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β-Carotene Prevents Bile Acid-Induced Cytotoxicity in the Rat Hepatocyte: Evidence for an Antioxidant and Anti-Apoptotic Role of β-Carotene *In Vitro*

ERIC GUMPRICHT, ROLF DAHL, MICHAEL W. DEVEREAUX, AND RONALD J. SOKOL

Pediatric Liver Center and Liver Transplantation Program, Section of Pediatric Gastroenterology, Hepatology and Nutrition, Department of Pediatrics, University of Colorado School of Medicine and The Children's Hospital, Denver, Colorado 80262, U.S.A.

ABSTRACT

Hydrophobic bile acids are implicated in the pathogenesis of cholestatic liver disorders through mechanisms involving oxidative stress and mitochondrial dysfunction. Antioxidants ameliorate bile acid–induced cytotoxicity in rat hepatocyte suspensions. The purpose of the current study was to evaluate the potential protective role of β-carotene (βC), a putative fat-soluble antioxidant that is reduced in patients with cholestasis, against bile acid–induced hepatotoxicity. In freshly isolated rat hepatocyte suspensions that were exposed to the toxic hydrophobic bile acid glycochenodeoxycholic acid (100 or 500 μM), βC (100 μM) decreased generation of reactive oxygen species by >50%, similar to the inhibition afforded by α-tocopherol. Commensurate with this antioxidant effect, 100 μM βC also protected hepatocytes against both glycochenodeoxycholic acid–induced cellular necrosis and apoptosis, which was associated with reduction in caspase 3 activation, inhibition of mitochondrial cytochrome c release in rat hepatocytes, and prevention of the mitochondrial permeability transition in both liver mitochondria and rat hepatocytes. A lower concentration of βC (50 μM) produced similar antioxidant and anti-apoptotic protection but with less inhibition against cell necrosis, suggesting that the higher concentration of βC may have conferred additional cytoprotection not directly related to its antioxidant function. These results demonstrate that the antioxidant effects of βC may provide hepatoprotection against cholestatic liver injury by preventing bile acid–induced oxidative stress and mitochondrial perturbations. **(*Pediatr Res* 55: 814–821, 2004)**

Abbreviations

α-TH, α-tocopherol
βC, β-carotene
CLD, cholestatic liver disorders
DCF-DA, dichlorofluorescin diacetate
DCFein, 2'7'-dichlorofluorescein
GCDC, glycochenodeoxycholic acid
KRH, Krebs Ringers HEPES
MPT, mitochondrial permeability transition
PI, propidium iodide
ROS, reactive oxygen species
Δψ, mitochondrial electrochemical gradient

The preventive roles of dietary antioxidants, such as vitamin E (including tocopherols and tocotrienols) and ascorbic acid, have been evaluated extensively as potential protective agents toward several diseases or conditions in humans, including cancer, heart disease, and cataractogenesis (see Refs. 1 and 2 for overviews). The mechanism of protection afforded by these agents is generally consistent with their antioxidant activities, although inhibition of cellular mediators, including protein kinases and eicosanoids, have also been implicated (3, 4). In addition to these antioxidant vitamins, β-carotene (βC) may function as a physiologically relevant antioxidant along with its well-defined role as a precursor to vitamin A (for review, see Ref. 5). However, despite the seminal report by Burton and Ingold (6), who found that βC functioned as an efficient antioxidant in chemical systems, recent large-scale human studies with the carotenoid have been disappointing. For example, βC failed to protect human subjects against lung cancer and coronary artery disease (7, 8). Consequently, the Panel on Dietary Antioxidants and Related Compounds of the Food and Nutrition Board of the Institute of Medicine determined that βC does not meet the definition of a dietary antioxidant and that βC supplementation should not be recommended (9). In contrast to the studies that evaluated βC's role in the preven-

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Correspondence: Ronald J. Sokol, MD, Department of Pediatrics, Box B290, The Children's Hospital, 1056 E. 19th Avenue, Denver, CO 80218, U.S.A.; e-mail: sokol.ronald@tchden.org
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tion of cancer and atherosclerosis, few studies have addressed the potential protective effects of βC in hepatobiliary diseases. Recent reports show that *1*) βC is malabsorbed in various hepatobiliary diseases (10–12); *2*) serum βC levels are very low in adults with the cholestatic liver disorders (CLD), primary biliary cirrhosis, and primary sclerosing cholangitis (12) and in children with cholestasis (13); and *3*) antioxidant therapy improved symptoms in patients with cholestasis in a recent pilot study (14). Thus, a lack of adequate βC may play a role in potentiating liver injury in CLD.

Recent studies from our laboratory demonstrate in both *in vivo* and *in vitro* models that oxidative stress is a key mediator for the hepatic injury observed in CLD. These diseases, characterized by an impairment of bile flow or secretion that results in increased concentrations of bile constituents within the liver and subsequent hepatotoxicity, include biliary atresia, neonatal hepatitis, progressive familial intrahepatic cholestasis, a variety of metabolic liver disorders, cystic fibrosis, and primary sclerosing cholangitis in childhood. Because treatment for many of these disorders is inadequate, liver transplantation is frequently required in affected children and adults (15). One primary factor implicated in cholestasis is the accumulation of hydrophobic bile acids within the liver (16), which have been demonstrated to be toxic to freshly isolated and cultured hepatocytes, liver mitochondria, and whole animals (17–20). Hepatocytes that are exposed to lower concentrations of hydrophobic bile acids (≤100 μM) typically undergo apoptotic cell death (20, 21), whereas at higher concentrations, these compounds promote cell necrosis through oxidative stress, ATP depletion, hepatocyte swelling, and disruption of the plasma membrane (17, 20). Both modes of bile acid–induced cell death are believed to be important in the pathogenesis of CLD through generation of oxidative stress (17, 22).

Mechanistic studies of bile acid–induced apoptosis and/or necrosis have also focused attention on induction of the mitochondrial permeability transition (MPT) as a critical event preceding cell death (19, 23–25). The MPT is characterized by large-amplitude swelling and loss of the electrochemical potential across the inner mitochondrial membrane caused by opening of a megachannel (MPT pore) spanning both the inner and the outer mitochondrial membranes (26, 27). Upon induction of the MPT, several intermembranous space proteins are released into cytosol, including the pro-apoptotic signal cytochrome c and several inhibitors of apoptosis (28). Of particular interest are findings correlating increased generation of reactive oxygen species (ROS) with stimulation of the MPT by bile acids (19, 22, 25), most likely *via* the oxidative modification of MPT pore proteins (29). Commensurate with their ability to inhibit the MPT and cytochrome c release from mitochondria, antioxidants such as α-tocopherol (α-TH), ebselen, and idebenone also reduce bile acid–induced cellular necrosis and apoptosis in rat hepatocytes (17, 25).

Because βC has been proposed as an antioxidant and levels of βC are low in cholestatic liver injury, the current study was performed to determine the potential antioxidant and cytoprotective effects of βC toward glycochenodeoxycholic acid (GCDC)-induced hepatocyte toxicity by addressing the following questions that are relevant to the pathogenesis of CLD: *1*) Can βC function as an antioxidant in rat hepatocytes that are exposed to a bile acid–induced oxidative stress? *2*) Can βC prevent bile acid–induced hepatocyte cytotoxicity? *3*) What are the mechanisms responsible for the cytoprotective effects afforded by βC against bile acid–induced hepatotoxicity?

METHODS

All chemicals were obtained in reagent-grade quality from suppliers. BSA-fraction V (BSA) and alcohol-soluble digitonin were obtained from Calbiochem (La Jolla, CA, U.S.A.). GCDC (Na^+ salt) and βC (type 1) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2,7-dichlorofluorescin diacetate (DCF-DA) was purchased from Eastman Kodak Co. (Rochester, NY, U.S.A.), and the fluorescent probe JC-1 was purchased from Molecular Probes (Eugene, OR, U.S.A.). R,R,R-(α-TH) was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). All other reagents unless stated were reagent grade or better.

Isolation of rat hepatocytes and liver mitochondria. Humane care was given to all experimental animals, and this study was approved by the Institutional Animal Care and Use Committee of the University of Colorado Health Sciences Center. Hepatocytes were isolated by a recirculating collagenase technique from 175- to 225-g male Sprague Dawley rats (Sasco, Inc., Omaha, NE, U.S.A.) maintained on a 12-h light-dark cycle and fed standard laboratory rat diet, as previously described (17). Initial hepatocyte viability measured by trypan blue exclusion was always >94%. Freshly isolated hepatocytes were resuspended in a Krebs Ringers HEPES (KRH) buffer containing 0.2% BSA (KRH/BSA) to a concentration of ~1×10^6/mL and preincubated for 30 min at 37°C with βC (50 or 100 μM, dissolved in DMSO), 100 μM α-TH, or both 100 μM βC and α-TH to determine whether the antioxidant effects were additive or synergistic. After the preincubation period, 100 or 500 μM GCDC was added and hourly aliquots of cell suspension were removed to determine their effects on ROS generation, cell necrosis, and apoptosis. For immunoblot analysis, aliquots were withdrawn after 3 h of incubation.

Measurement of ROS generation in rat hepatocytes. For determining whether βC could function as an antioxidant in this model, generation of ROS was measured by spectrofluorescence using the hydroperoxide detecting probe DCF-DA as previously described in detail (17). Briefly, hepatocytes were preloaded with DCF-DA for 30 min at 37°C before the addition of antioxidants. After an additional 30-min preincubation with antioxidants, hepatocytes were exposed to GCDC (100 or 500 μM) for 4 h and hourly aliquots were removed for analysis of ROS by measuring 2'7'-dichlorofluorescein (DCFein) fluorescence at 490-nm excitation and 520-nm emission. Results were expressed as picomoles per 10^6 cells based on a standard curve using DCFein.

Determination of hepatocyte apoptosis and necrosis. Hepatocyte apoptosis was quantified by determining the percentage of hepatocytes with nuclear morphologic changes of apoptosis detected by fluorescence microscopy of 4'6-diamidino-2-phenylindole–stained fixed hepatocytes, as previously described (21). Necrosis was determined by the release of

lactate dehydrogenase activity from cells, as previously described (21), and expressed as the percentage of total cellular activity released into the media.

Immunoblot analysis of cytochrome c release and caspase 3 activation. To determine the extent of mitochondrial cytochrome c release in isolated rat hepatocytes that were exposed to GCDC and βC, we used the protocol of selective digitonin permeabilization as originally described by Leist *et al.* (30) and as modified by Qiao *et al.* (31). Briefly, after 3 h of incubation with 100 μM GCDC with or without antioxidants, $4-6 \times 10^6$ cells were pelleted by centrifugation, washed once with KRH buffer (no BSA), and recentrifuged to obtain hepatocyte pellets. Hepatocytes were resuspended in a cell permeability buffer containing 75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, and 250 mM sucrose (pH 7.4) containing 350 μg/mL digitonin for 30 s and centrifuged at 13,000 × *g* for 1 min. The cytosolic supernatant fraction was collected, and protein concentration was determined by Lowry *et al.* (32). Approximately 15–25 μg of protein was loaded onto a 12% SDS-PAGE and transferred to nitrocellulose membrane and probed with 1–2 μg/mL purified mouse anti–cytochrome c MAb (BD Biosciences, San Diego, CA, U.S.A.). Cytochrome c was detected colorimetrically with stabilized 3,3',5,5'-tetramethylbenzidine (Promega, Madison, WI, U.S.A.) and quantified by densitometry using UN-SCAN-IT gel software (Silk Scientific, Orem, UT, U.S.A.).

For caspase 3 immunoblotting, a cytosolic fraction was obtained as follows: 2×10^6 cells were pelleted by centrifugation, washed once with KRH buffer (no BSA), and recentrifuged to obtain hepatocyte pellets. Cells were lysed by the addition of 200–300 μL of 3-[3-cholamidopropyl)dimethyl, ammonio]-1-propanesulfonate Cell Extract Buffer (Cell Signaling Technology, Beverly, MA, U.S.A,) containing 5 mM DTT and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was subjected to three freeze–thaw cycles before centrifugation at 13,000 × *g* for 5 min. The supernatants were collected, and protein concentration was determined by the Bradford assay (33). Electrophoresis and analysis were carried out as described for cytochrome c except that 50–100 μg of protein was loaded onto 12% SDS-PAGE, and the blots were incubated with 1:50 dilution of mouse monoclonal anti–caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Equal loading was confirmed in selected experiments by also probing blots against a 1:5000 dilution of anti-actin MAb followed by a 1:2000 dilution of goat anti-mouse IgM-specific horseradish peroxidase (Oncogene Research Products, Boston, MA, U.S.A.).

Flow cytometry. Flow cytometric analysis was performed to determine the effect of βC on GCDC-induced MPT in freshly isolated hepatocytes, as previously described in detail (34). Briefly, freshly isolated hepatocytes were pretreated with 50–100 μM βC for 30 min before exposure to 100 μM GCDC for 4 h. Hourly aliquots of cells were removed, loaded with 7.6 μM JC-1 or 3 μM propidium iodide (PI) for 15 min at 22°C in the dark, and washed with KRH buffer at 4°C before flow cytometry on a Becton Dickinson FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.) using CELLQuest software. In actively respiring mitochondria, JC-1 aggregates form and the intensity of their fluorescence at 590 nm is proportional to the mitochondria mitochondrial electrochemical gradient ($\Delta\psi$) and indicative of a closed MPT pore (βC alone had no effect on JC-1 fluorescence). Approximately 10,000 cells were analyzed for each time point and treatment. The fluorescence of JC-1 aggregates was determined only in live cells (as determined by PI staining) by gating on hepatocytes that showed no uptake of PI.

Measurement of the MPT. Rat liver mitochondria were isolated by differential centrifugation of liver homogenate through a Percoll gradient, and the MPT assay was performed as previously described in detail (22). Briefly, hepatic mitochondria (1.5–3.0 mL) in respiration buffer [125 mM sucrose, 100 mM NaCl, 10 mM MOPS (pH 7.4) treated with 1% Chelex-100] were incubated at 25°C for 5 min alone or in the presence of 10–250 μM βC. After the preincubation period, 100 μM $CaCl_2$, 5 mM sodium succinate, and 5 μM rotenone (in dimethylformamide) were added to mitochondria, and the absorbance at 540 nm was monitored for 5 min. The MPT was then induced by the addition of 100 μM GCDC, and absorbance was monitored continuously for an additional 5 min.

Statistical analysis. Mean and SEM were calculated for each time point. Comparisons among groups were performed by ANOVA and the Scheffe test or *t* test when appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

β-carotene reduces GCDC-stimulated ROS generation. Because the antioxidative function of βC in biologic systems is controversial (35), we first undertook an evaluation of the effect of βC on ROS generation under conditions of excess hydrophobic bile acids that may exist in the cholestatic liver. Freshly isolated rat hepatocyte suspensions that were exposed to either 100 or 500 μM GCDC generated increased ROS (hydroperoxides) in a time-dependent manner when compared with untreated cells (Fig. 1*a* and *b*). Pretreatment with βC led to a >50% reduction in ROS generation in GCDC-exposed hepatocytes at 4 h and was not dose dependent. The effects of βC were compared with that observed with α-TH, an antioxidant previously demonstrated to inhibit ROS generation in rat hepatocytes and liver mitochondria that were exposed to GCDC (17, 22). Figure 1*c* indicates that the antioxidative potencies of βC and α-TH were identical in preventing ROS generation in rat hepatocytes that were exposed to 100 μM GCDC and that no additive or synergistic effects were observed when the antioxidants were combined.

βC protects rat hepatocytes against GCDC-induced cell necrosis and apoptosis. We next sought to determine whether the observed antioxidative effect of βC was associated with cytoprotection against GCDC-induced necrosis and apoptosis. To assess the effects of varying concentrations of βC on GCDC-induced cell necrosis, rat hepatocyte suspensions were exposed to either 100 or 500 μM GCDC, and cell necrosis was quantified by release of lactate dehydrogenase from cells. By 4 h of incubation (Fig. 2*a* and *b*), GCDC caused significant cell necrosis at both GCDC concentrations when compared with untreated cells (14 ± 1% for control *versus* 35 ± 2% for 100 μM GCDC *versus* 47 ± 4% for 500 μM GCDC). Preincuba-

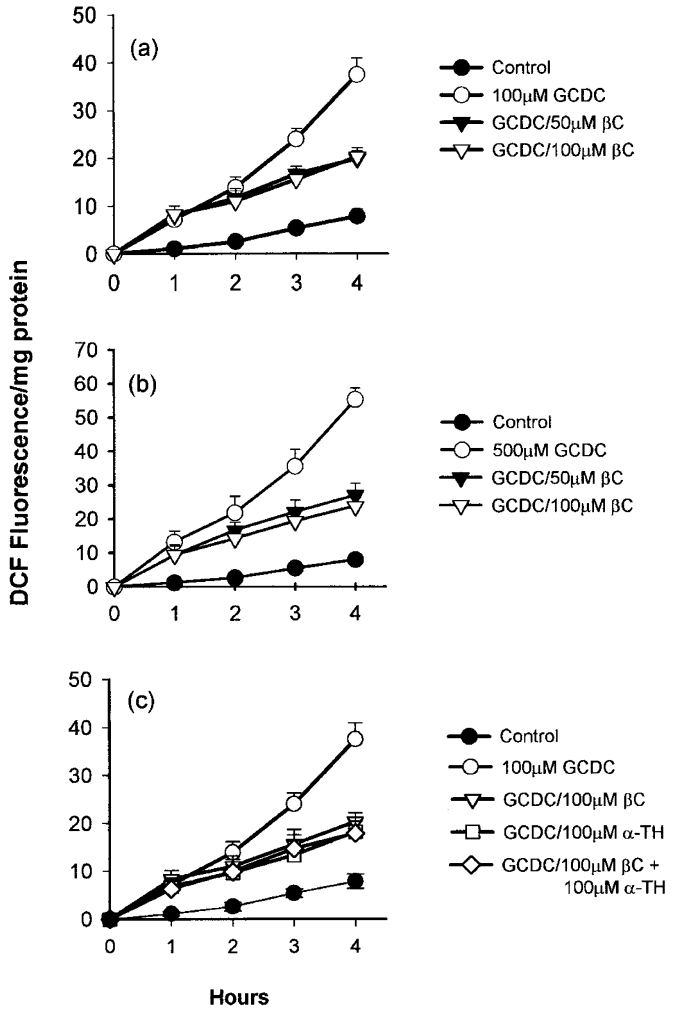

Figure 1. βC prevents generation of ROS in isolated rat hepatocytes that were exposed to GCDC. Hepatocytes (10^6/mL) were loaded with 10 μM DCF-DA for 30 min at 37°C and, where indicated, preincubated for an additional 30 min with βC (50–100 μM) or vehicle before the addition of either 100 μM (*a*) or 500 μM (*b*) GCDC. (*c*) The antioxidative effect of 100 μM βC was compared with that of 100 μM α-TH alone or in combination. Hourly aliquots were removed, and ROS generation was determined by fluorescence of DCFein, as described in "Methods." Results are expressed as mean ± SEM of at least four separate experiments.

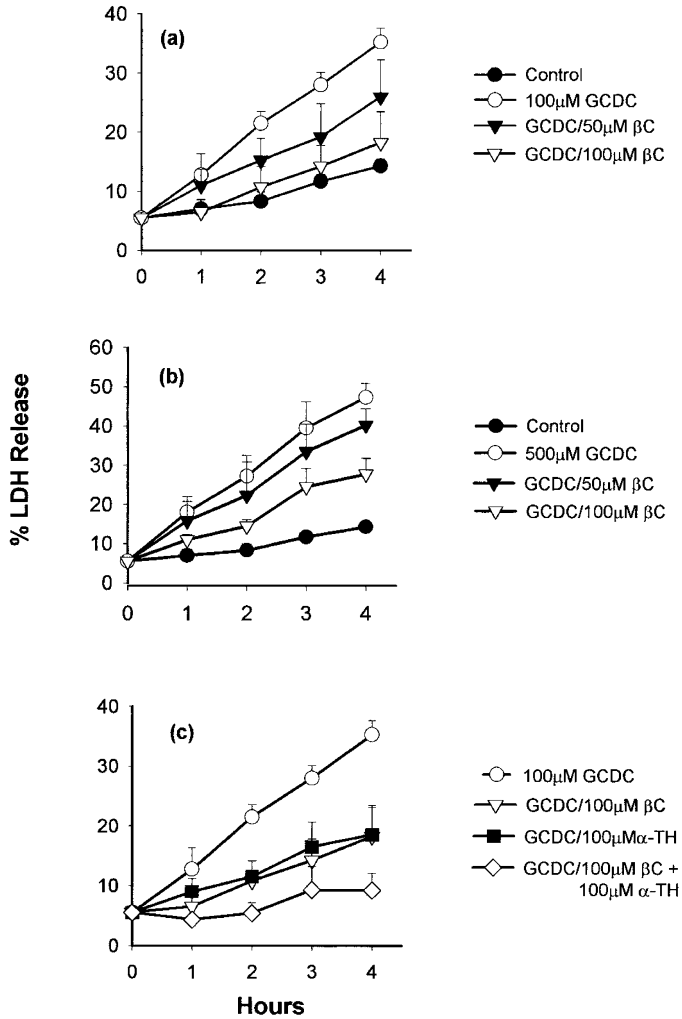

Figure 2. βC inhibits GCDC-induced cellular necrosis in isolated rat hepatocytes. Hepatocytes were preincubated for 30 min at 37°C with βC (50–100 μM) or vehicle before the addition of either 100 μM (*a*) or 500 μM (*b*) GCDC to promote cell necrosis. (*c*) The antioxidative effect of 100 μM βC was compared with that of 100 μM α-TH alone or in combination. Hourly aliquots were removed for determination of cell necrosis (% lactate dehydrogenase release), as described in "Methods." Results are expressed as mean ± SEM of at least four separate experiments.

tion of cells with 100 μM βC protected hepatocytes against cellular necrosis, whereas the lower concentration of βC (50 μM) protected hepatocytes against 100 μM GCDC but not 500 μM of the bile acid. When cells were exposed to 100 μM GCDC, a significant increase in protection was afforded by the combination of 100 μM βC and 100 μM α-TH compared with 100 μM βC or α-TH alone (Fig. 2*c*).

Isolated rat hepatocytes that are exposed to 100 μM GCDC undergo significant apoptosis by 2 h, which continues to increase by 4 h of incubation (Fig. 3; 43% of GCDC-treated cells *versus* 1% untreated cells at 4 h). Pretreating hepatocytes with 50 μM βC reduced bile acid–induced apoptosis by approximately 50% at 2 h and 35% at 4 h; 100-μM βC concentration was associated with somewhat better protection against apoptosis at 4 h. Pretreating cells with 100 μM α-TH led to a similar reduction of GCDC-induced apoptosis as that produced by 100 μM βC; 100 μM βC combined with α-TH had a mild additive protective effect (data not shown).

Inhibition by βC of GCDC-induced MPT in liver mitochondria. Induction of the MPT by a variety of toxic agents is believed to be a critical step in mediating cell death by necrotic and apoptotic pathways. GCDC (100 μM) led to a rapid decrease in absorbance of liver mitochondria at 540 nm, representative of mitochondrial swelling and induction of the MPT. βC exerted a dose-dependent inhibition of the MPT in this model (Fig. 4*a*). The MPT was verified by the complete inhibition of the GCDC-induced mitochondrial swelling by preincubation with cyclosporin A. A comparison of the dose-dependent effects of βC and α-TH on GCDC-induced MPT (Fig. 4*b*) shows that the profile of MPT inhibition afforded by each of the antioxidants is nearly identical.

Effect of βC on GCDC-induced cytochrome c release and caspase 3 levels. The suppression of bile acid–induced he-

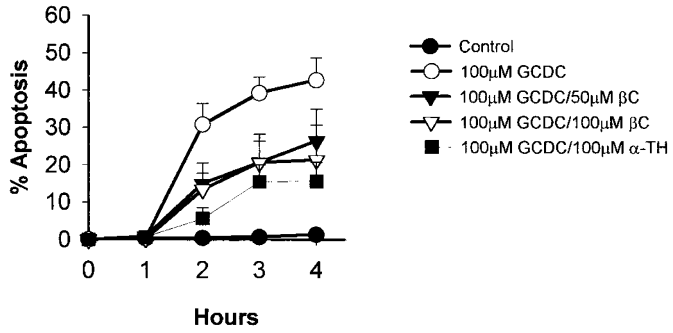

Figure 3. βC inhibits GCDC-induced apoptosis in isolated rat hepatocytes. Hepatocytes were preincubated for 30 min at 37°C with βC (50–100 μM), α-TH, or vehicle before the addition of 100 μM GCDC to induce cell apoptosis. Hourly aliquots were removed for determination of apoptosis as assessed by the percentage of cells with nuclear morphologic changes observed under fluorescence microscopy, as described in "Methods." Results are expressed as mean ± SEM of at least four separate experiments.

patocyte apoptosis by βC suggests that the carotenoid may alter critical steps in the induction of hepatocyte apoptosis. Therefore, we determined the effect of βC on two important events in the execution of hepatocytes apoptosis, release of cytochrome c from mitochondria and activation of caspase 3 (Fig. 5). Examination of postmitochondrial supernatant from hepatocytes that were treated with 100 μM GCDC indicated that exposure to GCDC for 3 h stimulated release of cytochrome c from mitochondria when compared with untreated hepatocytes (Fig. 5*a*). Pretreating cells with 100 μM βC, 100 μM α-TH, or the combination abrogated release of cytochrome c from mitochondria. To determine the effect of antioxidants on GCDC-stimulated caspase 3 activation, we analyzed the amount of procaspase 3 levels in cytosol of hepatocytes by immunoblotting (Fig. 5*b*). Results from these experiments demonstrated that procaspase 3 in cells exposed to 100 μM GCDC for 3 h was reduced, indicative of caspase 3 activation (Fig. 5*b*). Pretreating hepatocytes with βC, α-TH, or the combination prevented the reduction of procaspase levels, concomitant with protection against apoptosis (Fig. 3). Similar loading of lanes with cytosol was indicated by the immunoblots for β−actin (Fig. 5*c*).

βC prevents GCDC-induced mitochondrial depolarization in rat hepatocytes. Induction of the MPT is associated with increased oxidative stress in hepatocytes that were treated with hydrophobic bile acids. Inasmuch as βC reduced ROS generation and inhibited the MPT in isolated mitochondria, we next determined whether βC affected the MPT in live hepatocytes. To measure MPT in intact cells, we assessed mitochondrial $\Delta\psi$ in isolated hepatocytes by flow cytometric analysis using the probe JC-1 (Fig. 6). Exposure of hepatocytes to 100 μM GCDC caused a significant decrease in fluorescence of JC-1 aggregates in hepatocytes at 2 h, indicative of reduction of mitochondrial $\Delta\psi$ and opening of the MPT pore. Pretreating cells with 50–100 μM βC significantly prevented the reduction in JC-1 aggregate fluorescence at 3 and 4 h. These results demonstrate that βC blocked the MPT concurrent with inhibition of ROS generation and reduced activation of caspase 3 and inhibition of apoptosis.

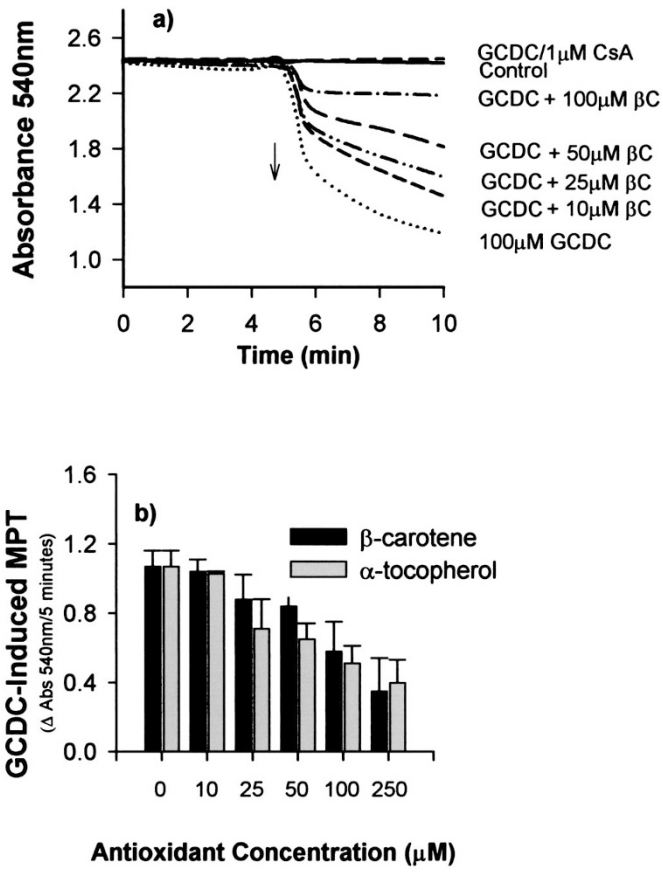

Figure 4. Dose-dependent inhibition by βC of GCDC-induced MPT in liver mitochondria. Suspensions of liver mitochondria were preincubated with 0–250 μM βC or α-TH, or 1 μM cyclosporin A (CsA) for 5 min before the addition of 100 μM GCDC to induce the MPT. Mitochondrial swelling was measured spectrophotometrically as a reduction in absorbance at 540 nm over 5 min. (*a*) A typical tracing demonstrates the dose-dependent inhibition of MPT induced by 100 μM GCDC in liver mitochondria and the complete blockage of MPT by CsA. (*b*) The dose-dependent protection against GCDC-induced MPT by βC and α-TH is shown. MPT is expressed as the change in absorbance during the 5 min of exposure of mitochondria to 100 μM GCDC. Results are expressed as mean ± SEM of at least four separate experiments.

DISCUSSION

Results from the current study using an *in vitro* model of cholestatic liver injury demonstrate that βC, a carotenoid of nutritional and physiologic significance, protects rat hepatocyte suspensions from hydrophobic bile acid–induced cell apoptosis by a mechanism consistent with its putative antioxidant activity. Concentrations of βC in human liver vary widely, with its concentration in nonsupplemented individuals ranging from 6 to 25 nmol/g liver (35, 36). Assuming $140{,}000 \times 10^6$ hepatocytes/g wet weight of liver, with 5 μL of water per 10^6 hepatocytes (37), the range of liver concentrations of βC are converted to 9–36 μM. However, in hepatobiliary disorders, concentrations of liver and serum βC are substantially decreased (10, 12, 13, 36). Therefore, the concentrations of βC chosen in this study are close to the range of normal values, particularly when considering the accelerated time course needed to achieve intracellular βC concentrations in isolated hepatocyte incubations.

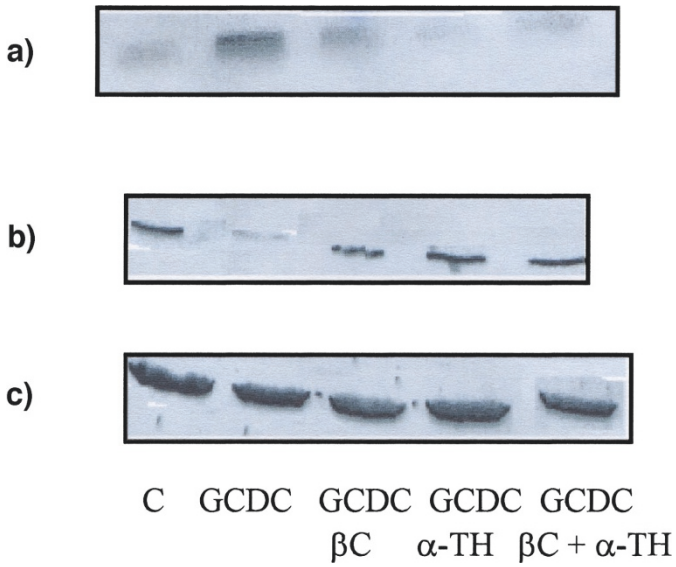

Figure 5. βC and α-TH prevent GCDC-induced cytochrome c release and caspase 3 activation in rat hepatocytes. The effects of βC (100 μM), α-TH (100 μM), both antioxidants, or no treatment (control-C) on GCDC-induced cytochrome c release from mitochondria into cytosol of hepatocytes (*a*) and caspase 3 activation (*b*) were determined after 3 h of incubation, as described in "Methods." Samples of cytosol from hepatocytes were immunoblotted against mouse anti–cytochrome c MAb in *a* or mouse anti–caspase 3 MAb in *b*. In selected experiments, blots that were probed with anti–caspase 3 antibodies were stripped and reprobed with anti-actin antibodies (*c*) to demonstrate similar loading. Results shown are representative of at least two separate experiments.

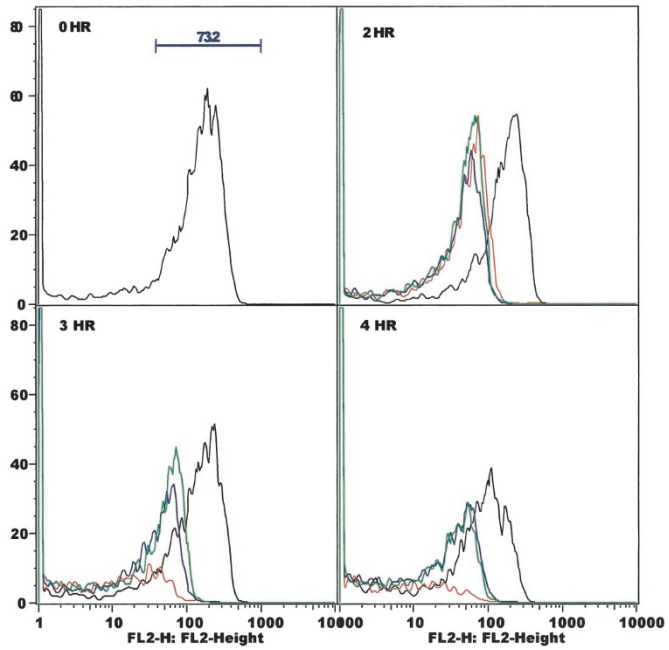

Figure 6. The effect of βC on GCDC-induced collapse of mitochondrial Δψ in rat hepatocytes. Isolated rat hepatocytes were exposed to 100 μM GCDC in the absence or presence of 50–100 μM βC. Hourly aliquots were removed, loaded with JC-1 and PI for 15 min at 22°C, and washed with KRH buffer before fluorescence determination at 590 nm for JC-1. JC-1 aggregate fluorescence in live cells (PI-negative), which represents an intact mitochondrial Δψ, was significantly reduced by 2 h of incubation with GCDC (red line) compared with untreated cells (black line). Preincubation of hepatocytes with either 50 μM βC (blue line) or 100 μM βC (green line) prevented the GCDC-induced reduction in JC-1 aggregate fluorescence, indicating that the bile acid–induced mitochondrial depolarization was inhibited by βC. βC alone failed to affect JC-1 aggregate fluorescence when compared with control cells (data not shown). The horizontal bar indicates the percentage of the population of cells exhibiting the designated fluorescence. Results shown are representative of at least two separate experiments.

The antioxidative effect and cytoprotection afforded by βC in these experiments were nearly identical to that of α-TH, an antioxidant previously reported to protect rat hepatocytes from bile acid–induced cell death (17, 18). The anti-apoptotic effect of βC was further characterized by demonstrating inhibition of both caspase 3 activation and loss of mitochondrial Δψ in cells that were exposed to the toxic bile acid and significant reduction of the MPT in liver mitochondria incubated with the bile acid. Taken together, these results support an antioxidative and anti-apoptotic role of βC under conditions that may exist in the cholestatic liver.

The cytoprotective effects of βC reported in this study provide evidence that βC may protect mammalian cells that are subjected to oxidative stress–dependent apoptosis. Although the antioxidative function of βC *in vivo* is a source of considerable controversy (38, 39), there is extensive evidence that βC functions as an antioxidant under a variety of *in vitro* conditions. In the original study by Burton and Ingold (6), βC prevented peroxidation of methyl linoleate in a unique manner when compared with previously characterized mechanisms of other antioxidants. The hypothesized mechanism of βC antioxidant protection against lipid oxidation was that of a chain-breaking antioxidant by efficient trapping of peroxyl radicals resulting in generation of lipid hydroperoxides. As hydrophobic bile acids have been shown to increase generation of hydroperoxides (17, 19), it is possible that βC functioned as an antioxidant in the current study by protecting membrane lipids from propagating oxidative damage through termination of peroxyl radical-mediated reactions. Inhibition of DCFein fluorescence, a measurement of intracellular peroxide generation, by βC would support this antioxidative mechanism. A second, less likely explanation for the reduction of ROS generation by βC was its role as a quencher of electronically excited singlet oxygen (40, 41). Although studies have reported generation of singlet oxygen in hepatocytes (42), no evidence exists that this photochemically generated intermediate is produced in bile acid–treated hepatocytes under the conditions used in this study (which did not use photosensitizing agents).

At the higher concentration of βC used in this study (100 μM), we observed a significant inhibition of GCDC-induced generation of ROS, reduction of cellular apoptosis, and reduced cell necrosis. It is interesting that when a lower concentration of the carotenoid was examined (50 μM), we observed nearly identical antioxidant and anti-apoptotic protection to that of the higher dose but a lesser degree of protection against cellular necrosis. A similar observation was noted when comparing the effects of a combination of βC and α-TH (100 μM each) toward ROS generation (Fig. 1*c*) and cellular necrosis (Fig. 2*c*), where the combination failed to decrease ROS generation further yet provided increased protection against necrosis, suggesting that once a maximal level of antioxidant protection is achieved, increased potentiation against cellular

necrosis continued. Explanations for the increased cytoprotection conferred by 100 μM βC include *1*) possible nonantioxidant functions for the carotenoid (43), *2*) increased concentrations of the carotenoid or a retinoid metabolite (44), and *3*) increased intra- or extracellular effects that increase cell stabilization against necrotic cell death. Such findings are not surprising given the myriad of structural and metabolic events implicated in bile acid–induced necrotic cell death, including alterations in Ca+2 homeostasis, ATP depletion, and activation of cellular proteolytic pathways (45). These data suggest that pathways involved in bile acid–induced hepatocyte apoptosis, such as activation of caspases and mitochondrial release of cytochrome c into the cytosol, may be more sensitive to cellular redox status compared with processes that are observed during necrosis.

The current finding of a potent anti-apoptotic effect of βC in a normal cell model is in contrast to studies using tumor cell models. For example, a study with human adenocarcinoma cells found that βC at concentrations up to 100 μM exhibited pro-oxidative and pro-apoptotic effects including decreased expression of the anti-apoptotic protein BCL-2 (46). βC's differential effects between freshly isolated rat hepatocytes and cultured tumor cells can be explained by a variety of experimental and biologic factors. First, tumor cells have an antioxidant defense mechanism and redox status that contrast with those of normal cells (47). In addition, the uptake and metabolism of βC can differ greatly depending on the cell type and mode of βC administration (44, 48). Other properties of βC (or other related carotenoids) that may contribute to its anti-apoptotic effects include an antiproliferative effect (49), stimulation of gap junctional communications (50), and regulation of cellular detoxification systems (51). Finally, the metabolism of βC to retinoids (48, 50) cannot be disregarded as a contributing factor to βC's observed anti-apoptotic effects. However, induction of gene transcription by retinoids through binding of nuclear receptor complexes would not likely occur in the short time frame of the current study using freshly isolated (not cultured) hepatocytes.

The protective role of βC in the current study is supportive of the hypothesis that oxidant stress is a crucial step in the pathogenesis of bile acid–induced toxicity. Many laboratories have linked generation of an oxidant stress to pro-apoptotic events in liver mitochondria and intact hepatocytes (19, 22). For example, we have demonstrated that GCDC induced the MPT in liver mitochondria and stimulated the release of cytochrome c from mitochondria into cytosol and loss of mitochondrial $\Delta\psi$ in hepatocytes (22, 25), all of which were blocked by antioxidants. These findings, now extended with the current results with βC, clearly implicate oxidative stress and mitochondrial dysfunction in the pathogenesis of bile acid–induced cytotoxicity. Although the evidence herein supports a role for the antioxidant effect of βC in this cytoprotection, as stated above, one cannot discount the possibility that βC also possesses nonantioxidant functions that are also operative.

In summary, the current study provides additional evidence that oxidative stress and mitochondrial dysfunction play critical roles in bile acid–induced hepatocyte toxicity, an important mediator of hepatocyte injury in the cholestatic liver (52). Moreover, the results demonstrate for the first time that βC can prevent biochemical and morphologic evidence of cell necrosis and apoptosis in rat hepatocyte suspensions that are exposed to toxic, hydrophobic bile acids. The protection afforded by this nutrient was consistent with its role as an antioxidant of biologic importance. Consideration of using βC in humans, as was recently done in a pilot study with patients with CLD (14), must take into account the possible adverse effects of the compound under certain circumstances. Questions of potential toxicity of βC have arisen during intervention studies in which βC supplementation in long-term cigarette smokers was associated with increased susceptibility to lung cancer and coronary artery disease (7, 8). Lieber's laboratory has also provided evidence in both cells and baboons suggesting that a toxic interaction may arise between βC and ethanol administration, perhaps by a mechanism involving common metabolic detoxification pathways (53, 54). Nevertheless, additional studies into the mechanisms and relevance of βC as a potential hepatoprotective agent in childhood cholestasis are warranted.

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