alter mitochondrial function (Cai et al., 1996; Chen et al., 1998; Klohn et al., 1998). Treatment with the liver-tumor promoter, ethinyl estradiol (Chen et al., 1998), and several peroxisome proliferators, including clofibrate, perfluorooctanoic acid, and ace-tylsalicylic acid (Cai et al., 1996), enhances the expression of several mitochondrial respiratory chain components, including NADH dehydrogenase (complex I) subunit I and cytochrome $c$ oxidase subunit I. Furthermore, enhanced levels of mitochondrial respiratory chain protein expression enhanced mitochondrial respiration (Chen et al., 1998). Enhanced mitochondrial function may provide these cells with a selective growth advantage or increased resistance to apoptosis. Recent studies by Klohn et al. (1998) demonstrated that mitochondria, isolated from rats fed the carcinogen, 2-acetylaminofluorene, are more resistant to the induction of the MPT by elevated Ca$^{2+}$ than mitochondria isolated from untreated rats. The present study demonstrated the role of the MPT in rotenone-induced apoptosis in rat liver cells. Since apoptosis is considered to counteract cell proliferation, and dysregulated apoptosis has been implicated in carcinogenesis (Bayly et al., 1995; Bursch et al., 1984; Roberts et al., 1995; Wylie et al., 1980) and since rotenone induces MPT, enhanced apoptosis through induction of MPT may be the mechanism for the anti-carcinogenicity of rotenone.

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