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# Ursodeoxycholic Acid Protects Against Secondary Biliary Cirrhosis in Rats by Preventing Mitochondrial Oxidative Stress

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**Ursodeoxycholic acid (UDCA) improves clinical and biochemical indices in primary biliary cirrhosis and prolongs survival free of liver transplantation. Recently, it was suggested that the cytoprotective mechanisms of UDCA may be mediated by protection against oxidative stress, which is involved in the development of cirrhosis induced by chronic cholestasis. The aims of the current study were 1) to identify the mechanisms involved in glutathione depletion, oxidative stress, and mitochondrial impairment during biliary cirrhosis induced by chronic cholestasis in rats; and 2) to determine the mechanisms associated with the protective effects of UDCA against secondary biliary cirrhosis. The findings of the current study indicate that UDCA partially prevents hepatic and mitochondrial glutathione depletion and oxidation resulting from chronic cholestasis. Impairment of biliary excretion was accompanied by decreased steady-state hepatic levels of  $\gamma$ -glutamyl cysteine synthetase and  $\gamma$ -cystathionase messenger RNAs. UDCA treatment led to up-regulation of  $\gamma$ -glutamyl cysteine synthetase in animals with secondary biliary cirrhosis and prevented the marked increases in mitochondrial peroxide production and hydroxynonenal-protein adduct production that are observed during chronic cholestasis. A population of damaged and primarily apoptotic hepatocytes characterized by dramatic decreases in mitochondrial cardiolipin levels and membrane potential as well as phosphatidylserine exposure evolves in secondary biliary cirrhosis. UDCA treatment prevents the growth of this population along with the decreases in mitochondrial cardiolipin levels and membrane potential that are induced by chronic cholestasis. In conclusion, UDCA treatment enhances the antioxidant defense mediated by glutathione; in doing so, this treatment prevents cardiolipin depletion and cell injury in animals with secondary biliary cirrhosis. (HEPATOLOGY 2004;39:711–720.)**

*Abbreviations:* PBC, primary biliary cirrhosis; ROS, reactive oxygen species; MPT, mitochondrial permeability transition; UDCA, ursodeoxycholic acid; mRNA, messenger RNA; GSH, reduced glutathione; BDL, bile duct ligation; Gln, glutamine; Gly, glycine; Ser, serine; Met, methionine; NAC, N-acetylcysteine; PCR, polymerase chain reaction;  $\gamma$ -GCS,  $\gamma$ -glutamyl cysteine synthetase; CT, threshold cycle; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNE, 4-hydroxynonenal; GSSG, oxidized glutathione; afu, arbitrary fluorescence unit.

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Cholestasis occurs in numerous chronic human diseases including primary biliary cirrhosis (PBC), primary sclerosing cholangitis, allograft rejection, iatrogenic obstruction of bile ducts, and biliary atresia.<sup>1</sup> Elucidation of the cellular and molecular mechanisms that lead to liver injury and fibrosis is critical in designing new interventional strategies for treating these disorders. Although the primary injury to the bile ducts may be immunologic, toxic, or genetic, progression of liver disease appears to be promoted by secondary chemical damage to hepatocytes as a result of toxic hydrophobic bile salts.<sup>2,3</sup> Cholestasis results in intrahepatic accumulation of potentially toxic bile acids, and this accumulation leads to hepatocyte apoptosis, necrosis, and, eventually, biliary fibrosis and cirrhosis.<sup>4</sup> *In vitro* exposure to high concentrations of hydrophobic bile acids has been linked to necrosis in freshly isolated hepatocytes and primary cultured hepatocytes<sup>5,6</sup>; this association explains the observation of cellular swelling,

disrupted plasma membrane integrity, and cytosolic enzyme release in cholestasis.<sup>7</sup>

Oxidative stress plays an important role in cell death<sup>8,9</sup> and has been linked to the development of cholestatic liver injury. Hydrophobic bile acids stimulate the generation of reactive oxygen species (ROS) in hepatocytes<sup>5,10</sup> and in liver mitochondria.<sup>5,11</sup> Evidence of oxidative injury has been found in rats receiving intravenously infused hydrophobic bile acids<sup>12</sup> and in the bile duct–ligated rat model of cholestasis.<sup>13,14</sup> Recently, Sokol et al.<sup>12</sup> proposed that hydrophobic bile acids accumulate intracellularly during cholestasis and interfere with normal mitochondrial electron transport, inhibiting the activity of respiratory complexes I and III and consequently reducing adenosine triphosphate synthesis.<sup>15</sup> Mitochondrial dysfunction is widely recognized as a key mechanism leading to apoptosis,<sup>16</sup> particularly through the mitochondrial permeability transition (MPT). Toxic bile salts can induce the opening of MPT pores, and inhibition of MPT prevents bile salt–induced hepatocyte cytotoxicity.<sup>17,18</sup>

Administration of ursodeoxycholic acid (UDCA) is the currently accepted method for treating cholestasis.<sup>19</sup> Combined analysis of the three largest randomized clinical trials of UDCA for PBC<sup>20</sup> indicated that UDCA improved clinical and biochemical indices and prolonged survival free of liver transplantation. Nonetheless, the cytoprotective mechanisms by which UDCA acts remain unclear. Experimental evidence suggests three primary mechanisms of action: protection against the cytotoxicity of hydrophobic bile acids,<sup>21</sup> stimulation of hepatobiliary secretion,<sup>22</sup> and protection of hepatocytes against bile acid–induced apoptosis.<sup>23</sup> Recently, UDCA was shown to have *in vitro* antioxidant activity in hepatocytes.<sup>24</sup> Pretreatment with UDCA prevented hydrogen peroxide–induced injury by increasing  $\gamma$ -glutamylcysteine synthetase messenger RNA (mRNA) levels and, consequently, levels of reduced glutathione (GSH).<sup>24</sup> Furthermore, GSH levels were higher in liver specimens perfused with UDCA than in those perfused with taurocholic acid. This effect is correlated with an increase in methionine S-adenosyl transferase activity.<sup>25</sup>

The aims of the current study were 1) to identify the mechanisms involved in GSH depletion, oxidative stress, and mitochondrial impairment during biliary cirrhosis induced by chronic cholestasis; and 2) to determine the mechanisms associated with the protective effect of UDCA against secondary biliary cirrhosis.

## Materials and Methods

**Animal Experiments.** Adult male Wistar rats weighing 220–260 g were held in cages in groups of 2, fed *ad*

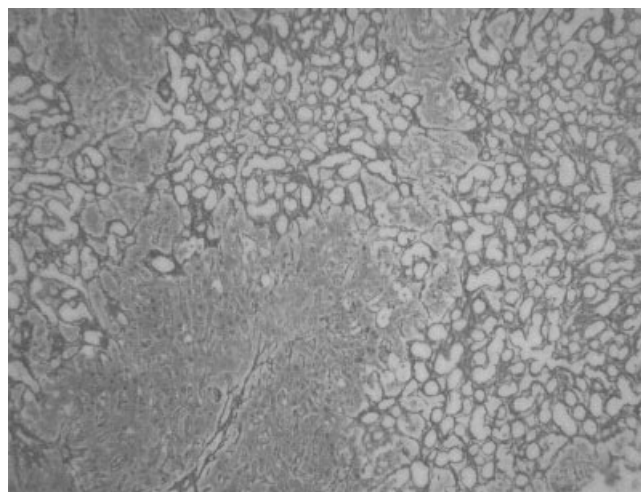


Fig. 1. Histochemical analysis of rat liver specimen, with staining for reticulin after 21 days of chronic cholestasis. Original magnification  $\times 100$ .

*libitum*, and given free access to water. All rats received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*.<sup>26</sup> The study was approved by the Research Committee of the University of Valencia School of Medicine (Valencia, Spain). Animals were randomly divided into three groups: a group that underwent sham operation, a group that underwent bile duct ligation (BDL), and a group that underwent BDL and treatment with UDCA (BDL + UDCA). BDL was performed using a modified version of the method described by Kountouras et al.<sup>27</sup> Anesthesia was induced with ketamine (100 mg/kg body weight) and acepromazine (2.5 mg/kg body weight) administered intraperitoneally. The peritoneal cavity was opened and the common bile duct exposed via a right paramedian incision measuring 1.5 cm in length. BDL was achieved as follows: the common bile duct was clamped with a surgical microclamp, ligated twice (silk suture, 5/0), and cut through between the ligations. Control animals underwent a sham operation that consisted of exposure of the common bile duct without ligation. The sham operation and BDL groups received standard nourishment, and the BDL + UDCA group received the same nourishment supplemented with 2.5 g/kg UDCA (Mucedola, Milan, Italy). Food intake and body weight were monitored throughout the 28-day experimental period.

Animals were sacrificed under anesthesia and rapidly had their livers removed 21 or 28 days after surgery. Development of biliary cirrhosis was confirmed by histochemical methods, using staining for reticulin (Fig. 1). Histologic analysis of reticulin via the silver nitrate technique was performed with a commercial kit (D.D.K. Italia, Milan, Italy).

**Measurement of Peroxide Production in Liver Mitochondria.** Liver mitochondria were isolated as described previously.<sup>28</sup> The rate of peroxide production in liver mitochondria also was determined according to the method described by Miñana et al.,<sup>28</sup> which was a modified version of the method described by Barja.<sup>29</sup>

**Isolation and Incubation of Hepatocytes.** Hepatocytes were isolated from rats that had fasted for 48 hours, as described by Romero and Viña.<sup>30</sup> Cells (10–15 mg [dry weight] per mL) were incubated in Krebs–Henseleit bicarbonate buffer containing 5 mM glutamine (Gln), 2 mM glycine (Gly), 1 mM serine (Ser), and 0.5 mM methionine (Met) or containing 5 mM Gln and 2 mM Gly plus 0.5 mM *N*-acetylcysteine (NAC). A gas atmosphere of O<sub>2</sub> and CO<sub>2</sub> (O<sub>2</sub>:CO<sub>2</sub> ratio, 19:1) was used. GSH levels were measured spectrophotometrically, as described by Miñana et al.<sup>28</sup>

**Flow Cytometric Analysis.** Flow cytometric analysis was performed using an EPICS ELITE cell sorter (Coulter Electronics, Miami, FL). Fluorochromes were excited with an argon laser tuned to 488 nm. Forward-angle and right-angle light scattering were measured. Samples were acquired for 20,000 individual cells. Mitochondrial membrane potential was measured using the fluorescent dye Rhodamine-123<sup>31</sup> at 525 ± 5 nm fluorescence emission. This potential is the driving force for Rhodamine-123 uptake. Mitochondrial cardiolipin content was measured with nonylacridine orange (final concentration, 10 µg/mL; 525 ± 5 nm fluorescence emission<sup>32</sup>), which binds to mitochondrial membranes regardless of their membrane potential. Therefore, mitochondrial uptake of this metachromatic dye does not depend on mitochondrial energy status.<sup>33</sup> Phosphatidylserine exposure was measured with annexin V (Alexa Fluor 488 conjugate; Molecular Probes, Eugene, OR) and used as an index of apoptosis.

Cell viability was determined using the fluorescent dye propidium iodide (final concentration, 5 µg/mL) at 630 nm fluorescence emission. There were no statistical differences in the viability of hepatocytes among the different treatment groups. The percentage of viable hepatocytes was 94 ± 2.1% in the control group, 87 ± 4.8% in the BDL group, and 90 ± 3.8% in the BDL + UDCA group.

**RNA Extraction and the Real-Time Polymerase Chain Reaction (PCR).** Liver tissue stored at –80 °C was homogenized (Ultra-Turrax disperser; Janke and Kunfel, Staufen, Germany), and total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A PCR master mix containing the specific primers ( $\gamma$ -glutamyl cysteine synthetase [GCS] heavy subunit: forward, 5'-ATC CTC

CAG TTC CTG CAC ATC TAC-3'; reverse, 5'-GAT CGA AGG ACA CCA ACA TGT ACT C-3';  $\gamma$ -GCS light subunit: forward, 5'-TGG AGT TGC ACA GCT GGA CTC T-3'; reverse, 5'-CCA GTA AGG CTG TAA ATG CTC CAA-3'; cystathionase: forward, 5'-TTT CCT GGA GTC TAA TCC CCG-3'; reverse, 5'-TTG AGG AAG ACC TGA GCA TGC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5'-CCT GGA GAA ACC TGC CAA GTA TG-3'; reverse, 5'-GGT CCT CAG TGT AGC CCA AGA TG-3') then was added, along with AmpliTaq Gold DNA polymerase (Applied Biosystems; Foster City, CA). Real-time quantitation of  $\gamma$ -GCS heavy subunit mRNA,  $\gamma$ -GCS light subunit mRNA, and cystathionase mRNA relative to GAPDH mRNA was performed using SYBR Green I (Molecular Probes, Eugene, OR), and mRNA levels were evaluated using the iCycler detection system (Biorad, Hercules, CA). The threshold cycle (CT) was determined, and relative gene expression levels subsequently were calculated as follows: fold change =  $2^{-\Delta(\Delta CT)}$ , where  $\Delta CT = CT_{\text{target}} - CT_{\text{housekeeping}}$ , and  $\Delta(\Delta CT) = \Delta CT_{\text{treated}} - \Delta CT_{\text{control}}$ .

**Immunohistochemistry for 4-Hydroxynonenal (HNE)-Protein Adducts.** HNE-protein adducts were detected in 4% formalin-fixed, paraffin-embedded sections. Sections measuring 5 µm in thickness were incubated with an anti-HNE-histidine rabbit polyclonal antibody (Alpha Diagnostic International, San Antonio, TX). Peroxidase-linked secondary antibody and diaminobenzidine were used to detect specific binding. No positive staining was detected when tissue from experimental liver samples was processed without primary antibody; this finding indicated the absence of nonspecific binding under the conditions used in the current study.

**Assays.** Mitochondrial and liver homogenate levels of GSH and oxidized glutathione (GSSG) were measured as described previously.<sup>28</sup> Western blot analysis was performed using the Phototope-HRP Western blot kit (Cell Signalling, Beverly, MA) and a specific rabbit antibody against the heavy subunit of  $\gamma$ -GCS (NeoMarkers, Fremont, CA). Total protein concentration was determined using the Lowry micromethod kit (Sigma-Aldrich, St. Louis, MO).

**Statistical Analysis.** Data were expressed as mean values ± standard deviations of the mean (SDM). Because the data were not paired, differences between means were analyzed using one-way ANOVA after Gaussian distribution evaluation with the Kolmogorov–Smirnov test. The Tukey multiple comparisons test for all pairs of columns was applied as a post-test. In all instances,  $P < .05$  was taken as an indication of significance. A commercial soft-

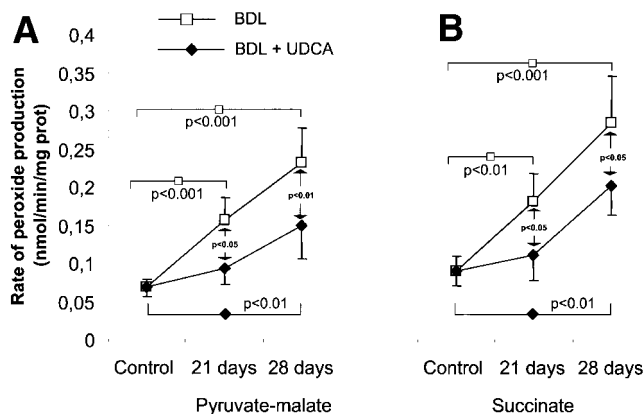


Fig. 2. Peroxide production by rat liver mitochondria during secondary cirrhosis: protective effects of UDCA treatment. (A) Rate of peroxide production in mitochondria incubated with 5 mM pyruvate plus 2.5 mM malate. (B) Rate of peroxide production in mitochondria incubated with 10 mM succinate. Liver mitochondria were isolated from control group rats, BDL group rats, and UDCA + BDL group rats after 21 and 28 days of chronic cholestasis. Four to eight experiments were performed in each case. Statistical differences were assessed using ANOVA and Tukey-Kramer multicomparison tests.

ware package (GraphPad Prism Software, San Diego, CA) was used to perform all statistical analyses.

## Results

**Prevention of Increased Mitochondrial Peroxide Production by UDCA Treatment.** Mitochondrial peroxide production, which may be a major contributor to both mitochondrial and intracellular oxidative stress, was assessed in animals with secondary biliary cirrhosis. Peroxide production by liver mitochondria was measured using pyruvate and malate as complex I-linked substrates and using succinate as a complex II-linked substrate. The rate of peroxide production in liver mitochondria 21 days after the induction of cholestasis was twice as great as it was in sham-operated rats for both complex I-linked and complex II-linked substrates ( $P < .01$ ; Fig. 2). Peroxide production increased further, to a level 3.5 times greater than in sham-operated rats, at 28 days after BDL ( $P < .001$ ; Fig. 2). UDCA treatment completely prevented the increase that would have been induced by cholestasis at 21 days ( $P < .05$ ). The rate of peroxide production in liver mitochondria from animals that received BDL and were treated with UDCA for 28 days was significantly lower than the corresponding rate in untreated rats, although it was greater than the corresponding rate in control animals ( $P < .01$  for the comparison with control animals).

**Partial Prevention of Hepatic and Mitochondrial Glutathione Depletion and Oxidation During Chronic Cholestasis by UDCA.** GSH and GSSG levels were measured in liver tissue and in hepatic mitochondria

from rats in the sham, BDL, and BDL + UDCA groups. Hepatic GSH levels (in  $\mu\text{mol}$  per gram of liver tissue) were significantly lower in BDL group rats compared with sham-operated rats at 21 and 28 days after the induction of cholestasis ( $P < .001$  at 21 and 28 days; Fig. 3A); UDCA treatment partially reversed this effect. Furthermore, GSSG levels were significantly higher in BDL group rats than in sham-operated rats ( $P < .001$  at 21 and 28 days; Fig. 3B); this effect also was partially reversed by UDCA treatment.

The same changes noted in hepatic glutathione levels also were observed when mitochondrial GSH and GSSG levels were measured. Specifically, BDL caused a significant decrease in GSH levels and an increase in GSSG levels. These changes also were prevented by UDCA treatment (Fig. 4).

**Effect of UDCA Administration on Cholestasis-Induced Impairment of GSH Synthesis due to Decreased Expression of  $\gamma$ -GCS and  $\gamma$ -Cystathionase.** To elucidate the mechanisms responsible for GSH depletion dur-

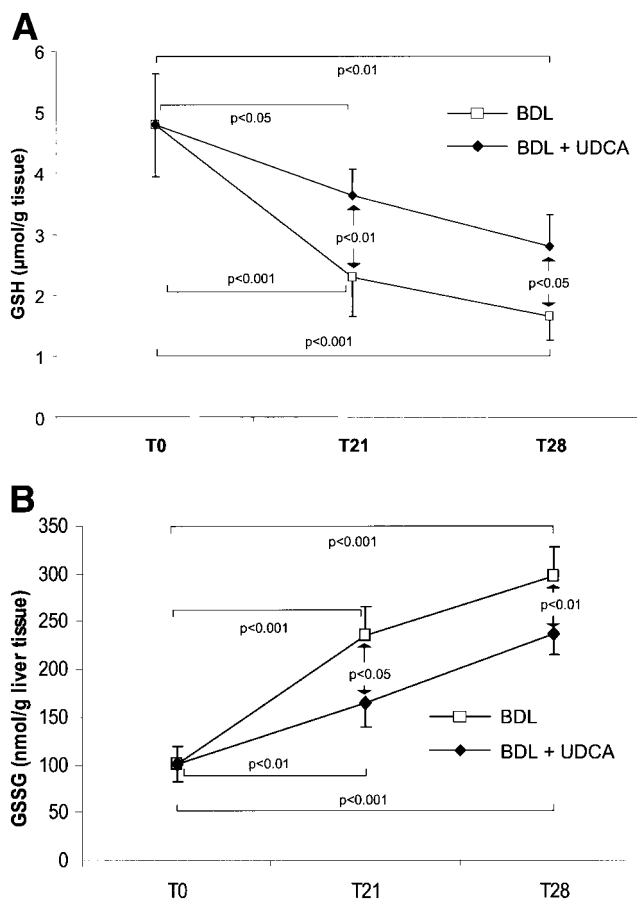


Fig. 3. Effects of chronic cholestasis and UDCA treatment on hepatic glutathione redox status. (A) GSH and (B) GSSG levels in liver homogenates from control group rats, BDL group rats, and UDCA + BDL group rats after 21 and 28 days of chronic cholestasis. Five to eight experiments were performed in each case. Statistical differences were assessed using ANOVA and Tukey-Kramer multicomparison tests.

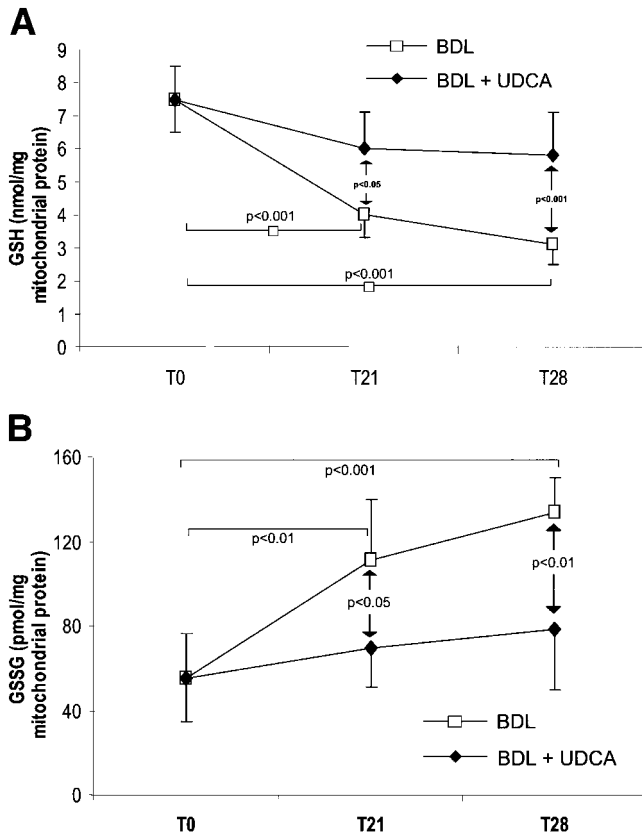


Fig. 4. Effects of chronic cholestasis and UDCA treatment on mitochondrial glutathione redox status. (A) GSH and (B) GSSG levels in liver mitochondria from control group rats, BDL group rats, and UDCA + BDL group rats after 21 and 28 days of chronic cholestasis. Five to eight experiments were performed in each case. Statistical differences were assessed using ANOVA and Tukey-Kramer multicomparison tests.

ing chronic cholestasis, GSH synthesis was examined in hepatocytes isolated from rats that had fasted for 48 hours and were distributed into three treatment groups (sham operation, BDL, and BDL + UDCA). GSH synthesis was measured using two sets of substrates: 1) 5 mM Gln, 2 mM Gly, 1 mM Ser, and 0.5 mM Met; and 2) 5 mM Gln and 2 mM Gly plus 0.5 mM NAC. For both substrates, the rate of GSH synthesis was diminished in hepatocytes from BDL group rats compared with sham-operated rats (Fig. 5). UDCA treatment prevented the decrease in the rate of GSH synthesis from the Gln + Gly + NAC substrates, but not from the Gln + Gly + Ser + Met substrates.

In the liver, the major rate-limiting factors with respect to GSH synthesis are the activity of  $\gamma$ -cystathionase, which determines cysteine availability, and the activity of  $\gamma$ -GCS, which has regulatory (light [30 kD]) and catalytic (heavy [73 kD]) subunits.<sup>34–36</sup> Synthesis of GSH from Ser and Met involves  $\gamma$ -cystathionase and  $\gamma$ -GCS, whereas synthesis of GSH from NAC depends only on  $\gamma$ -GCS. Therefore, subsequent experiments were conducted to

determine whether UDCA influenced the expression of these enzymes' mRNAs in the liver during chronic cholestasis.

BDL led to decreased expression of the mRNAs encoding  $\gamma$ -GCS (both the catalytic and regulatory subunits;  $P < .01$ ; Fig. 6A,C) and  $\gamma$ -cystathionase ( $P < .01$ ; Fig. 6D). UDCA prevented the decrease in expression of the mRNAs encoding  $\gamma$ -GCS ( $P < .01$  and  $P < .05$  for the catalytic and regulatory subunits, respectively; Fig. 6A,C), but not the decrease in  $\gamma$ -cystathionase mRNA expression. UDCA also protected against the decrease, observed in BDL group rats, in expression of the  $\gamma$ -GCS heavy subunit at the protein level (Fig. 6B).

**HNE-Protein Adducts in Secondary Biliary Cirrhosis.** HNE-protein adduct levels have been measured by immunohistochemical methods as an index of oxidative damage to proteins in secondary biliary cirrhosis induced by chronic cholestasis. Figure 7B shows the presence of HNE-protein adducts in rat liver samples 21 days after the induction of cholestasis. The distribution pattern of HNE adducts in BDL group rats was diffuse throughout the liver parenchyma, with strong staining for diffuse cytosolic deposits in hepatocytes and in epithelial cells from bile ducts and ductules. Staining for HNE adducts was far less intense in liver samples from BDL + UDCA group rats (Fig. 7C).

**Protection by UDCA Against the Evolution of a Population of Damaged Hepatocytes in Secondary Biliary Cirrhosis.** To assess whether the antioxidant effects of UDCA treatment led to protection against cell injury, we investigated the hepatocyte populations in animals with secondary biliary cirrhosis. First, the effects of chronic cholestasis and UDCA treatment on hepatocyte size were studied by flow cytometry, using forward-angle

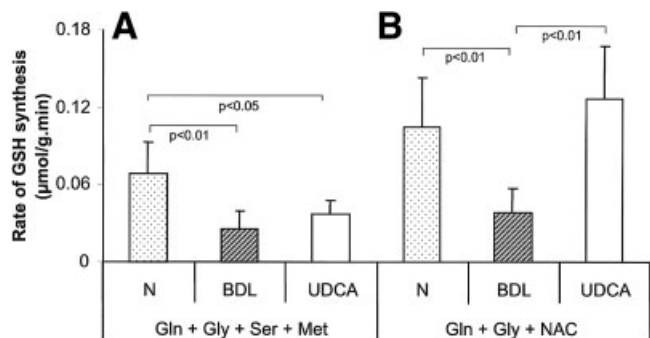


Fig. 5. Effects of chronic cholestasis and UDCA treatment on glutathione synthesis in hepatocytes. The rates of GSH synthesis were measured in isolated hepatocytes from control group rats, BDL group rats, and UDCA + BDL group rats after 21 days of chronic cholestasis. Rats were subjected to 48 hours of fasting before isolation of hepatocytes. Hepatocytes were incubated with one of two sets of glutathione precursors: (A) 5 mM Gln, 2 mM Gly, 1 mM Ser, and 0.5 mM Met; or (B) 5 mM Gln and 2 mM Gly plus 0.5 mM NAC.

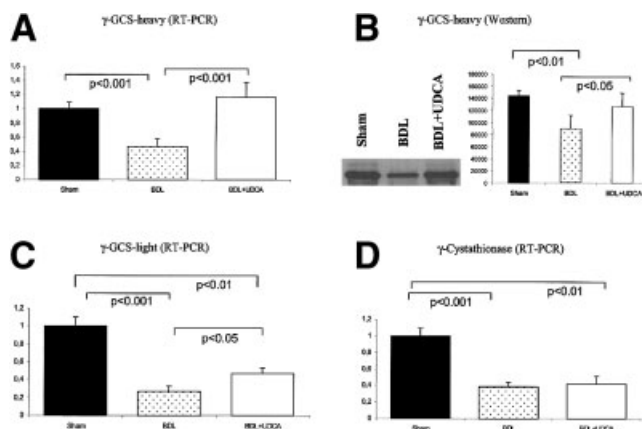


Fig. 6. Effects of chronic cholestasis and UDCA treatment on expression of  $\gamma$ -GCS and  $\gamma$ -cystathionase in rat liver samples. mRNA levels were measured using real-time reverse-transcription PCR. Protein expression of the heavy subunit of  $\gamma$ -GCS was measured using Western blot analysis. Gene expression of the  $\gamma$ -GCS (A) heavy and (C) light subunits and of (D)  $\gamma$ -cystathionase was lower in BDL group rats than in control group rats ( $P < .01$ ,  $P < .001$ , and  $P < .001$ , respectively). Treatment with UDCA restored expression of the  $\gamma$ -GCS heavy subunit at the (A) mRNA and (B) protein levels ( $P < .05$  for BDL group rats vs. BDL + UDCA group rats) and partially restored gene expression of the  $\gamma$ -GCS light subunit ( $P < .05$  for BDL group rats vs. BDL + UDCA group rats;  $P < .01$  for BDL + UDCA group rats vs. control group rats). Real-time quantitation of  $\gamma$ -GCS heavy subunit,  $\gamma$ -GCS light subunit, and cystathionase mRNA expression relative to GAPDH mRNA expression was performed. PCR data are shown as mean values  $\pm$  standard deviations for each gene relative to an arbitrary value of 1, which was assigned to expression levels in control group animals. Densitometry data from Western blots are presented as mean values  $\pm$  standard deviations and are shown together with representative experimental images in (B). Three to five different experiments were performed in each case.

scattering. Figure 8 shows representative assays of hepatocytes isolated from sham, BDL, and BDL + UDCA group rats. Two distinct populations were found in hepatocytes from BDL group rats: one of normal-size cells (similar to hepatocytes from sham-operated rats) and another of smaller cells. The same two populations were found in hepatocytes from BDL + UDCA group rats, but the proportion of smaller cells was much lower ( $P = .0119$ ) in this group than in the BDL group (Fig. 8).

Mitochondrial cardiolipin content in smaller hepatocytes was approximately three times lower than in normal-size hepatocytes from BDL group rats ( $167 \pm 23$  arbitrary fluorescence units [afu] vs.  $64 \pm 13$  afu;  $n = 3$ ;  $P < .01$ ) and five times lower than in hepatocytes from control rats ( $302 \pm 46$  afu vs.  $64 \pm 13$  afu;  $n = 3-4$ ;  $P < .01$ ). Mitochondrial membrane potential in smaller hepatocytes was three times lower than in normal-size hepatocytes from BDL group rats ( $75 \pm 20$  afu vs.  $22 \pm 6.1$  afu;  $n = 3$ ;  $P < .05$ ) and six times lower than in hepatocytes from control rats ( $148 \pm 25$  afu vs.  $22 \pm 6.1$  afu;  $n = 3-4$ ;  $P < .01$ ).

Annexin V was used to detect translocation of phosphatidylserine to the outer leaflet of the plasma membrane; such translocation is a marker of the apoptotic process in isolated hepatocytes. We found that at least half of the population ( $58\% \pm 8\%$ ;  $n = 3$ ) of small hepatocytes from BDL group rats exhibited high levels of fluorescence with annexin V (Fig. 9); this elevated fluorescence is characteristic of apoptotic hepatocytes. The lack of propidium iodide fluorescence confirmed that these hepatocytes still were viable. The marked decreases in cardiolipin levels and mitochondrial membrane poten-

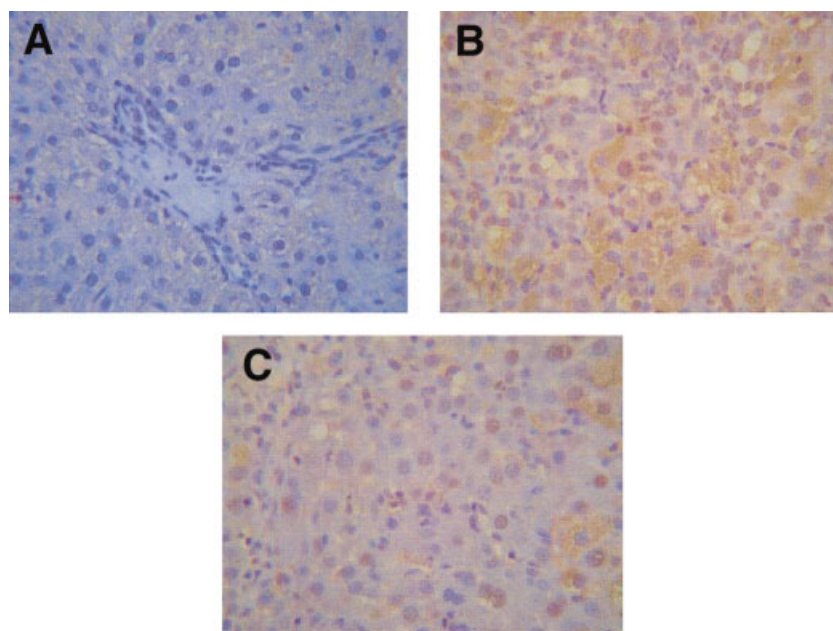


Fig. 7. Immunohistochemical staining for HNE-protein adducts in secondary biliary cirrhosis: protective effects of UDCA treatment. HNE-protein adducts were measured as an index of oxidative damage to proteins in (A) control group, (B) BDL group, and (C) UDCA + BDL group rats. Each image is representative of three different experiments. Original magnification  $\times 200$ .



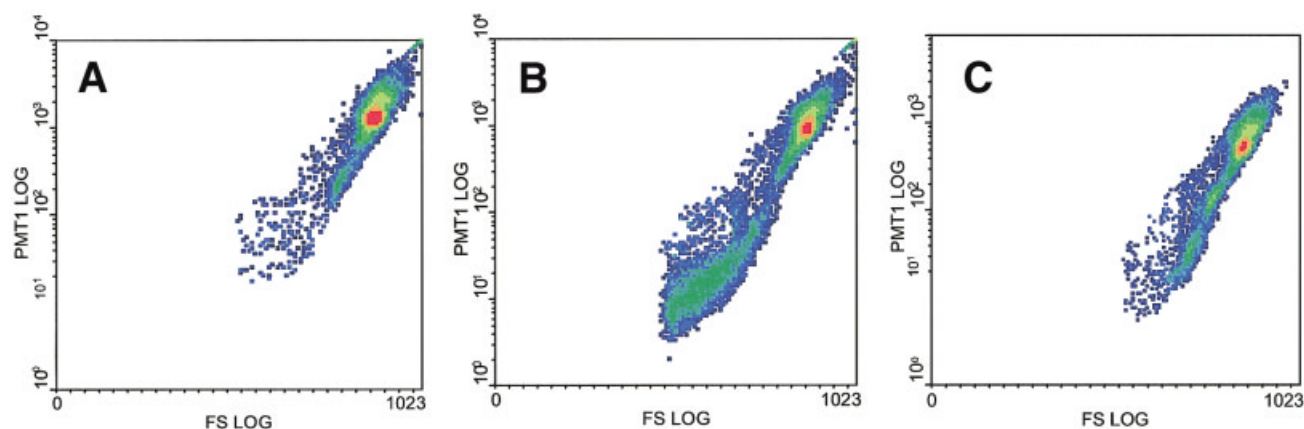


Fig. 8. Representative experiments showing hepatocyte populations in secondary biliary cirrhosis and the protective effects of UDCA treatment. Hepatocyte size was measured by flow cytometry using forward-angle scattering. Hepatocytes were isolated from (A) control group, (B) BDL group, and (C) UDCA + BDL group rats. Two separate populations were found in hepatocytes from BDL group rats, according to size. The percentage of hepatocytes with smaller size and less morphologic complexity was higher in BDL group rats than in control group rats. UDCA treatment significantly prevented the cholestasis-induced change in hepatocyte size.

tial that were observed in small hepatocytes from BDL group rats appeared to render these cells susceptible to apoptosis. Thus, we can conclude that the population of small hepatocytes was formed by the damaged and primarily apoptotic hepatocytes that are present in the liver during chronic cholestasis.

UDCA treatment almost completely prevented the development of a cell population formed by damaged hepatocytes in animals with chronic cholestasis (Fig. 8). Two distinct populations were found in hepatocytes from BDL + UDCA group rats, but the proportion of hepatocytes with decreased cardiolipin content, decreased membrane potential, and small size was much lower than the corresponding proportion in BDL group rats. The distribution of hepatocytes was as follows:  $54\% \pm 13\%$  normal-size

cells and  $30\% \pm 8\%$  small cells in BDL group rats, compared with  $78\% \pm 9\%$  normal-size cells and  $12\% \pm 3\%$  small cells in BDL + UDCA group rats ( $P = .0119$ ).

**Prevention by UDCA of Cholestasis-Induced Decreases in Mitochondrial Cardiolipin Levels and Membrane Potential.** Oxidative stress lowers cardiolipin levels and membrane potential in mitochondria.<sup>37,38</sup> Consequently, we investigated these parameters using flow cytometry in hepatocytes isolated from rats subjected to chronic cholestasis and treatment with UDCA. We found that cardiolipin levels in normal-size hepatocytes from BDL group rats were significantly lower ( $P < .05$ ) than cardiolipin levels in hepatocytes from sham-operated rats (Fig. 10A). Treatment with UDCA completely prevented the decrease in mitochondrial cardiolipin levels that would have been induced by chronic cholestasis ( $P < .05$  for the comparison with BDL group rats).

Mitochondrial membrane potential was significantly lower in normal-size hepatocytes from BDL group rats than in hepatocytes from sham-operated rats ( $P < .05$ ). UDCA treatment completely restored normal membrane potential ( $P < .01$  for comparison with BDL group rats; Fig. 10B).

## Discussion

**Cholestasis-Induced Oxidative Stress Caused by Increased Mitochondrial Production of ROS and by Increased Glutathione Oxidation.** Chronic cholestasis induced by BDL in rats is associated with oxidative stress, as evidenced by increased lipid peroxide levels and low antioxidant enzyme activity in the liver.<sup>39</sup> Liver mitochondria also exhibit increased levels of thiobarbituric

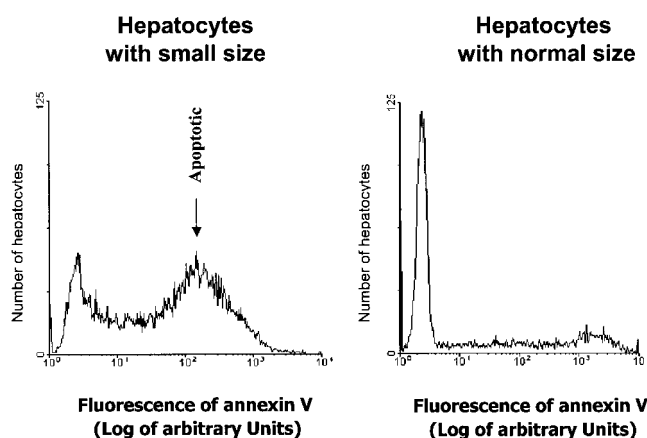


Fig. 9. Representative experiment showing detection of phosphatidylserine exposure in small hepatocytes from BDL group rats. Phosphatidylserine exposure was measured by flow cytometry, using annexin V as a fluorochrome.



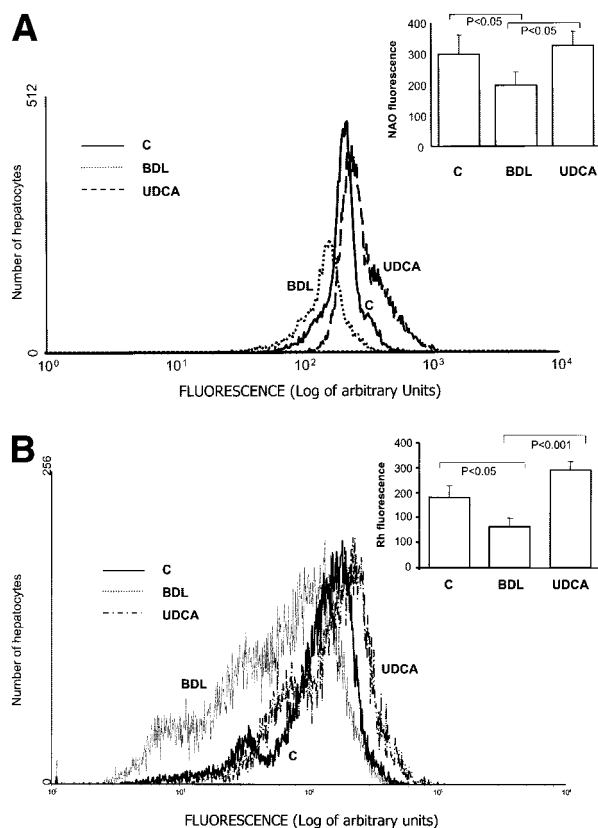


Fig. 10. Representative histograms showing (A) mitochondrial cardiolipin content and (B) mitochondrial membrane potential in secondary biliary cirrhosis, along with the protective effects of UDCA treatment. Mitochondrial cardiolipin content was measured in isolated rat hepatocytes by flow cytometry, using nonylacridine orange as a fluorochrome. Mitochondrial membrane potential was determined by flow cytometry in isolated hepatocytes using rhodamine 123 at 525 nm fluorescence emission. Hepatocytes were isolated from control group (C), BDL group, and BDL + UDCA group rats after 21 days of chronic cholestasis. Nonylacridine orange and rhodamine 123 fluorescence levels were significantly lower ( $P < .05$ ) in BDL group rats compared with control group rats and BDL + UDCA group rats. No significant difference was found between control group rats and BDL + UDCA group rats. Insets show plots for three to four experiments.

acid-reactive substances and diminished levels of GSH during experimental cholestasis.<sup>40</sup> In the current study, we found several indicators of oxidative stress, such as increased GSSG levels and increased levels of HNE-protein adducts, in BDL group rats.

A noteworthy increase in mitochondrial ROS production occurs during chronic cholestasis in rats. It has been reported that hydrophobic acids enhance ROS production in liver mitochondria from control rats.<sup>5,10</sup> Nonetheless, to our knowledge, the current study is the first to assess the rate of peroxide production in liver mitochondria from rats with cirrhosis under State 4 conditions using physiologic substrates linked to complexes I and II. These conditions are much more similar to *in vivo* conditions in the livers of rats with cirrhosis.

Our data support the hypothesis of Sokol et al.,<sup>12</sup> who proposed that bile acids accelerate superoxide generation at complex I and ubiquinone, overwhelming the endogenous mitochondrial antioxidant defense. In another study, Sokol et al.<sup>5</sup> reported that hydrophobic bile acids enhanced the generation of hydroperoxides in isolated mitochondria from control rats. The results of the current study indicate that chronic cholestasis leads to increased peroxide production via mitochondrial complexes I and III, as enhancement was noted with both pyruvate/malate and succinate substrates. The observed threefold increase in the rate of mitochondrial peroxide production suggests that the contribution of mitochondria to oxidative damage in biliary cirrhosis may have been underestimated. The high level of mitochondrial production of ROS also accounts for the oxidative damage and glutathione oxidation observed in the liver (particularly in mitochondria) during biliary cirrhosis and may be responsible for mitochondrial impairment.<sup>41</sup> These effects are exacerbated in chronic cholestasis, because peroxide production via mitochondrial complexes increases as biliary cirrhosis progresses.

Under physiologic conditions, cysteine availability is the limiting factor for GSH synthesis in the liver,<sup>36</sup> particularly in cases of cirrhosis, in which the transsulfuration pathway appears to be critically impaired. In fact, methionine adenosyltransferase and cystathionine  $\beta$ -synthase exhibit decreased activity and mRNA levels in cirrhosis.<sup>42–44</sup> We now report that  $\gamma$ -cystathionase mRNA levels are low in chronic cholestasis. Accordingly, in rats with cirrhosis, synthesis of GSH from methionine, which involves the transsulfuration pathway, is diminished. Furthermore, the rate of synthesis of GSH from NAC was diminished in hepatocytes from BDL group rats; this finding was in agreement with the finding of decreased  $\gamma$ -GCS expression.

Cardiolipin may modulate apoptotic processes by inhibiting MPT. In fact, it provides an increased pool of negative ions that nonspecifically bind  $\text{Ca}^{2+}$ , thereby preventing it from binding to protein sites that induce the opening of mitochondrial pores.<sup>45</sup> Lieser et al.<sup>46</sup> found that mitochondrial cardiolipin content increased 10 days after BDL and thus protected against cell death by increasing the threshold for MPT. Nonetheless, this adaptive mechanism appears to function only during the first days of cholestasis, as we observed a decrease in mitochondrial cardiolipin content after 21 days of chronic cholestasis. This decrease in cardiolipin levels may be due to increased mitochondrial peroxide production induced by chronic cholestasis, as ROS lead to oxidative cardiolipin damage and decreased mitochondrial cardiolipin content.<sup>38</sup>

We also found two different populations of hepatocytes in rats with cirrhosis: one of these populations exhibited cell damage that was characterized by dramatic losses in mitochondrial cardiolipin content and membrane potential. We postulate that increased mitochondrial ROS production and decreased cardiolipin content create a vicious cycle, because these two effects potentiate each other. In fact, diminished mitochondrial cardiolipin content leads to decreased mitochondrial complex I activity<sup>38</sup> and thus may account for the decreased mitochondrial membrane potential and increased leakage of ROS through the mitochondrial electron transport chain in chronic cholestasis.

**UDCA Treatment Restores GSH Synthesis From Cysteine and Diminishes Oxidative Stress in Chronic Cholestasis.** UDCA is a hydrophilic bile acid that is increasingly used to treat various cholestatic disorders. It normally is present in human bile, albeit at a low concentration, accounting for only approximately 3% of total bile acid. To date, UDCA has been used widely to treat PBC, for which it is the only drug approved by the United States Food and Drug Administration. Although UDCA represents the current and primary treatment for cholestatic disorders, the mechanisms underlying its effects are in the process of being elucidated.

It should be noted that UDCA treatment significantly increased expression of  $\gamma$ -GCS, but not expression of  $\gamma$ -cystathionase. Consequently, UDCA increased the rate of GSH synthesis when cysteine availability was not a limiting factor. This finding is in agreement with previous *in vitro* data indicating that UDCA could induce  $\gamma$ -GCS expression in hepatocytes that were experiencing chemical oxidative stress.<sup>24</sup> Furthermore, our results suggest that cotreatment with NAC, which would prevent limitations in cysteine availability due to low  $\gamma$ -cystathionase activity during biliary cirrhosis, should be recommended.

The positive effect on  $\gamma$ -GCS expression contributes to the protective effects of UDCA against oxidative stress in liver homogenates as well as in mitochondria, as evidenced by diminished levels of GSSG and HNE-protein adducts. The antioxidant defense mediated by GSH is enhanced by UDCA treatment in secondary biliary cirrhosis; this defense prevents the development of cell injury during chronic cholestasis. In fact, a population of damaged and primarily apoptotic hepatocytes, which exhibited dramatic losses in mitochondrial cardiolipin content and membrane potential, as well as phosphatidylserine exposure, evolved during chronic cholestasis. UDCA treatment prevented the growth of this cell population.

It has been established that oxidative stress is an early event in apoptosis<sup>47</sup> and that mitochondrial oxidative stress renders hepatocytes much more susceptible to eth-

anol-induced apoptosis.<sup>28</sup> Therefore, the beneficial effects of UDCA against chronic cholestasis appear to be mediated, at least in part, by protection against mitochondrial oxidative stress. Because oxidative stress may lead to oxidation and depletion of cardiolipin, the inhibitory effects of UDCA against mitochondrial peroxide production and oxidation of glutathione may be responsible for the subsequently observed maintenance of cardiolipin levels. This effect also would provide protection against cell death.

The effect of UDCA on the progression of liver fibrosis in PBC recently was defined.<sup>48</sup> According to Corpechot et al.,<sup>48</sup> UDCA therapy significantly delays the progression of liver fibrosis in early-stage PBC (*i.e.*, PBC without extensive fibrosis). Although UDCA protects against oxidative stress, we observed no significant difference in terms of degree of fibrosis between BDL group rats and BDL + UDCA group rats in our model of established cirrhosis, in which all compensatory actions had proved insufficient (results not shown). Thus, although UDCA is able to protect against mitochondrial and overall cellular oxidative stress, additional treatment should be recommended to effectively reduce the rate of fibrogenesis during the late stages of cirrhosis.

In conclusion, we have demonstrated that UDCA treatment up-regulates  $\gamma$ -GCS expression and consequently increases the rate of GSH synthesis in secondary biliary cirrhosis. UDCA treatment also was found to prevent the marked increases in mitochondrial peroxide production and oxidative protein damage that normally occur during chronic cholestasis. These antioxidant effects led to the prevention of cardiolipin depletion and cellular injury in secondary biliary cirrhosis.

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