Altered mitochondrial function and overgeneration of reactive oxygen species precede the induction of apoptosis by 1-O-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine in p53-defective hepatocytes

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ABSTRACT **The mechanism of induction of apoptosis by the novel anti-cancer drug 1-O-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) was investigated in p53-defective SV40 immortalized rat hepatocytes (CWSV1). Exposure to 12 μM ET-18-OCH_3 for 36 h induced apoptosis as determined using classical morphological features and agarose gel electrophoresis of genomic DNA. Increased levels of reactive oxygen species (ROS) were detected spectrophotometrically using a nitroblue tetrazolium (NBT) assay in cells treated with ET-18-OCH_3. Both the increased generation of ROS and the induction of apoptosis were inhibited when cells were treated concurrently with ET-18-OCH_3 in the presence of the antioxidant α-tocopherol. Similar results were achieved when cells were switched acutely to choline-deficient (CD) medium in the presence of the antioxidant. The possible role of mitochondria in the generation of ROS was investigated. Both ET-18-OCH_3 and CD decreased the phosphatidylcholine (PC) content of mitochondrial and associated membranes, which correlated with depolarization of the mitochondrial membrane as analyzed using 5,5′,6,6′-tetramethylbenzimidazolcarbocyanine iodide (JC-1), a sensitive probe of mitochondrial membrane potential. Rotenone, an inhibitor of the mitochondrial electron transport chain, significantly reduced the intracellular level of ROS and prevented mitochondrial membrane depolarization, correlating with a reduction of apoptosis in response to either ET-18-OCH_3 or CD. Taken together, these results suggest that the form of p53-independent apoptosis induced by ET-18-OCH_3 is mediated by alterations in mitochondrial membrane PC, a loss of mitochondrial membrane potential, and the release of ROS, resulting in completion of apoptosis.—Vrablic, A. S., Albright, C. D., Craciunescu, C. N., Salganik, R. I., Zeisel, S. H. Altered mitochondrial function and overgeneration of reactive oxygen species precede the induction of apoptosis by 1-O-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine in p53-defective hepatocytes. *FASEB J.* 15, 1739–1744 (2001)**

Key Words: apoptosis · cancer · edelfosine · mitochondria · phosphatidylcholine · reactive oxygen species

THE ANTICANCER DRUG 1-O-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3), a synthetic ether phospholipid, inhibits the activity of CTP:phosphocholine cytidylyltransferase, the rate-limiting step in the synthesis of phosphatidylcholine (PC) (1). It exhibits selective cytotoxicity against many tumor cell types (2) and is known to induce apoptosis (3). Choline deficiency (CD) limits the availability of phosphocholine, thus effectively limiting the synthesis of PC by CTP: phosphocholine cytidylyltransferase, and triggers a p53-independent form of apoptosis in cultured hepatocytes (4). The form of apoptosis induced by acute CD appears to be mediated by generation of reactive oxygen species (5). Although the anti-tumor mechanism of action of ET-18-OCH_3 is unclear, we hypothesized that this drug kills cells in a manner similar to CD.

Evidence suggests that ET-18-OCH_3 is capable of generating increased oxidant stress. Thus, in L1210 leukemia cells, ET-18-OCH_3 increased the generation of lipid-derived free radicals before the appearance of cytotoxic effects (6), although the generation of free radicals required iron supplementation and prior enrichment of cellular components with polyunsaturated fatty acid. Similarly, ET-18-OCH_3 increased lipid peroxidation in nude mice inoculated with MDA-MB-231 breast carcinoma cells and fed a diet high in fish oils; unfortunately, the effects on apoptosis were not studied (7). In contrast, overexpression of bcl-2 or bcl-X_L, two gene products capable of blocking mitochondrial ROS-mediated apoptosis (8–10), abrogated ET-18-OCH_3 apoptosis in human leukemia cells.

Mitochondria are the richest source of reactive oxygen species (ROS) in the cell, converting 1–2% of reduced oxygen into superoxide (11). Inhibition of the mitochondrial electron transport chain, resulting in subsequent release of ROS, is an early event in many forms of apoptosis (e.g., ceramide, dexamethasone,

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and bleomycin) (8, 12, 13). However, it is not clear whether all forms of apoptosis are mediated by ROS. This study provides evidence that the anti-cancer drug ET-18-OCH_3 shares some biochemical features with CD and causes a form of p53-independent apoptosis that is mediated by ROS derived from mitochondria.

MATERIALS AND METHODS

Cell culture

SV40-immortalized CWSV1 rat hepatocytes were obtained from Dr. Harriet C. Isom (Department of Microbiology, The Pennsylvania State University College of Medicine, Hershey, PA). This cell line was derived from male Fisher 344 rat liver and was immortalized with the SV40 large T antigen that inactivates p53 protein (14–17). The cells retain expression of liver-specific proteins and are routinely grown in a serum-free defined media (16, 17). CWSV1 hepatocytes were maintained in 70 μM choline (CS), serum-free RPMI 1640 medium (American Bioganics, Inc., Niagara Falls, NY) as described previously (4). All experiments used CWSV1 cells at passage 32–34.

ET-18-OCH_3 and apoptosis

A stock solution (10 mM; 100×) of ET-18-OCH_3 (Calbiochem, La Jolla, CA) was prepared in sterile water and stored at −20°C. For cell treatment, ET-18-OCH_3 stock was added to cell culture medium to achieve a final concentration of 12 μM. The antioxidant α-tocopherol succinate (Sigma, St. Louis, MO) was dissolved in sterile absolute ethanol at a concentration of 10 mM (100×) and stored at −20°C. For cell treatment, α-tocopherol was added to cell culture medium to achieve a final concentration of 10 μM. Cells were plated at a density of 2×10^4 cells per well in 6-well plates (Falcon, Franklin Lakes, NJ) and grown for 2 days. Subconfluent cultures (≈ 70% confluent) were treated with ET-18-OCH_3-containing medium for 36 h. Additional cultures of CWSV1 cells were switched for 48 h from CS (70 μM choline) to 0 μM choline.

Assessment of apoptosis

Apoptosis was assessed at the end of treatment in attached cells fixed in 70% ethanol and stained with hematoxylin as described previously (4). The percentage of cells undergoing apoptosis was determined from counts of the number of cells with classical apoptotic morphology in 10 randomly selected microscope fields (10× ocular, 40× objective).

DNA fragmentation associated with apoptosis was also assessed. High and low molecular weight DNA from 2×10^6 cells was extracted using DNAzol™ (Life Technologies, Inc., Gaithersburg, MD) and separated on a 1.8% agarose slab gel containing 1 μg/ml ethidium bromide (Sigma).

Quantification of reactive oxygen species

A modified version of a previously described assay for the intracellular conversion of nitro blue tetrazolium (NBT) to formazan by superoxide anion was used to measure the generation of reactive oxygen species (18). Briefly, 50 μM NBT (Sigma) was added to the media at the end of the treatment periods; cells were incubated for an additional 15 min at 37°C, fixed in absolute ethanol, and allowed to air dry. The formazan content of the cells was then solubilized with 960 μl 2M KOH and 1120 μl DMSO, and the absorbance at $OD_{630\ nm}$ was measured spectrophotometrically. A standard curve was prepared by adding KOH and DMSO to known amounts of NBT. As a positive control, 100 μM hydrogen peroxide was added to cells and the amount of formazan formed was measured.

Isolation of mitochondria and associated membranes

Mitochondria and associated membranes were isolated from cells at the end of the treatment as described previously (19). Briefly, 1×10^7 cells were homogenized in 5 volumes buffer A (20 mM HEPES-KOH, pH 7.5; 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, 0.1 mM PMSF, 250 mM sucrose, 10 μg/ml leupeptin, 5 μg/ml pepstatin, and 2 μg/ml aprotinin), and the homogenate was subjected to centrifugation at 750 *g* for 10 min at 4°C. The supernatant was collected and subjected to centrifugation at 750 *g* for 10 min at 4°C, then the resulting supernatant was transferred and subjected to centrifugation at 10,000 *g* for 15 min at 4°C. The resulting mitochondrial pellet was resuspended in buffer A and stored frozen at −80°C until analyzed. Two pellets were processed for transmission electron microscopy (TEM) as described previously (20).

Measurement of mitochondrial PC

Protein content of isolated mitochondria and associated membranes was determined by the Lowry method (21). Total phospholipids were extracted by the method of Bligh and Dyer (22), separated by thin-layer chromatography, and phosphatidylcholine mass was determined by inorganic phosphorous determination (23).

Inhibition of mitochondrial function

Rotenone (Sigma), an inhibitor of complex I of the mitochondrial electron transport chain (24), was dissolved in tissue

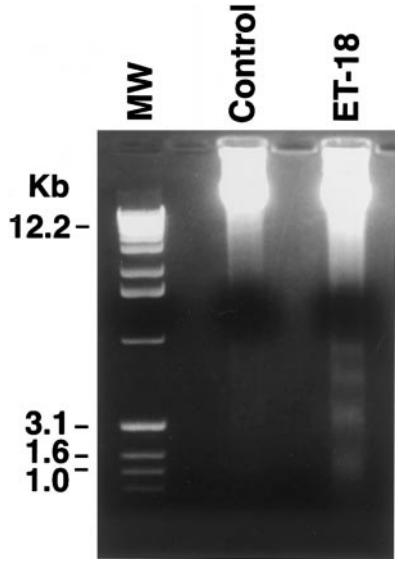

Figure 1. ET-18-OCH_3 causes DNA fragmentation. CWSV1 hepatocytes were cultivated in RPMI-defined medium containing 70 μM choline until 70% confluent and then switched for 36 h to the same medium containing 12 μM ET-18-OCH_3. DNA fragmentation was analyzed after electrophoresis in a 1.8% agarose slab gel stained with ethidium bromide. This experiment was replicated three times.

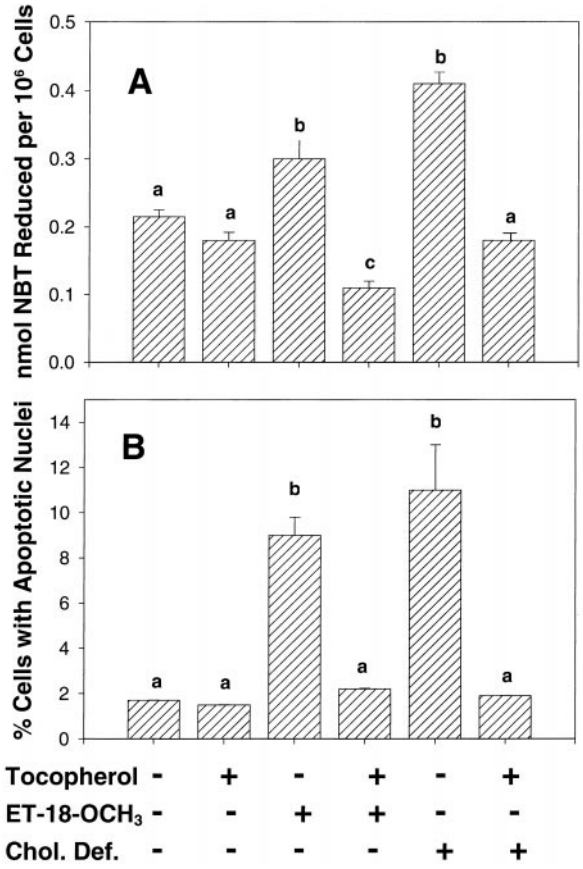

Figure 2. ET-18-OCH_3 treatment or CD increase generation of intracellular reactive oxygen species and induce apoptosis, which can be abrogated by α-tocopherol. *A)* CWSV1 hepatocytes were cultivated as described in Materials and Methods until 70% confluent and then switched for 36 h to RPMI-defined medium containing 70 μM choline (control) and 10 μM α-tocopherol succinate or 12 μM ET-18-OCH_3; additional cultures were treated for 48 h with RPMI-defined medium containing 0 μM choline (CD). Generation of intracellular ROS was measured by NBT assay. Data are expressed as mean nmol NBT reduced/10^6 cells ± SE. $n = 5$/point. ANOVA with Scheffé's F test: bars with different letter superscripts differ by $P < 0.05$. This experiment was replicated twice. *B)* Cells were fixed with 70% ethanol, stained with hematoxylin, and cells with classical apoptotic nuclear morphology were counted. Data are expressed as percent of cells with apoptotic morphology ± SE. $n = 6$/point. ANOVA with Scheffé's F test: bars with different letter superscripts differ by $P < 0.01$.

culture grade dimethyl sulfoxide (Sigma) to achieve a final concentration of 1 mM (1000×). For cell treatments, rotenone was added to cultures at a final concentration of 50 nM. The solvent concentration in media never exceeded 0.001%.

JC-1 (5,5′,6,6′-tetramethylbenzimidazolcarbocyanine iodide; Molecular Probes, Inc., Eugene, OR), a lipophilic, cationic molecule that is capable of crossing the plasma membrane and specifically binding to the mitochondrial membrane (25), was dissolved in tissue culture grade dimethyl sulfoxide at a concentration of 1 mg/ml. After treatments, cells were probed with JC-1 and visualized as described (26). When taken up by normal cells, JC-1 exists as a monomer that emits at 527 nm (green fluorescence) after excitation at 490 nm; as the mitochondrial membrane polarization changes, JC-1 forms aggregates that are associated with a large shift in emission to 590 nm (red fluorescence) (25, 27). The potassium ionophore valinomycin was used as a positive control to collapse the mitochondrial membrane potential (26).

Statistics

Multiple comparisons analysis of variance was performed using Statview 512™ software (Brain Power, Inc., Calabasas, CA).

RESULTS

ET-18-OCH_3, ROS, and apoptosis

ET-18-OCH_3 treatment of CWSV1 cells produced characteristic apoptotic morphology in attached cells that was accompanied by cleavage of internucleosomal DNA to produce a typical apoptosis-associated DNA ladder (**Fig. 1**). Both ET-18-OCH_3 and CD induced overgen-

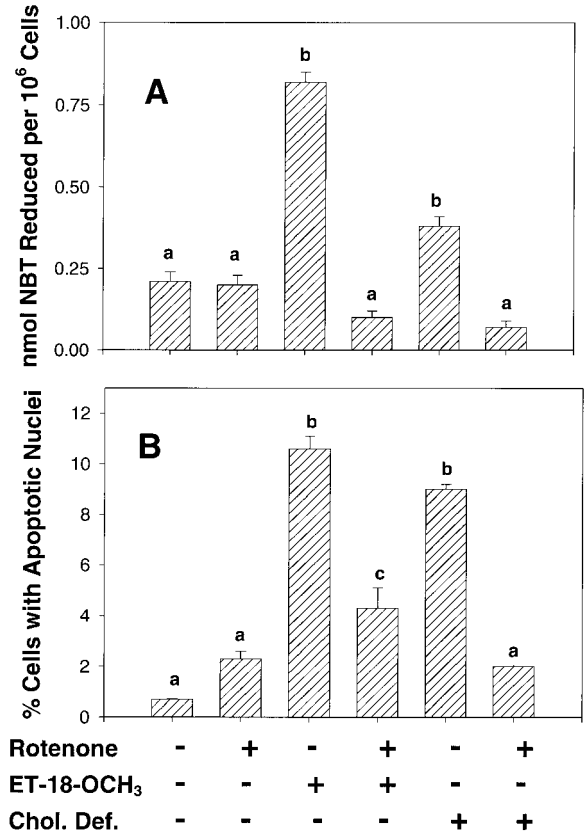

Figure 3. Inhibition of mitochondrial respiration reduces oxidative stress and abrogates apoptosis. *A)* CWSV1 hepatocytes were cultivated as described in Materials and Methods until 70% confluent, then switched for 36 h to RPMI-defined medium containing 70 μM choline (control) and 50 nM rotenone or 12 μM ET-18-OCH_3 plus rotenone; additional cultures were treated with CD (0 μM choline) in the presence or absence of 50 nM rotenone. Generation of intracellular ROS was measured by NBT assay. Data are expressed as mean nmol NBT reduced/10^6 cells ± SE. $n = 5$/point. ANOVA with Scheffé's F test: bars with different letter superscripts differ by $P < 0.01$. This experiment was replicated once. *B)* Cells were fixed and analyzed for the induction of apoptosis as described in the legend to Fig. 2. $n = 6$/point. ANOVA with Scheffé's F test: bars with different letter superscripts differ by $P < 0.01$.

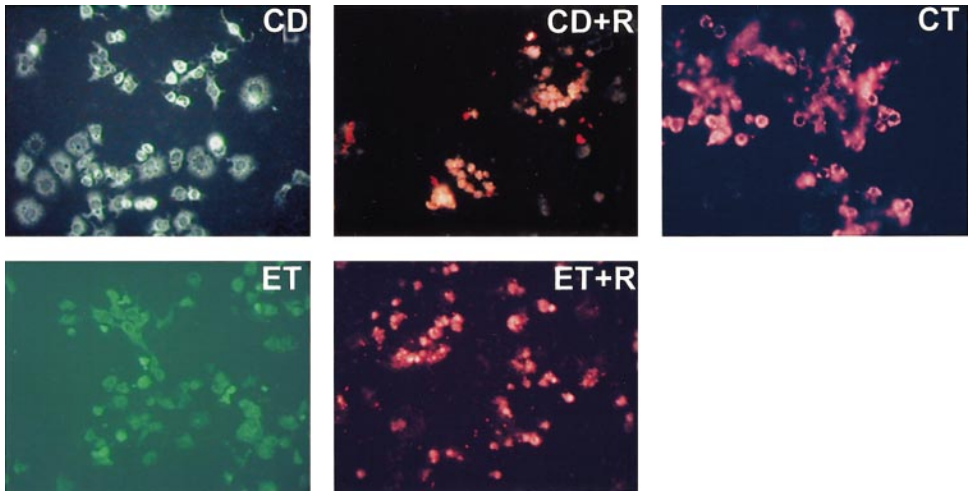

Figure 4. Mitochondrial membrane potential is reduced in ET-18-OCH_3 or CD-treated cells; this effect is abrogated by rotenone. CWSV-1 cells were loaded with JC-1 as described in Materials and Methods. They were treated for 48 h with 0 μM choline (CD), 0 μM choline plus 25 nM rotenone (CD+R), control medium (CT), 12 μM ET-18-OCH_3 (ET), or 12 μM ET-18-OCH_3 plus 25 nM rotenone (ET+R). Normally polarized mitochondria appear orange; depolarized mitochondria appear green. This experiment was replicated three times. 400×.

eration of ROS that was reduced significantly by simultaneous treatment with the antioxidant α-tocopherol (**Fig. 2*A***). In addition, in the presence of α-tocopherol, ET-18-OCH_3 and CD-induced apoptosis were reduced by 73% and 86%, respectively (Fig. 2*B*).

Mitochondria, ROS, and apoptosis

Studies were undertaken to determine whether mitochondria were the source of ROS in this model system. Compared to cells treated with ET-18-OCH_3 or CD alone, treatment with rotenone led to a nearly 85% reduction in the generation of ROS caused by these agents (**Fig. 3*A***). This effect of rotenone was accompanied by a 59% (ET-OCH_3) and 74% (CD) reduction in the level of apoptosis (Fig. 3*B*).

Mitochondrial membrane potential

Compared with cells maintained in 70 μM choline, which exhibited orange-red JC-1 fluorescence (**Fig. 4**, panel CT), CWSV1 hepatocytes treated with CD (0 μM choline) medium (Fig. 4, panel CD) or ET-18-OCH_3 (Fig. 4, panel ET) exhibited green JC-1 fluorescence, which is consistent with a loss of mitochondrial membrane polarization. Cells treated with valinomycin alone (positive control) exhibited green fluorescence consistent with a loss of mitochondrial membrane polarization (data not shown). Treatment with rotenone (25 nM) in the presence of CD (Fig. 4, panel CD+R) or ET-18-OCH_3 (Fig. 4, panel ET+R) resulted in a shift in JC-1 fluorescence to orange-red, consistent with a restoration of normal mitochondrial membrane polarization.

Mitochondria and PC

To determine the role of PC synthesis in induction of apoptosis, the PC content of mitochondria and associated membranes was measured in cells that were treated for 24 h with either ET-18-OCH_3 or CD. These agents reduced PC content of mitochondria and associated membranes by 31% and 24%, respectively, compared with controls (70 μM choline) (**Table 1**). TEM analysis showed that the mitochondrial preparations contained intact mitochondria and associated endoplasmic reticulum (data are not shown).

DISCUSSION

Since PC plays an important role in maintaining the structural integrity of cell membranes, perturbation of PC synthesis could be an early event in apoptosis signal transduction. We show that the anti-cancer drug ET-18-OCH_3 inhibits PC synthesis and induces generation of ROS, resulting in apoptotic cell death in p53-defective hepatocytes; these observations are similar to those described in acute CD (4, 5, 28). Previous reports showed that ET-18-OCH_3 inhibited PC synthesis in nonhepatocyte-type cells resulting in an inhibition of cell proliferation (1) and increased apoptosis (29). However, these studies did not examine whether changes in PC within a plasma membrane or some other membrane were a critical variable. Since both ET-18-OCH_3 and CD decreased mitochondrial PC lev-

TABLE 1. *PC content of mitochondria and associated membranes after 24 h treatment with ET-18-OCH_3 or CD (0 μM choline)*

Treatment	nmol PC/mg protein
Control	226 ± 19
ET-18-OCH_3	156 ± 13*
CD	173 ± 7*

* $P < 0.05$ vs. control by ANOVA and Tukey-Kramer HSD test.

els and induced apoptosis in CWSV1 cells, and rotenone inhibited both forms of apoptosis, the present data demonstrate an important connection between mitochondrial PC synthesis and the participation of mitochondria in apoptosis signaling.

Recent reports demonstrate that perturbations in mitochondrial respiration can occur early in the apoptotic process and that the mitochondrion itself may serve as a control switch for some forms of apoptosis (30, 31). For example, loss of mitochondrial membrane potential and opening of the mitochondrial membrane megachannel (the so-called permeability transition) have been documented in apoptotic signaling (25, 32, 33). The occurrence of this permeability transition permits the release of proapoptotic proteins (e.g., cytochrome *c*, apoptosis-inducing factor) into the cytosol resulting in activation of caspases and endonucleases and the completion of apoptosis (9, 34, 35).

Some well-controlled studies have shown that complex I of the mitochondrial electron transport chain is an important site for leakage of electrons and subsequent generation of ROS, resulting in completion of apoptosis. To understand the possible role of mitochondria in apoptosis, we modulated the levels of CD and ET-18-OCH_3 in the presence or absence of rotenone, an inhibitor of complex I (NADH-quinone oxidoreductase) of the mitochondrial electron transport chain (36). Complex I contains a single large inhibitor binding pocket (37), and binding of different types of inhibitors, including rotenone, prevent overgeneration of ROS and apoptosis-associated DNA cleavage (38, 39). Rotenone is capable of exerting diverse dose-dependent effects on cell survival. In stem-like liver epithelial cells high doses of rotenone (0.5–5 μM) induced apoptosis (40, 41). However, lower doses of rotenone ($\leq$50 nM) inhibited hydrogen peroxide production, internucleosomal DNA fragmentation, and the morphological appearance of apoptosis in response to C6-ceramide or overexpression of PKC_δ (13, 42). CD apoptosis is associated with generation of ceramide (43) and activation of PKC_δ (44). Thus, our observations with CD- and ET-18-OCH_3-treated cells are consistent with the previously established apoptosis inhibitory effects of rotenone.

Rotenone itself does not influence PC synthesis directly (45), so the inhibition of apoptosis we observed must have occurred downstream of the change in PC. The ability of both ET-18-OCH_3 and CD to collapse the mitochondrial membrane potential in CWSV1 cells suggests that mitochondrial generation of ROS per se plays a central role in the completion of both CD and ET-18-OCH_3 apoptosis. Mitochondria are a potential source and target of ROS in cells treated with a variety of proapoptotic agents. In the present study, both ET-18-OCH_3 and CD caused overgeneration of ROS and apoptosis, which were decreased significantly when cells were exposed simultaneously to rotenone, an inhibitor of the mitochondrial electron transport chain. Thus, it is likely that mitochondria are the source of ROS in this model system.

In summary, this study shows that the anti-cancer drug ET-18-OCH_3 causes apoptosis in p53-defective hepatocytes as a consequence of generation of ROS and the loss of mitochondrial membrane potential. Taken together, the results suggest that the induction of structural changes in the mitochondrial membrane because of decreased PC synthesis may play an important role in this form of apoptosis. FJ

Supported by NIH grant AG09525 (S.H.Z.) and DK55865 (S.H.Z.), NIH fellowship GM14840 (A.S.V.), the Elsa U. Pardee Foundation (C.D.A.), and AICR grant 99B074 (C.D.A.).

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Received for publication May 11, 2000.
Revised for publication April 5, 2001.