Increased susceptibility of fat-laden Zucker-rat hepatocytes to bile acid–induced oncotic necrosis: An in vitro model of steatocholestasis

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Metabolic liver disorders cause chronic liver disease and liver failure in childhood. Many of these disorders share the histologic features of steatosis and cholestasis, or steatocholestasis. In this study we sought to (1) develop an in vitro model of steatocholestasis, (2) determine the mechanisms of cell death in this model, and (3) determine the role of mitochondrial disturbances in this model. Methods: Hepatocytes were isolated from 8-week-old obese (fa/fa) and lean Zucker rats. Cell suspensions were treated with glycochenodeoxycholic acid (GCDC), after which reactive oxygen species (ROS) generation, oncotic necrosis, apoptosis, and ATP content were assessed. Isolated liver mitochondria were exposed to GCDC and analyzed for ROS generation, mitochondrial membrane–permeability transition (MPT), and cytochrome c release. Oncotic necrosis was significantly increased and apoptosis reduced in fa/fa hepatocytes exposed to GCDC compared with that in lean hepatocytes. Necrosis occurred by way of an ROS- and MPT-dependent pathway. Basal and dynamic ATP content did not differ between fa/fa and lean hepatocytes. GCDC stimulated ROS generation, MPT, and cytochrome c release to a similar extent in purified mitochondria from both fa/fa and lean rats. These findings suggest that fat-laden hepatocytes favor a necrotic rather than an apoptotic cell death when exposed to low concentrations of bile acids. The protective effects of antioxidants and MPT blockers suggest novel therapeutic strategies for the treatment of steatocholestatic metabolic liver diseases. (J Lab Clin Med 2005;145: 247–262)

Abbreviations: ALT = alanine aminotransferase; AP = alkaline phosphatase; AST = aspartate aminotransferase; BSA = bovine serum albumin; CSA = cyclosporin A; $\Delta\Psi$ = mitochondrial membrane potential; DAPI = 4,6-diamino-2-phenylindole; DCF = 2,7′-dichlorofluorescein; DCF-DA = dichlorofluorescein diacetate; DAPI = 4,6-diamino-2-phenylindole; EGTA = ethylene glycol-*bis*-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid; GCDC = glycochenodeoxycholic acid; HEPES = *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; *KRH* = Krebs-Ringer's-HEPES buffer; LDH = lactate dehydrogenase; MOPS = 3-(*N*-morpholino) propane–sulfonic acid; *MPT* = mitochondrial permeability transition; *ROS* = reactive oxygen species; UDCA = ursodeoxycholic acid

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Metabolic liver disorders are a common cause of chronic liver disease and liver failure in infancy and childhood, accounting for more than 30% of all liver transplants in children.[1] Many metabolic diseases, such as cystic fibrosis, galactosemia, hereditary tyrosinemia, mitochondrial hepatopathies, Wilson's disease, and congenital defects of glycosylation, share the histologic features of micro- or macrovesicular steatosis in combination with cholestasis,[2] or steatocholestasis. Although the genetic origins of many of these metabolic disorders have been elucidated in recent years, the mechanisms responsible for liver injury and fibrogenesis have not been established. Hepatic steatosis was once considered a benign component of the pathophysiology of metabolic disorders, perhaps caused by malnutrition.[3] It is now believed that steatosis in these disorders is caused, at least in part, by the accumulation of toxic intermediates or the absence of essential cofactors, leading to impaired transport or β-oxidation of fatty acids in mitochondria or altered mitochondrial respiratory-chain activity. In recent years, it has become evident that steatosis plays an important role in the biochemical pathogenesis of other liver disorders, such as nonalcoholic steatohepatitis.[4,5] Because simple steatosis may be well tolerated by hepatocytes, it has been proposed that a "second hit"—most likely the induction of oxidative stress, is necessary to trigger cellular injury in the fat-laden hepatocyte.[5] Oxidative stress in the fatty liver leads to lipid peroxidation, which may then promote chemotaxis and inflammation through activation of nuclear factor-κB, which, in turn, up-regulates expression of proinflammatory cytokines, including tumor necrosis factor-α and interleukin-8,[4–6] and inducers of fibrogenesis.

Cholestasis in metabolic disorders of childhood may result from the accumulation of toxic metabolites or cytokines that down-regulate or interfere with the function of bile-acid and phospholipid canalicular transport proteins or reduce mitochondrial oxidative phosphorylation and impair ATP-dependent bile-acid secretion,[7] the result being retention of bile acids in hepatocytes. In normal hepatocytes, toxic hydrophobic bile acids promote both oncotic necrosis at high concentrations ($>$100 μmol/L) and apoptosis (programmed cell death) at low concentrations (25–100 μmol/L),[8–10] both of which are ROS-dependent.[9–13] We propose that in steatocholestatic metabolic disorders, hepatocellular retention of toxic bile acids[14] in fat-laden hepatocytes may provide a second hit through stimulation of ROS generation, triggering hepatocellular injury.[8–12]

We are unaware of previous attempts to characterize the mechanisms underlying the hepatocyte response to concurrent bile-acid toxicity and steatosis. Consequently, in this study we sought to (1) develop an in vitro model of hepatocellular steatocholestasis, using freshly isolated rat hepatocytes from the Zucker rat, in which spontaneous hepatic steatosis develops, exposed to varying concentrations of the hydrophobic bile acid GCDC; (2) characterize the cell-death pathways involved in this model; and (3) determine whether mitochondrial disturbances are important factors in this model. In these experiments we used freshly isolated rat hepatocytes in suspension, rather than cultured or hepatoma cell lines, so that normal kinetics of physiologic bile-acid uptake[15] and endogenous antioxidant pathways[16] were maintained to better reflect in vivo conditions and so that physiologic apoptotic pathways remained intact. Our findings demonstrated that isolated fat-laden rat hepatocytes show a markedly increased susceptibility to GCDC-induced cellular necrosis (with decreased apoptosis) compared with that seen in lean hepatocytes that is dependent on ROS generation and mitochondrial dysfunction.

METHODS

Materials. All chemicals were obtained from Sigma-Aldrich (St Louis, Mo) and were of analytical grade unless otherwise noted. BSA (fraction V) was obtained from Calbiochem (La Jolla, Calif), and CSA was purchased from Alexis Biochemicals (San Diego, Calif). Z-IETD-FMK (caspase-8 inhibitor) and Z-LEHD-FMK (caspase-9 inhibitor) were purchased from Enzyme Systems Products (Livermore, Calif). Idebenone was a gift from Takeda Industries (Osaka, Japan).

Animals. Homozygous obese (fa/fa) male Zucker rats and their corresponding lean (Fa/Fa or Fa/fa) male littermates were obtained at 6 to 7 weeks of age from Charles River Breeding Laboratories (Wilmington, Mass) and housed for 1 to 2 weeks in polyethylene cages and supplied Purina Lab Chow (Ralston-Purina Co, Chicago, Ill) and water provided ad libitum. All animals received humane care in compliance with the guidelines of the Laboratory Animal Care and Use Committee of the University of Colorado Health Sciences Center.

Isolation and treatment of rat hepatocytes. Freshly isolated rat hepatocytes were taken, after an overnight fast, from rats with the use of a recirculating collagenase–perfusion technique, described previously,[11] and then maintained in suspension for the 4-hour duration of the experiments. Rats were anesthetized with pentobarbitol (50 mg/kg), sacrificed by exsanguination, and blood was obtained for the determination of serum AST, ALT, total bilirubin, and AP concentrations with the use of an automated chemical analyzer. A small lobe of the liver, removed when each animal was killed, was processed for light microscopy in buffered formalin and for triglyceride content, which we determined by means of enzymatic measurement of glycerol using a commercially available kit (Sigma A-320; Sigma-Aldrich). Liver sections were stained with hematoxylin and eosin and trichrome, then subjected to histologic examination. Frozen liver sections were also stained for fat with the oil red O stain. Liver