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The Role of Lipid Peroxidation and Glutathione on the Glycochenodeoxycholic Acid-Induced Cell Death in Primary Cultured Rat Hepatocytes

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Intracellular accumulation of bile acids in the hepatocytes during cholestasis is thought to be pathogenic in cholestatic liver diseases. The objective of this study was to determine the role of lipid peroxidation and glutathione on the bile acid-induced hepatic cell death mechanism in primary cultured rat hepatocytes. To induce hepatic cell death, we incubated primary cultured rat hepatocytes with glycochenodeoxycholic acid (GCDC; 0~400 μM) for 3 hours. In electron microscopic examination and agarose gel electrophoresis, low concentration of GCDC treatment mainly induced apoptotic feature. Whereas 400 μM GCDC treated cells demonstrated both apoptosis and necrosis. Lipid peroxidation was increased dose-dependently in GCDC treated hepatocyte. And this was also accompanied by decreased glutathione. Therefore, oxygen free radical damage may play a partial role in GCDC-induced hepatic cell death.

Key Words: Hepatocyte, Apoptosis, Glycochenodeoxycholic acid, Lipid peroxidation, Glutathione

INTRODUCTION

Cholestasis, the impairment of bile flow, is the common feature of many liver diseases (Scharschmidt, 1990). Among many pathological processes resulted in cholestasis, one of the final common pathways leading to cholestatic liver injury is the intracellular accumulation of hydrophobic bile acids (Greim et al, 1973; Festi et al, 1983; Attini et al, 1986; Hofmann & Popper, 1987). Although bile acid induced cytotoxicity is related to the detergent properties and hydrophobicity of bile acids (Armstrong & Carey, 1982; Attini et al, 1986), it is unlikely that they reach levels sufficient for the detergent properties to lead to massive solubilization and disruption of cell membranes (Attini et al, 1986). It has been also proposed that other properties of hydrophobic bile acids are responsible for cytotoxicity.

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Free radicals are molecules with an unpaired electron including superoxide, hydrogen peroxide, hydroxyl radical and possibly, singlet oxygen that may cause tissue injury by reacting with membrane lipids, thiol proteins or nucleic acids (Halliwell, 1991). Clinical evidence of free radical generation during cholestasis includes the demonstration of increased plasma and erythrocyte levels of lipid peroxides, end-products of oxidative modification of polyunsaturated fatty acids, in children with chronic cholestasis (Lubrano et al, 1989). An extensive system of cytosolic and membrane-bound enzymatic and non-enzymatic antioxidants functions to scavenge free radicals produced in physiological process and in pathological conditions (Halliwell, 1991). The liver is richly endowed with cytoprotective mechanisms such as antioxidants, scavenging enzymes and repair processes, which act to counteract the effects of free radicals production. In addition, levels of Cu · Zn-superoxide dismutase and catalase, two of the free radical-scavenging enzymes in liver, have been reported to be low in the diseased human liver, thereby the susceptibility of the liver to injury by oxygen- derived free radicals potentially increased (Togashi et al,

1990).

Although it is clinically important, the mechanism of hydrophobic bile acid-induced hepatocellular injury remains unclear. The objectives of this study were to investigate the role of lipid peroxidation and glutathione during glycochenodeoxycholic acid (GCDC)-induced hepatic cell death in primary cultured rat hepatocytes.

METHODS

Isolation and primary culture of rat hepatocytes

Rat hepatocytes were isolated and cultured according to Seglen (1976) as we have previously described (Chu et al, 1999).

Bile acid treatment

GCDC was treated on hepatocytes up to 400 μM concentration for 3 hours because the intracellular concentration of chenodeoxycholic acid and its conjugates are elevated up to 800 μM at the state of cholestasis (Spivey et al, 1993). Freshly isolated and cultured rat hepatocytes were used to avoid the dedifferentiation of hepatocytes that causes decrease of intrahepatic bile salt uptake in the long term cultures (Kwo et al, 1995).

Electron microscopic examination

Cultured hepatocytes (5×10^5 cells/ml) were harvested with gentle scraping and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C for 2 hour. After rinsed for 30 min in three changes of 0.1 M phosphate buffer, the cells were followed by a 1 hour postfix in phosphate-buffered 1% OsO_4. And then rinsed again in three changes of distilled water, the cells were dehydrated in progressive concentrations of ethanol followed by 100% propylene oxide, and embedded in Epon 812. Sections were cut on an LKB Ultratome III (Mager Scientific, USA), placed on 200 nm mesh copper grids, stained with 2% uranyl acetate and lead citrate and micrographs were taken.

Agarose gel electrophoresis (DNA fragmentation)

Cultured hepatocytes (5×10^5 cells/ml) were harvested with 0.25% trypsin and by gentle scraping. After centrifugation, 20 μl of lysis buffer (20 mM EDTA, 100 mM Tris, pH 8.0, 0.8% SDS) and 10 μl of RNase (1 mg/ml in 10 mM tris-HCl, pH 7.5, 15 mM NaCl) were added to the cell pellets. After incubation of 1 hour at 37°C, 10 μl of proteinase K (20 mg/ml) was added. The mixtures were incubated at 50°C for 5 hours. And then 5 μl of loading buffer (10% glycerol, 10 mM Tris, pH8, 0.1% bromophenol) was added. Gel electrophoresis was performed at 30 V for 5 hours on a 1.5% agarose gel using a TAE buffer (40 mM tris, 20 mM acetic acid, 1 mM EDTA, pH 8.4). The gel was stained with ethidium bromide and photographed under ultraviolet light (IBI, Germany, Kodak).

Measurement of lipid peroxidation

For the measurement of lipid peroxidation, thiobarbituric acid reacting substances (TBARS) method was used (Ohkawa et al, 1979). Briefly, 0.2 ml of hepatocytes suspension was added to a tube containing 0.2 ml of SDS (8%, w/v) and 0.4 ml of acetic acid (20%, w/v, pH 3.5), 0.4 ml of thiobarbituric acid (0.8%, w/v). The mixture was vortexed well and boiled for 1 hour. After cooling, the mixture was centrifugated and the absorbance of the supernatant was determined at 532 nm using a spectrophotometer (Uvikon 860, Kontron Instruments, Switzerland). A standard curves were prepared using 1,1,3,3-tetraethoxypropane. Final values were expressed as the relative percent of the control group.

Measurement of cellular glutathione

Cellular glutathione (GSH) concentrations were measured using mBCl with an adaptation of the method of Fernadez-Checa & Kaplowitz (1990). After 3 hour incubation with GCDC, cultured hepatocytes were incubated with 100 μM mBCl for 10 min at 37°C. The unreacted mBCl was removed by washing with PBS. The remained cells were lysed with 0.2% Triton X-100 in PBS. After centrifugation, the supernatant was used for quantification of fluorescence at excitation and emission wavelength of 400 nm and 480 nm, respectively, in a fluoroscence spectrophotometer (model 650-60, Hitachi, Japan). Standard curves were prepared by incubating GSH in the presence of rat liver glutathione-s-transferase (20 U/ml) for 10 min at 37°C. Final values were expressed

as the relative percent of the control group.

Statistical analysis

All the data are summerized as mean±S.E.. To analyze the data statistically, we performed a one-way analysis of variance (ANOVA). Then Dunnett's multiple range *t*-test was used to determine which means were significantly different from the mean of the control group. We considered the differences was significant at $p<0.005$.

RESULTS

Morphological features of apoptosis

In the control group, the euchromatin is distributed throughout the nucleus and the nucleolus was observed. 200 μM GCDC treated cells suggested apoptosis demonstrating the membrane-enclosed nuclear fragments containing sharply segregated, compact chromatin and condensed cytoplasm with preservation of the integrity of organelles. Whereas 400 μM GCDC treated cells demonstrated both apoptosis and necrosis. The ill-defined edges of the small chromatin

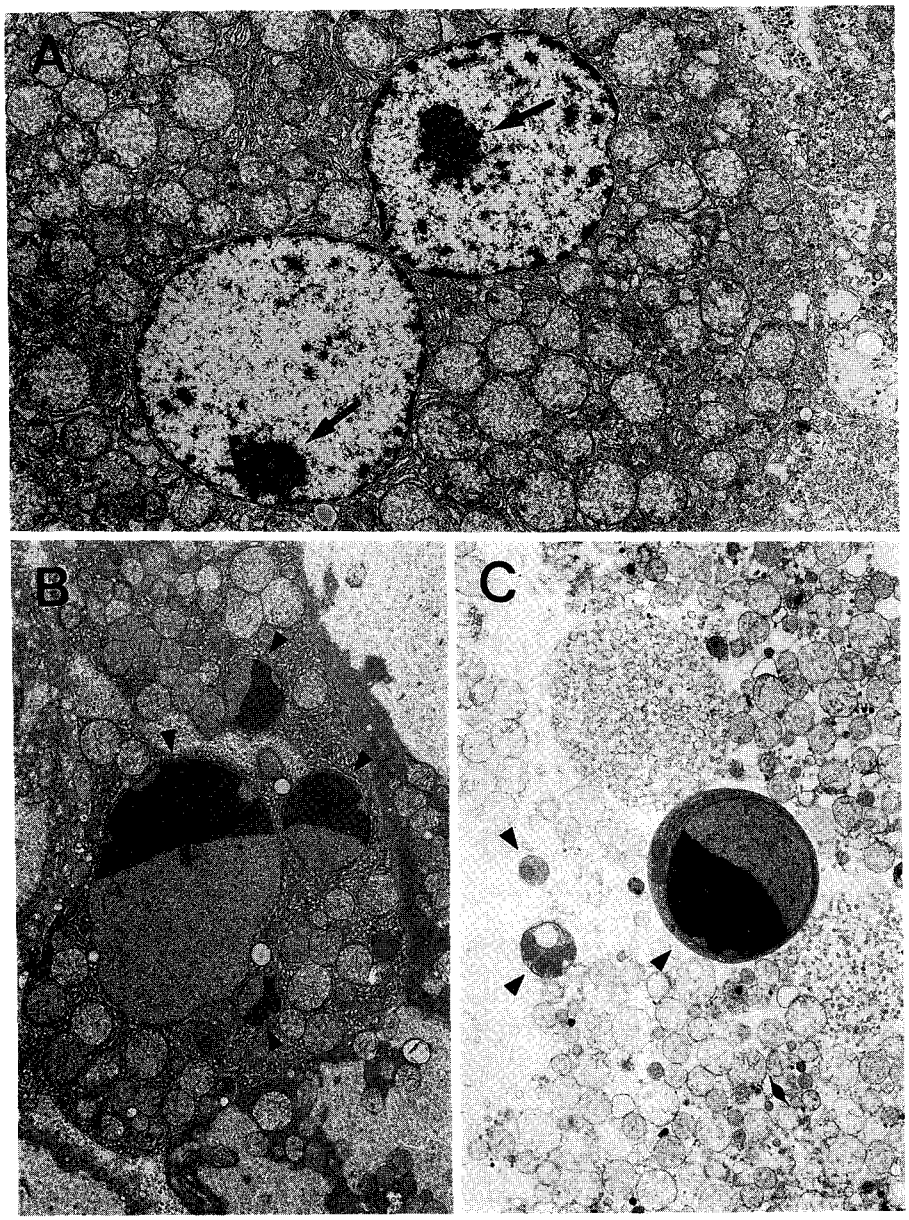

Fig. 1. Electron microscopy of cultured rat hepatocytes treated with glycochenodeoxycholic acid for 3 hours. In the control group the euchromatin is distributed throughout the nucleus and the nucleolus (arrow) was observed. 200 μM glycochenodeoxycholic acid (GCDC) treated cells demonstrated the membrane-enclosed nuclear fragments containing sharply segregated, compact chromatin (arrow head). 400 μM GCDC treated cells demonstrated both apoptosis and necrosis. (A) Control (×4500) (B) 200 μM GCDC (×4000) (C) 400 μM GCDC (×3700)

clumps and the preservation of the overall configuration of the cells despite extensive breakdown of their membrane as well as the membrane-enclosed nuclear fragments were observed (Fig. 1).

Biochemical features of apoptosis

GCDC treated hepatocytes induced DNA fragmentation, which resulted in a typical DNA ladder pattern on agarose gel electrophoresis under the concentrations of 200 μM after 3 hour of incubation with GCDC (Fig. 2).

Lipid peroxidation

After 3 hours GCDC treatment, lipid peroxidation was increased dose-dependently (Fig. 3). Generation of TBARS increased significantly to 141.7% ($p<0.005$), 193.0% ($p<0.0001$) of the control value at the concentrations of 200 μM and 400 μM respectively.

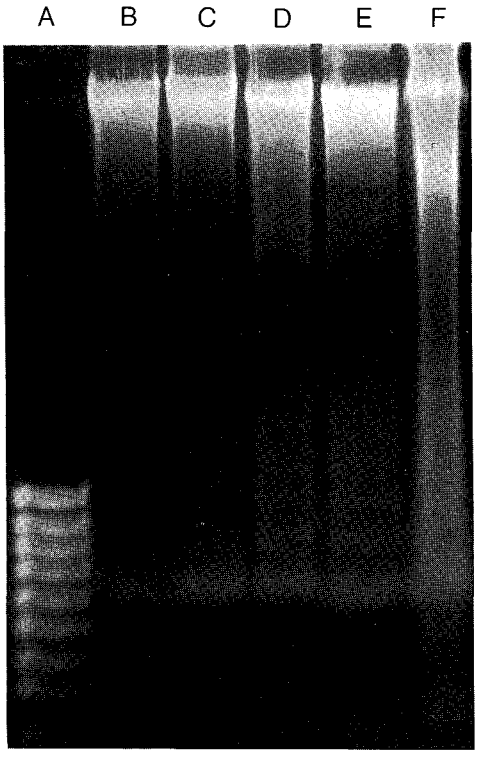

Fig. 2. An ethidium bromide-stained agarose gel after electrophoresis of hepatocyte DNA. The left lane contains a molecular weight marker. DNA was estracted from cultured hepatocytes treated with glychochenodeoxycholic acid (GCDC) for 3 hours. A ladder-like DNA fragmentation pattern was well demonstrated in the 100 μM and 200 μM GCDC treated group. A: DNA-STANDARD, B: 0 uM GCDC, C: 50 uM GCDC, D: 100 uM GCDC, E: 200 uM GCDC, F: 400 uM GCDC

Intracellular glutathione

Cellular GSH concentrations in hepatocytes exposed to GCDC were unchanged from basal levels

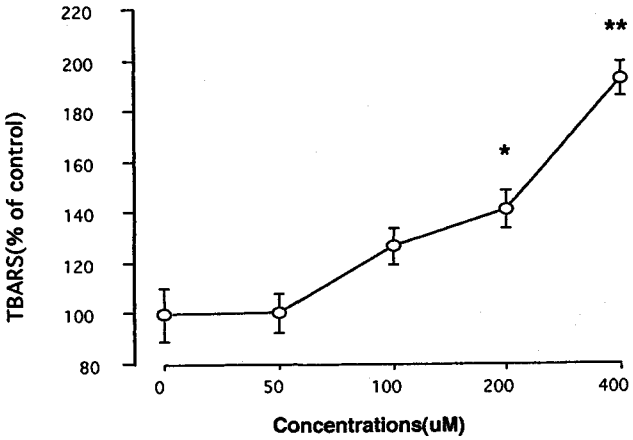

Fig. 3. Thiobarbituric acid reacting substances (TBARS) levels in isolated hepatocytes incubated for 3 hours with GCDC. Significant increase in TBARS levels were seen in cultured hepatocytes treated with 200 μM and 400 μM GCDC compared with control (n=24, *$p<0.005$, **$p<0.0001$). Results are expressed as the relative percent of the control group (mean$\pm$S.E.).

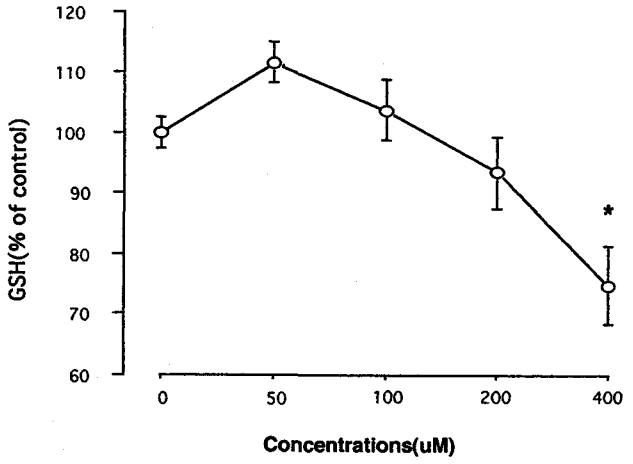

Fig. 4. Intracellular glutathione (GSH) concentrations of cultured hepatocytes treated with GCDC for 3 hours. Significant decrease of intracellular GSH concentration was detected at 400 μM (n=50, *$p<0.005$). Results are expressed as the relative percent of the control group (mean$\pm$S.E.).

and were >90% of those in controls under the concentration of 200 μM (Fig. 4). However, at the concentration of 400 μM, celluar concentration of GSH decreased significantly to 75.0% ($p<0.005$) compared with the control value.

DISCUSSION

In human cholestatic liver diseases, toxic bile salts are retained within the liver because biliary excretion of these compounds is impaired (Hofmann, 1989). Despite the clinical importance and implications for future therapeutic approaches of bile salt-induced cytotoxicity, its mechanisms remain incompletely understood. This study was designed to determine the mechanism of GCDC-induced hepatic cell death.

In this study, we wanted to begin by confirming the occurrence of apoptosis in GCDC-induced hepatic cell death. In electron microscopic examination demonstrated that 200 μM GCDC treated cells suggest apoptosis whereas 400 μM GCDC treated cells demonstrated both apoptosis and necrosis. And apoptosis was accompanied by a typical DNA ladder pattern on agarose gel electrophoresis. Thus after exposure to GCDC, the resulting cell death pattern depended on the concentration. High GCDC concentration (400 μM) rapidly induced significant necrosis as well as apoptosis, whereas low GCDC concentrations ($\leq$200 μM) were followed by apoptosis. In high concentrations, bile acid are biophysically active molecules that act as detergents, and hepatocellular injury has been attributed to direct membrane damage (Heuman, 1993). Recently apoptosis has been the subject of intense investigation of bile acid-induced cell death. Although less is known about the intracellular signals that trigger the effectors of apoptosis, several signaling mechanisms have received attention in bile acid-induced apoptosis including ATP depletion, increase of cytosolic free calcium (Spivey et al, 1993), increase of magnesium (Patel et al, 1994), activation of mitogen-activated protein kinase (Webster & Anwer, 1998) and activation of protein kinase C (Jones et al, 1997). The increased nuclear endoclease and cytosolic protease activity (Kwo et al, 1995) and the involvement of cathepsin B as one of the proteases (Lewis et al, 1997) have been reported in bile acid-induced hepatocellular injury.

Although bile acids are not oxidants per se, bile acids have been implicated in causing oxidant damage both *in vitro* and *in vivo* (Sokol et al, 1991; Sokol et al, 1993). Sokol et al (1995) reported that intracellular hydroperoxide generation appeared to precede hepatocytes injury on exposure to taurochenodeoxycholic acid. Rodrigues et al (1998) demonstrated that increased production of superoxide anion during exposure to deoxycholic acid. In this study, we demonstrated lipid peroxidation was increased dose-dependently. Therefore, we can suggest that lipid peroxidation can occur both in apoptosis and necrosis. The earlier literature on cell death and lipid peroxidation focused on decomposition of membrane lipids by massive lipid peroxidation as a mechanism of necrosis (Rosser & Gores, 1995). We realized that low levels of oxidative stress with lipid peroxidation can result in apoptosis. Indeed, lipid peroxidation by oxidative stress is widely recognized as an induction mechanism for apoptosis.

GSH is known to be broadly distributed and participate in numerous cellular processes, particularly in the detoxification processes (Meister, 1989). GSH protects cells from the toxic effects of many oxygen free radicals as a substrate in the removal of intermediates such as hydrogen peroxide and other hydroxy peroxides by GSH peroxidase. In the liver, GSH is a more important reducing agent against hydrogen peroxide than catalase (Shan et al, 1990). In this study, hepatocellular GSH concentrations exposed to GCDC were unchanged from basal levels under the concentration of 200 μM whereas at the concentration of 400 μM, cellular concentration of GSH decreased significantly. Thus, during low concentration of GCDC-induced hepatocellular apoptosis ($\geq$200 μM), lipid peroxidation occurred without changing intracellular GSH levels whereas during high concentration of GCDC-induced hepatocellular necrosis (400 μM), lipid peroxidation and concomitant intracelluar GSH depletion occurred. Recent study (Morales et al, 1997) also suggested that low concentration of oxygen free radical generator induced GSH synthesis whereas high concentration of radical generator depleted intracellular GSH. However, Patel & Gores (1997) reported that during GCDC-induced apoptosis, cellular GSH levels were unchanged from basal levels and were >95% of those in controls, suggesting that cellular lipid peroxidation occurs without a concomitant depletion in GSH during GCDC-induced hepatocellular injury.

In conclusion, low concentration of GCDC ($\geq$200

μM) mainly induced hepatocellular apoptosis followed by increased lipid peroxidation without changing intracellular GSH level whereas high concentration of GCDC (400 μM) mainly induced hepatocellular necrosis occurred with increased lipid peroxidation and concomitant GSH depletion. Thus, we suggest that oxygen free radical damage may play a partial role in GCDC-induced hepatocellular injury.

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