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Original Contribution

Mitochondrial genome depletion in human liver cells abolishes bile acid-induced apoptosis: Role of the Akt/mTOR survival pathway and Bcl-2 family proteins

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ABSTRACT

Acute accumulation of bile acids in hepatocytes may cause cell death. However, during long-term exposure due to prolonged cholestasis, hepatocytes may develop a certain degree of chemoresistance to these compounds. Because mitochondrial adaptation to persistent oxidative stress may be involved in this process, here we have investigated the effects of complete mitochondrial genome depletion on the response to bile acid-induced hepatocellular injury. A subline (Rho) of human hepatoma SK-Hep-1 cells totally depleted of mitochondrial DNA (mtDNA) was obtained, and bile acid-induced concentration-dependent activation of apoptosis/necrosis and survival signaling pathways was studied. In the absence of changes in intracellular ATP content, Rho cells were highly resistant to bile acid-induced apoptosis and partially resistant to bile acid-induced necrosis. In Rho cells, both basal and bile acid-induced generation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide anion, was decreased. Bile acid-induced proapoptotic signals were also decreased, as evidenced by a reduction in the expression ratios Bax-α/Bcl-2, Bcl-xS/Bcl-2, and Bcl-xS/Bcl-xL. This was mainly due to a downregulation of Bax-α and Bcl-xS. Moreover, in these cells the Akt/mTOR pathway was constitutively activated in a ROS-independent manner and remained similarly activated in the presence of bile acid treatment. In contrast, ERK1/2 activation was constitutively reduced and was not activated by incubation with bile acids. In conclusion, these results suggest that impaired mitochondrial function associated with mtDNA alterations, which may occur in liver cells during prolonged cholestasis, may activate mechanisms of cell survival accounting for an enhanced resistance of hepatocytes to bile acid-induced apoptosis.

The accumulation of some molecular species of bile acids in the liver during cholestasis causes parenchymal cell death, leading to impaired hepatic function and—eventually—liver failure [1]. A central feature observed during chronic cholestasis is mitochondrial dysfunction [2], which is evidenced by a reduction in mitochondrial membrane potential, decreased activity of respiratory chain complexes, and subsequent impairment of ATP production [3,4]. Cholestasis-induced cell death may be due to necrosis or apoptosis. The latter is activated by either mitochondrial damage or the interaction of proapoptotic effectors with membrane death receptors [5]. Apoptosis is the predominant mechanism during acute cholestasis, whereas necrosis is the major form of hepatocyte death during chronic cholestasis. After bile duct ligation in the rat, biphasic changes in processes leading to hepatocyte death occur, which include: (i) a transient increase in caspase-3 activity during the first week, followed by (ii) a decrease in caspase-3 activity during the second week. This suggests that prolonged cholestasis induces adaptive changes in hepatocytes that may result in a certain resistance to bile acid-induced activation of apoptosis. Thus, if the chemical stress is high enough and the

Abbreviations: ABC, ATP-binding cassette; Akt, protein kinase B; BCRP, Breast cancer resistance protein; BSEP, Bile salt export pump; DCA, Deoxycholic acid; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; ERK, Extracellular regulated kinase; EtBr, Ethidium bromide; GCDCA, Glycochenodeoxycholic acid; GSH, glutathione; HE, Hydroethidine; LDH, Lactate dehydrogenase; MDR, Multidrug resistance protein; MMP, Mitochondrial membrane potential; MRP, Multidrug resistance-associated protein; mtDNA, Mitochondrial DNA; MT-ND1, Mitochondrially encoded NADH dehydrogenase subunit 1; mTOR, Mammalian target of rapamycin; Nox, NADPH oxidase; nDNA, Nuclear DNA; PTEN, Phosphatase and tensin homolog; Rh123, Rhodamine 123; ROS, Reactive oxygen species

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exposure is maintained, cell death is characterized by an increased proportion of necrotic cells [6,7]. Indeed, oncotic necrosis has been proposed as the predominant form of cell death in rodent models of long-term cholestasis [8].

Bile acid-induced hepatocellular injury has been associated with the ability of these steroids to induce ROS[1] generation [9]. However, ROS can have both pro- and antiapoptotic effects, depending on the type of stimulus as well as on the amount and duration of ROS production [10]. Thus, the toxic effects of bile acids can be modified by the rate of ROS generation; i.e., high ROS production may induce toxicity due to an irreversible loss of mitochondrial membrane potential and the release of cytochrome *c* into the cytosol. In contrast, lower ROS levels may play a protective role through the activation of antiapoptotic pathways such as those involving ERK1/2 and Akt [10]. Because mitochondria are a major site of ROS generation and are critical targets of bile acid-induced toxicity, these organelles have received much attention with a view to elucidating the mechanism underlying hepatotoxicity associated with cholestatic liver diseases and to exploring novel strategies for their treatment [5,11]. Mitochondrial function depends on proteins that are encoded by both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA), which contains the genes coding for 13 components of the respiratory chain [12]. MtDNA damage is tightly associated with mitochondria-related pathology, including cholestatic liver disease [13,14]. Thus, persistent mitochondrial oxidative stress caused by bile acid accumulation may lead to a decline in mitochondrial respiratory function, with a decrease in the number of mtDNA copies per mitochondrion [13,14].

Chemical stress-induced dysfunctional mitochondria may trigger retrograde signaling from these organelles to the nucleus, which causes changes in gene expression, such as the upregulation of proteins involved in diverse cellular processes, including the control of metabolism, nutrient sensing, stress response, drug sensitivity, life span, and the development of resistance to apoptosis [15].

In previous studies, using an in vitro model of partial mtDNA depletion in mouse liver cells, we have reported that retrograde regulation may be involved in the defense response of mouse liver cells against the chemical stress induced by toxic compounds such as paracetamol and bile acids [16]. Thus, in addition to the control mediated by direct activation of nuclear receptors, retrocontrol mechanisms permitting the cross talk between mitochondrial and nuclear genomes could constitute an important mechanism in the overall regulation of hepatocyte survival and function. The aim of this study was to investigate the effect of complete mtDNA depletion in human liver cells on the mechanisms accounting for the development of resistance to bile acid-induced apoptosis and necrosis. To carry out the chemical insult, glycochenodeoxycholic acid (GCDCA) and deoxycholic acid (DCA), two major bile acids in the human bile acid pool, were selected. These are primary (GCDCA) and secondary (DCA) bile acids with marked ability to induce oxidative stress and apoptosis in hepatocytes [5].

Materials and methods

Cell lines and culture conditions

The human hepatoma SK-Hep-1 cell line HTB-52 was obtained from the American Type Culture Collection (Manassas, VA, USA). SK-Hep-1 cells were cultured in Minimum Essential Medium Eagle (Sigma–Aldrich, Madrid, Spain), containing 10% fetal calf serum (TDI S.A., Madrid, Spain) and 1% antibiotic–antimycotic solution (Invitrogen, Barcelona, Spain) and supplements reported by the supplier, in a humidified atmosphere in 5% CO_2 at 37 °C. SK-Hep-1 Rho cells totally depleted of mtDNA were derived from wild-type (WT) SK-Hep-1 cells by culturing them in the presence of 100 ng/ml ethidium bromide (EtBr) for more than 20 generations [17]. To compensate for the impairment of respiratory metabolism and to support cell growth, the auxotrophy of Rho cells for uridine and pyruvate [18] required the culture medium to be supplemented with 100 μg/ml pyruvate and 50 μg/ml uridine. To carry out the experiments, the cells were plated and incubated for 24 h in the absence of any of the compounds tested and then treated with DCA (25 to 500 μM) or GCDCA (25 to 2000 μM) (Sigma–Aldrich) for 0.5 or 8 h. In some experiments, Akt phosphorylation was blocked using the selective phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (Sigma–Aldrich). For flow cytometry and gene expression studies, cells were detached with trypsin–EDTA solution and then pelleted by centrifugation at 250 g for 10 min and washed once with phosphate-buffered saline (PBS).

Determination of gene expression levels and mtDNA copy number

The depletion of mtDNA was confirmed by real-time PCR amplification using specific primers for the mitochondrion-encoded genes 16S rRNA and NADH dehydrogenase subunit 1 (MT-ND1). Total cellular DNA (nDNA and mtDNA) was extracted using the QIAamp DNA blood mini kit from Qiagen (Izasa, Barcelona, Spain). DNA was then quantified fluorimetrically with the PicoGreen DNA quantitation kit (Invitrogen). To determine mRNA levels by real-time RT-QPCR, total RNA was isolated from cell lysates using the Illustra RNAspin mini RNA isolation kit (GE Healthcare, Barcelona, Spain). RNA was then quantified fluorimetrically with the RiboGreen RNA quantitation kit (Invitrogen). The SuperScript VILO cDNA synthesis kit (Invitrogen) was used to synthesize cDNA from total RNA. Real-time QPCR was then performed using AmpliTaq Gold polymerase (Applied Biosystems, Madrid, Spain) in an ABI Prism 7300 sequence detection system (Applied Biosystems). The thermal cycling conditions were as follows: a single cycle at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and at 60 °C for 60 s. Detection of the amplification products was carried out using SYBR Green I or TaqMan probes (Applied Biosystems). As a calibrator, total RNA from human liver was used. The results of mRNA abundance for the target genes in each sample were normalized on the basis of human GAPDH mRNA or 18S rRNA abundance. The mtDNA copy number was measured from total DNA using the primer and TaqMan probe oligonucleotides listed in Supplementary Table S1. The TaqMan ribosomal RNA control reagents kit (Applied Biosystems) was used to measure DNA encoding 18S rRNA. The same sets of primers and probes were used to measure the absolute abundance of the rRNAs or mRNAs corresponding to these genes. Absolute quantification of mtDNA and RNAs was carried out using standard curves generated by plotting the threshold cycle (C_t) versus log10 of the copy number of cDNA fragments obtained by conventional PCR and quantified with the PicoGreen detection kit, as previously described in detail [19]. The primer oligonucleotide sequences and the conditions to carry out quantitative PCR of human MRP1, MRP2, MRP3, MRP4, MDR1, MDR3, BSEP, and BCRP have been described previously [20]. The primer oligonucleotide sequences to carry out quantitative PCR of human Akt1, Bax-α, Bcl-2, Bcl-xL, and Bcl-xS are listed in Supplementary Table S1. All primers (obtained from Sigma-Genosys, Madrid, Spain) were designed with the assistance of Primer Express software (Applied Biosystems), and their specificity was checked using BLAST.

Cell death, ROS generation, mitochondrial membrane potential (MMP), ATP, and glutathione (GSH) levels

Cell death was determined by flow cytometry (FACSort flow cytometer; BD Biosciences, San Jose, CA, USA) after staining with propidium iodide (Sigma–Aldrich). The amount of attached cells