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Mitochondrial and Microsomal Derived Reactive Oxygen Species Mediate Apoptosis Induced by Transforming Growth Factor-β1 in Immortalized Rat Hepatocytes

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Abstract Transforming growth factor-β1 (TGFβ1) is a multifunctional cytokine that is over expressed during liver hepatocytes injury and regeneration. SV40-transformed CWSV-1 rat hepatocytes that are p53-defective undergo apoptosis in response to choline deficiency (CD) or TGFβ1, which mediates CD-apoptosis. Reactive oxygen species (ROS) are essential mediators of apoptosis. We have shown that apoptosis induced by TGFβ1 is accompanied by ROS generation and the ROS-trapping agent *N*-acetylcysteine (NAC) inhibits TGFβ1-induced apoptosis. While persistent induction of ROS contributes to this form of apoptosis, the source of ROS generated downstream of TGFβ1 is not clear. The mitochondria and the endoplasmic reticulum both harbor potent electron transfer chains that might be the source of ROS essential for completion of TGFβ1-apoptosis. Here we show that CWSV-1 cells treated with cyclosporine A, which prevents opening of mitochondrial membrane pores required for ROS generation, inhibits TGFβ1-induced apoptosis. A similar effect was obtained by treating these cells with rotenone, an inhibitor of complex 1 of the mitochondrial electron transfer chain. However, we demonstrate that TGFβ1 induces cytochrome P450 1A1 and that metyrapone, a potent inhibitor of cytochrome P450 1A1, inhibits TGFβ1-induced apoptosis. Therefore, our studies indicate that concurrent with promoting generation of ROS from mitochondria, TGFβ1 also promotes generation of ROS from the cytochrome P450 electron transfer chain. Since inhibition of either of these two sources of ROS interferes with apoptosis, it is reasonable to conclude that the combined involvement of both pathways is essential for completion of TGFβ1-induced apoptosis. J. Cell. Biochem. 89: 254–261, 2003. © 2003 Wiley-Liss, Inc.

Key words: apoptosis; cytochrome P4501A1; hepatocytes; mitochondria; reactive oxygen species; transforming growth factor-β1

Transforming growth factor-β1 (TGFβ1) is one member of a large family of growth factors (e.g., activin, bone morphogenic protein, decapentaplegic, TGFβ1-3 isoforms), which play a regulatory role in cell migration, proliferation, and apoptosis during tissue injury and repair. Normal epithelial cells respond to TGFβ1 receptor-mediated signal transduction by upregulating the expression of one or several cell cycle regulatory proteins (e.g., p15Ink4B, p16, p21Waf1/Cip1, p27Kip1) [Datto et al., 1995; Li et al., 1995; Markowitz and Roberts, 1996; Albright and Zeisel, 1997; Albright et al., 1999], resulting in cell growth arrest or apoptosis that depend on the generation of reactive oxygen species (ROS) and activation of caspases [Herrera et al., 2001; Albright et al., 2002]. Although TGFβ1 is an important physiological death signal for hepatocytes undergoing apoptosis, the TGFβ1 apoptosis signaling pathway is not completely understood.

Reactive oxygen species (ROS) are one of several important components of the complex apoptotic cell death process. ROS can be generated from several intracellular locations,

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including microsomal pathways (cytochrome P450 and NADPH-dependent) [Rao and Cederbaum, 1997], and the mitochondrial electron transport chain [Kukielka et al., 1994; Thannickal and Fanburg, 1995]. Previously we demonstrated that neutralization of TGFβ1 secreted by cultured CWSV-1 hepatocytes or scavenging of intracellular ROS protected these cells against apoptosis [Zeisel et al., 1997]. In the present study, we show that treatment with an antioxidant or inhibition of microsomal and mitochondrial sources of ROS block induction of TGFβ1-apoptosis in rat hepatocytes.

MATERIALS AND METHODS

Cell Culture

SV40 large T-antigen immortalized CWSV-1 rat hepatocytes were obtained from Dr. Harriet C. Isom (Department of Microbiology, The Pennsylvania State University College of Medicine, Hershey, PA). p53 function is inactivated in cells which are transfected with SV40 large T-antigen [Lane and Crawford, 1979]. CWSV-1 cells are routinely grown in serum-free medium, express liver specific proteins, and are non-tumorigenic at the low passages used in these studies [Woodworth et al., 1986, 1988]. CWSV-1 cells were maintained in serum-free RPMI 1640 medium (Atlanta Biologicals, Norcross, GA) supplemented with 70 μM choline as described previously [Albright et al., 1996].

Assessment of Apoptosis

For morphological assessment of apoptosis, cells were seeded at 2.2×10^4 cells/cm^2 in 6-well multiwell plates in 70 μmol/L choline-sufficient (CS) RPMI 1640 medium for 4 days before rinsing with phosphate-buffered saline (PBS) and switching them to fresh, 70 μmol/L CS, or choline-deficient (CD: 5 μmol/L choline) medium for an additional 2 days as described in the figures. Cells with classical morphological features of apoptosis [Wyllie, 1980] were detected in hematoxylin and eosin stained plates as described previously [Albright et al., 1996]. The presence of apoptotic cell morphology correlates with the occurrence of TUNEL labeling and an apoptotic DNA ladder [Shin et al., 1997; Zeisel et al., 1997]. The percent of cells with classical features of apoptosis was determined by counting at least 300 cells in 3–6 replicates per experiment.

Quantitation of DNA Synthesis

Cells grown in LabTek® chamber slides on day 6 in culture were incubated with 100 μmol/L 5-bromo-2′-deoxyuridine (BrdU) (Sigma, St. Louis, MO) as described previously [Albright et al., 1990] and then fixed in ice-cold 70% ethanol. Sites of BrdU incorporation were visualized using an indirect streptavidin-peroxidase immunocytochemical method and a commercially available kit (Zymed, South San Francisco, CA), following by counterstaining with Gill hematoxylin. The BrdU labeling index was determined by counting at least 300 cells in 4–6 replicates per experiment. The effects of treatment conditions on cell proliferation were confirmed by determining the mitotic index as described previously [Albright et al., 1990].

TGFβ1 Treatment

Cells on day 4 in culture in 70 μmol/L choline were switched to medium (70 or 5 μmol/L choline) containing 0–40 pmol/L TGFβ1 protein (purity >98% by SDS–PAGE; inhibits [^{3}H]-thymidine incorporation in human and rat epithelial cells; Boehringer Mannheim, Indianapolis, IN) or 0–10 μl/ml TGFβ1 neutralizing antibody (clone V; Santa Cruz Biotechnology, Santa Cruz, CA). This antibody recognizes amino acids 328–353 within the carboxy terminus of human and rat TGFβ1, and, according to the supplier, does not cross-react with TGFβ2 or TGFβ3.

Assessment of Cytochrome P450

For cytochrome P450 determinations, cultured cells maintained as described above were probed with a goat primary anti-cytochrome P450 polyclonal antibody (1:100 dilution) (CYP1A1, clone G-18; recognizes an epitope near the carboxy terminus of CYP1A1 of mouse, rat and human origin; Santa Cruz Biotech.). Negative controls were incubated with non-immune serum; positive controls were treated as described previously with 25 μmol/L β-naphthoflavone, a known inducer of CYP1A1 in hepatocytes [Meunier et al., 2000]. Sites of primary antibody localization were determined as describe above. In order to confirm induction of CYP1A1, hepatocyte microsomal proteins were resolved on SDS–PAGE gels, electrotransferred to nitrocellulose membrane and detected by Western blotting using a CYP1A1 antibody (Santa Cruz) and an enhanced

chemiluminescence method (Amersham Pharmacia Biotechnology, Piscataway, NJ).

Intracellular Mitochondrial Membrane Potential

Cells grown on glass chamber slides were treated with or without TGFβ1 (40 pmol/L for 48 h), after which time the medium was aspirated and replaced with fresh JC-1 staining solution (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide, 10 μg/ml; Molecular Probes, Pitchford, OR) in warm (37°C) cell culture medium as described previously [Smiley et al., 1991; Vrablic et al., 2001]. After 15 min, cells were rinsed in JC-1-free cell culture medium and mounted in aqueous mounting medium, and observed on an Olympus inverted fluorescence microscope equipped with epifluorescence optics and a 100-W mercury lamp. The green fluorescence of JC-1 was visualized using a 450–490 nm exciter filter and a 515–565 nm barrier filter; red fluorescence of JC-1 aggregates was visualized using a 590-nm barrier filter. Fluorescent images were captured using Scion Image as described previously [Albright et al., 1999].

Generation of ROS in CWSV-1 Cells

Protein carbonyl content was assayed to measure the induction of ROS-mediated damage. Briefly TCA precipitated cell lysate protein (0.5–1.0 mg) was treated with 0.2% dinitrophenylhydrazine (DNPH) in 2 N HCl for 15 min at room temperature, samples were then reprecipitated with TCA and the dried pellets were dissolved in 6 M guanidium HCl, and protein carbonyls were quantified colorimetrically as DNPH incorporated [Oliver et al., 1989]. In addition, ROS formation by cultured cells was measured using the fluorescent dye dihydroethidium (Molecular Probes) as described previously [Schlezinger et al., 1999]. In order to inhibit microsomal generation of ROS, cells were treated with metyrapone (Mty; 0–1 mmol/L; 2-methyl-1,2-di-3-pyridyl-1-propanone; Sigma), an inhibitor of cytochrome P450 activity [O'Donnell et al., 1995], contemporaneous with exposure to CD or TGFβ1 protein. In order to inhibit mitochondrial generation of ROS, cells were treated with rotenone (Rtn; 0–50 nM) (Sigma), an inhibitor of complex I of the mitochondrial electron transport chain [Fukami et al., 1967]. In order to investigate the possible role of mitochondrial membrane potential in apoptosis, cells were treated as indicated in the figures with the protonophore m-chlorophenylhydrazone carbamyl cyanide (mCCCP; 10 μmol/L; Sigma) dissolved in acetone (final acetone concentration $<0.005\%$), or with cyclosporine A (CycA) (0–50 μmol/L; cyclosporine from *T. inflatum*, Sigma) dissolved in absolute ethanol (final ethanol concentration $<0.0001\%$) [Zamzami et al., 1996].

Statistical Analysis

We used analysis of variance and appropriate multiple comparisons procedures to determine statistical significance between the treatment groups (JMP Version 2, SAS, 1989) [Zeisel et al., 1997].

RESULTS

Effect of TGFβ1 on Apoptosis

Choline deficient (5 μmol/L choline) CWSV-1 cells exhibited a 4–5-fold increase in apoptotic cells (8.9 ± 1.4 vs. 1.7 ± 0.4 percent in CD vs. CS; n = 6/point; $P < 0.01$). Antibody neutralization of endogenous, secreted TGFβ1 reduced the rate of apoptosis in CD hepatocytes to control levels (1.8 ± 0.4 vs. 1.7 ± 0.4 percent), but had no effect on cells maintained in 70 μmol/L choline (not shown) (correlation of apoptotic morphology with TUNEL and DNA ladders in CD is shown in previously published reports [Albright et al., 1996; Zeisel et al., 1997]. Most cells undergoing TGFβ1-apoptosis exhibited nuclear localization of p27Kip1 cyclin dependent kinase inhibitor protein (data not shown), consistent with alterations to cell proliferation.

Effect of TGFβ1 on Proliferation

Compared to controls (70 μmol/L choline), CWSV-1 cells made acutely choline deficient exhibited an approximate 50% reduction in the rate of cell proliferation (BrdU labeling: 14.6 ± 1.4 vs. 7.9 ± 1.5 percent in CS vs. CD; n = 6/point; $P < 0.01$). A nearly 65% reduction in the BrdU labeling index occurred when TGFβ1 (40 pmol/L) was added to CWSV-1 cells maintained in 70 μmol/L choline, consistent with the effects of CD alone on DNA synthesis, whereas acutely deficient hepatocytes were less affected by added TGFβ1 (Table I). However, in cells maintained in 5 μmol/L choline-containing medium, antibody-neutralization of endogenous, secreted TGFβ1 restored DNA synthesis to nearly control levels (Table I).

TABLE I. Effect of Endogenous TGFβ1 on BrdU Incorporation in CWSV-1 Hepatocytes

Treatment	BrdU labeling index (%)[a]	
	CS (70 μmol/L)	CD (5 μmol/L)
TGFβ1 [pmol/L]		
0.0	14.3 ± 1.7	7.7 ± 3.3
0.4	13.7 ± 2.0	6.6 ± 1.0
4.0	11.0 ± 0.8*	6.8 ± 0.8
40	5.1 ± 0.9*	4.2 ± 1.9*,**
TGFβ1 antibody [ng/ml]		
0.1	15.2 ± 0.9	7.8 ± 1.2
1.0	13.8 ± 1.4	14.2 ± 1.7***

[a]Data are mean ± SE (n = 4/point).
*$P < 0.01$ vs. CS (no TGFβ1).
**$P < 0.05$ vs. CD (no TGFβ1).
***$P < 0.01$ vs. CD (no TGFβ1) (ANOVA). This experiment was replicated twice with similar results.

Influence of ROS on TGFβ1 Signaling

Choline deficiency, as well as added TGFβ1, increased the generation of ROS (measured by formation of protein carbonyl groups), which were decreased by the simultaneous addition of the antioxidant *N*-acetylcysteine (NAC) (Fig. 1). Similarly, antibody-neutralization of secreted TGFβ1 reduced the level of superoxide generation (Fig. 2), consistent with the effects of NAC on protein oxidation. Simultaneous treatment

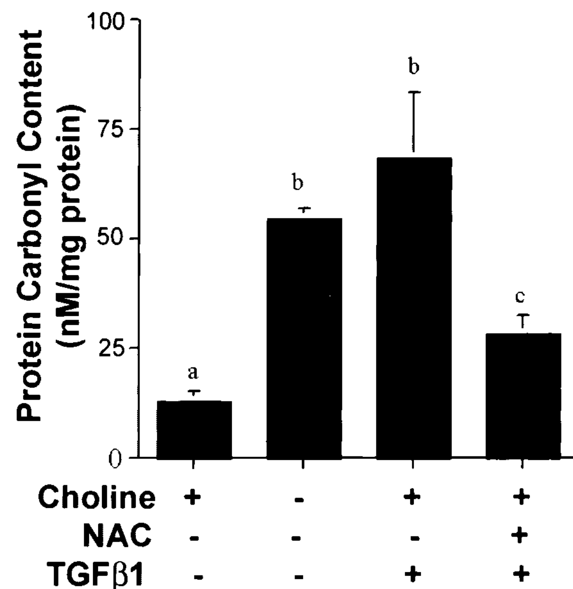

Fig. 1. CD (5 μmol/L choline chloride) and 40 pmol/L TGFβ1 induce reactive oxygen species (ROS) in CWSV-1 cells. Cells were maintained for 2 days in experimental medium and the generation of ROS was measured using an assay for the formation of protein carbonyl groups, a marker of oxidative stress. 0.25 mmol/L *N*-acetylcysteine (NAC) significantly reduces the induction of oxidant stress by TGFβ1 (n = 3/point; columns with different letters are significantly different ($P < 0.01$ by ANOVA and Tukey–Kramer test)).

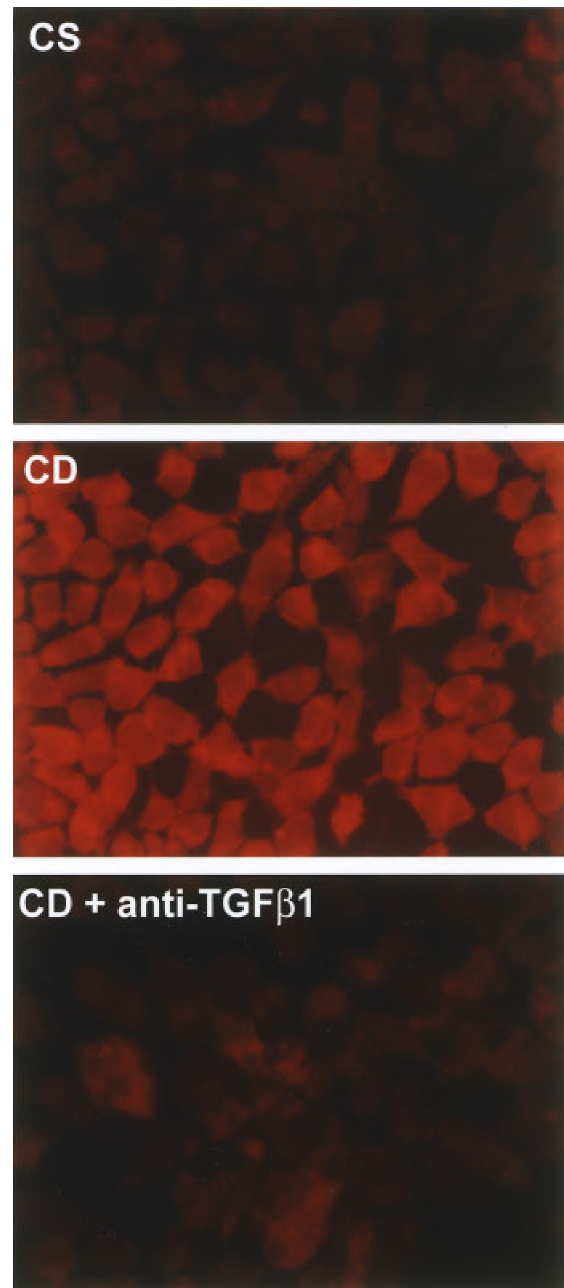

Fig. 2. TGFβ1 mediates CD-induced generation of ROS in CWSV-1 cells. Cells were loaded with dihydroethidium as described in the Materials and Methods. Cells were treated with (**A**) choline supplementad medium (CS, 70 μmol/L); (**B**) choline deficient medium (CD, 5 μmol/L); or (**C**) CD plus 10 μL/ml TGFβ1 neutralizing antibody. Dihydroethidium is sensitive to oxidation by superoxide anion generated following uncoupling of mitochondrial oxidative phosphorylation. Normal resting cells appear blue whereas upon generation of ROS cells appear red due to oxidation of dihydroethidium which then intercalates into nuclear DNA. (Magnification 400×). [Color figure can be viewed on the online issue, which is available at www.interscience.wiley.com.]

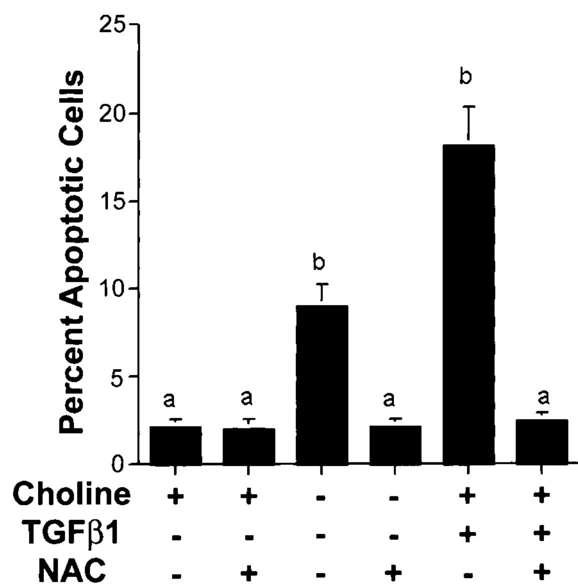

Fig. 3. ROS are mediators of both CD- and TGFβ1-apoptosis. Cells were maintained for two days in experimental medium and the role of ROS in apoptosis was investigated. 0.25 mmol/L NAC prevented both CD- and 40 pmol/L TGFβ1-apoptosis, but had no effect on cells maintained in CS medium alone (n = 3/point; columns identified with different letters are significantly different ($P < 0.01$ by ANOVA and Tukey–Kramer test)).

with NAC and CD reduced the rate of apoptosis to control levels, and abrogated the effects of TGFβ1 on apoptosis in control cells (Fig. 3). Cells treated with TGFβ1 in the presence of NAC exhibited rates of cell proliferation (BrdU labeling) within the range observed in control cells (no TGFβ1, data not shown). Cells treated with TGFβ1 plus NAC also increased their mitotic rate compared to TGFβ1 alone (mitotic index $= 1.95 \pm 0.28$ vs. 0.65 ± 0.6 percent with TGFβ1 alone ($n = 6, P < 0.01$)).

TGFβ1, Cytochrome P450, Mitochondrial Signaling, and Apoptosis

Several cytochromes P450 [Dai et al., 1993; Shertzer et al., 1998] and mitochondrial pathways [Boveris and Chance, 1972] participate in the generation of ROS. Cells in control medium (no TGFβ1 or BNF) exhibited very low levels of CYP1A1, whereas TGFβ1 induced high levels of CYP1A1 that were equal to or greater than the levels induced by β-naphthoflavone (BNF) alone (Fig. 4). Both TGFβ1-induced expression of CYP1A1 (Fig. 4) and apoptosis (Fig. 5) were reduced in the presence of metyrapone.

Cells in control medium exhibited very low rates of apoptosis (Fig. 6) and highly polarized mitochondria (Fig. 7). Treatment with rotenone

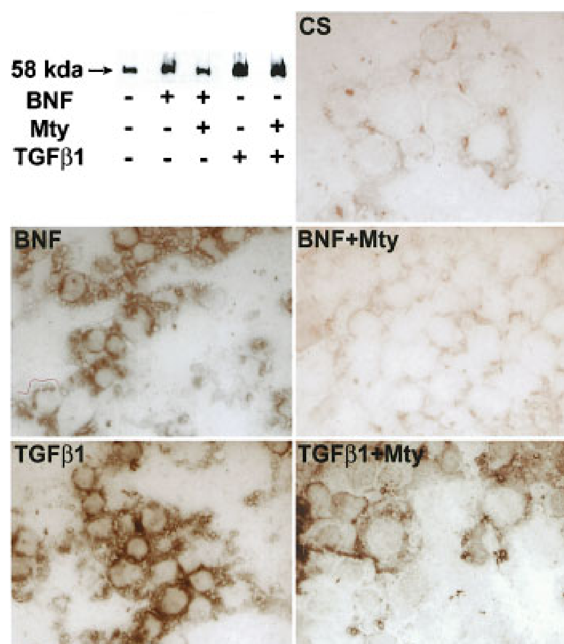

Fig. 4. Participation of CP450 in TGFβ1-apoptosis in CWSV-1 cells. Treatment with 40 pmol/L TGFβ1 or 25 μmol/L BNF (β-naphthoflavone, positive control) for 48 h increased the expression of CP4501A1 in CWSV-1 cells, detected by immunohistochemistry. Western blot analysis shows that 1 mmol/L metyrapone (Mty), a specific inhibitor of microsomal enzymes, decreased the level of expression of CYP1A1 in presence of TGFβ1 or BNF, although the inhibitory effect of Mty was much greater on the latter. [Color figure can be viewed on the online issue, which is available at www.interscience.wiley.com.]

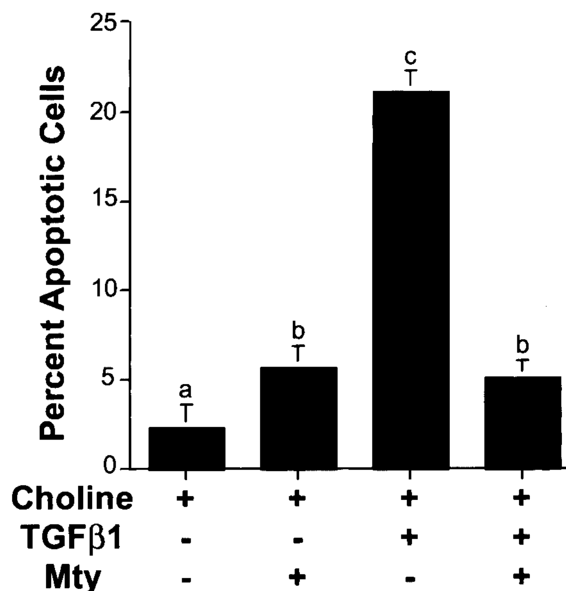

Fig. 5. Metyrapone significantly reduced TGFβ1-apoptosis in CWSV-1 cells. Cells in the log phase of growth were treated for 48 h with control medium, or control medium plus metyrapone (Mty, 1 mmol/L) in the presence or absence of TGFβ1 (40 pmol/L) (n = 5–6/point; columns with different letters are significantly different ($P < 0.01$ by ANOVA and Tukey–Kramer test).

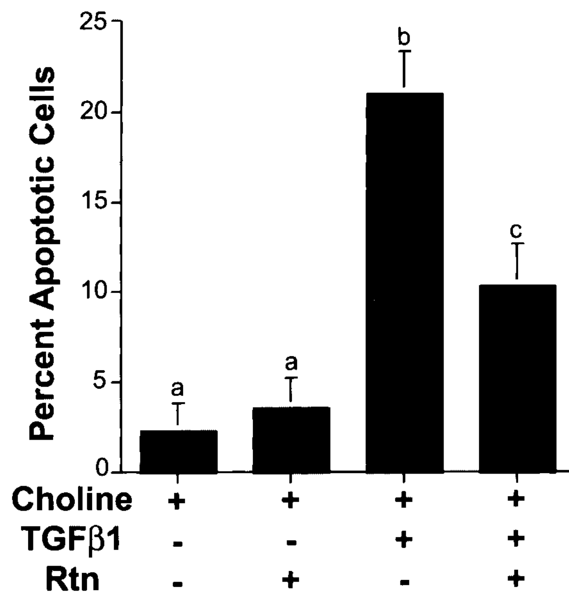

Fig. 6. Participation of mitochondrial alterations in TGFβ1-apoptosis in CWSV-1 cells. Cells in the log phase of growth were treated for 48 h with control medium, or control medium plus rotenone (Rtn, 50 nmol/L) in the presence or absence of TGFβ1 (40 pmol/L). Rtn, a specific inhibitor of complex I of the mitochondrial electron transport chain significantly inhibited TGFβ1-apoptosis, whereas Rtn alone had no apparent effects on control cells (columns identified with different letters are significantly different ($P < 0.01$ by ANOVA and Tukey–Kramer test).

in the presence of TGFβ1 significantly reduced the rate of apoptosis (Fig. 6) and restored mitochondrial membrane polarization (Fig. 7) in CWSV-1 hepatocytes. In order to confirm the

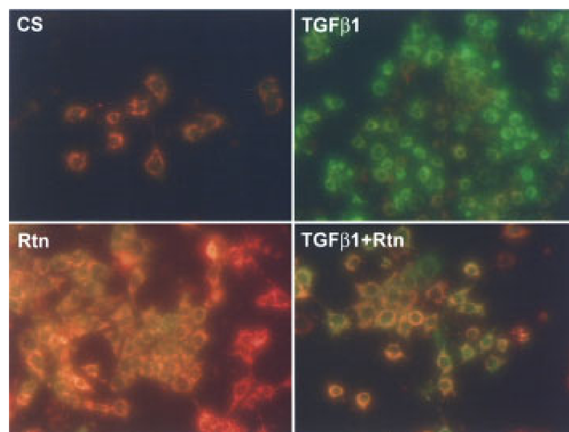

Fig. 7. Mitochondrial membrane potential is reduced in TGFβ1-treated cells and is restored by rotenone. CWSV-1 cells were loaded with JC-1 as described in the Materials and Methods. Cells were treated for 48 h with control medium (CS), CS plus 50 nmol/L rotenone (Rtn), 40 pmol/L TGFβ1, or TGFβ1 plus Rtn. Mitochondria appear orange when normally polarized and green when depolarized. (Magnification 400×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

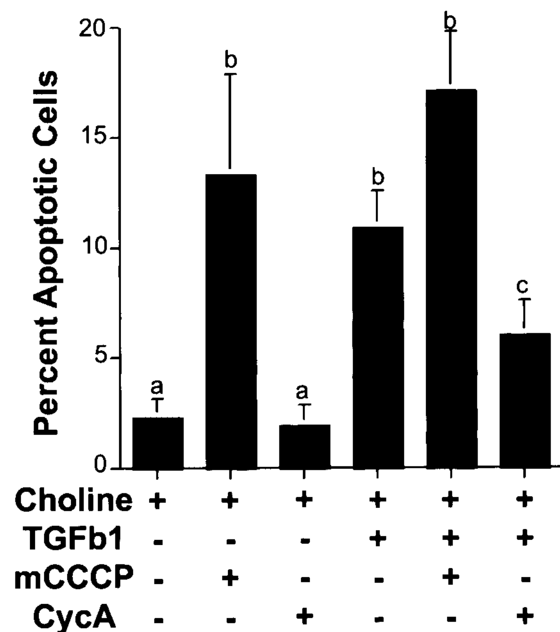

Fig. 8. Mitochondrial membrane potential and apoptosis in CWSV-1 cells. Cells maintained for two days in CS medium were treated with TGFβ1 (40 pmol/L) in the presence or absence of mCCCP (10 μmol/L) or CycA (10 μmol/L). The protonophore mCCCP acclerated TGFβ1-apoptosis whereas CycA, which prevents the opening of mitochondrial membrane pores associated with a loss of membrane potential [Zamzami et al., 1995], prevented TGFβ1-apoptosis (n = 4/point; columns with different letters are significantly different ($P < 0.01$ by ANOVA and Tukey–Kramer test)).

role of mitochondrial signaling in TGFβ1-mediated apoptosis, CWSV-1 cells were treated with mCCCP or CycA in the presence or absence of TGFβ1 (Fig. 8). As expected, the protonophore mCCCP accelerated apoptosis in the presence of TGFβ1, whereas CycA, which prevents the opening of mitochondrial membrane pores with resulting loss of membrane potential, diminished TGFβ1-apoptosis.

DISCUSSION

Choline is important for membrane integrity and signaling function, and withdrawal of choline from the diet caused apoptosis and an increase in the expression of TGFβ1 and related signaling proteins in hepatocytes in vivo [Zeisel, 1993; Albright and Zeisel, 1997; Zeisel et al., 1997]. The present studies confirm these findings and further show that CD causes an increased expression of TGFβ1 and p27Kip1 proteins in CWSV-1 hepatoctyes with morphological manifestations of apoptosis. This is consistent with previous studies showing that

CD caused apoptosis in immortalized rat hepatocytes in vitro [Albright et al., 1996; Shin et al., 1997]. It is known that NAC, a general scavenger of free radicals, as well as neutralization of TGFβ1 can inhibit CD-apoptosis [Zeisel et al., 1997]. This led us to postulate that ROS may be important mediators of apoptosis triggered by TGFβ1. We now demonstrate that TGFβ1-apoptosis in these p53-defective CWSV-1 cells is due to an increase in ROS derived from both mitochondrial and microsomal sources.

Alterations to choline metabolism may have diverse effects that culminate in the generation of oxidant stress. A combined CD-methionine deficient diet was shown to decrease both selenium-dependent GSH peroxidase and microsomal GSH peroxidase activity in hepatocytes in vitro [Tan et al., 1987], generating lipid peroxidation and 8-hydroxyguanosine residues in DNA [Ghoshal et al., 1987; Nakae et al., 1990]. Thus, the ability of an inhibitor (rotenone) of complex I of the mitochondrial electron transport chain, and metyrapone, an inhibitor of microsomal cytochrome P450 activity, to inhibit both generation of ROS and apoptosis in our studies is consistent with some of the known biochemical effects of choline availability.

Previously, we found that the mitochondria in CD CWSV-1 cells were not dysfunctional, as they retained the ability to reduce tetrazolium to formazan, an ATP-dependent process [Albright et al., 1996]. However, mitochondria have a higher content of phosphatidylcholine in their membranes [de Kroon et al., 1997], and the decrease in membrane phosphatidylcholine content that occurs in response to CD [Shin et al., 1997] could result in a leakage of electrons from the mitochondrial electron transport chain and the generation of ROS. Studies also link choline metabolism to other cytosolic membrane sources in the generation of ROS. In rodent lung, activity of choline phosphotransferase, the final enzyme in the de novo synthesis of phosphatidylcholine via the CDP-choline pathway, was identified in both mitochondria and microsomes [Das et al., 1993], and linked synthesis of phosphatidylcholine involving mitochondrial and microsomal enzymes can occur [Ardail et al., 1993]. Interestingly, administration of phosphatidylcholine prevented induction of lipid peroxidation in rat liver [Lieber et al., 1997], and the antioxidant vitamin E prevented the decrease in mitochondrial phosphatidylcholine caused by alcohol [Nazarov and Lider, 1996]. These reports suggest a link between the level of membrane phosphatidylcholine and the generation of ROS.

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