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Original article

ER stress contributes to alpha-naphthyl isothiocyanate-induced liver injury with cholestasis in mice

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ABSTRACT

Endoplasmic reticulum (ER) stress is involved in the development of several liver diseases and tumors. This study investigated the underlying mechanisms of α-naphthyl isothiocyanate (ANIT)-induced liver injury with cholestasis in mice and found ER stress contributes to the injury. All animals were randomly divided into three groups. In the ANIT-intoxicated group, mice were intragastrically given 100 mg/kg ANIT (dissolved in corn oil), while the other groups received an equal volume of vehicle as control. After 24 and 48 h of ANIT administration, blood samples and liver tissues of all animals were collected for serum biochemistry and hepatic histopathological examinations to evaluate liver injuries with cholestasis. Hepatocellular apoptosis was assessed by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The expression of hepatic ER stress-related markers was determined by real-time PCR, immunohistochemical assay and Western blot. ANIT was found to significantly induce liver injury with cholestasis compared with control mice as evidenced by the increase of serum transaminases and total bilirubin (TBil), and histopathological changes in mice. ANIT remarkably induced hepatocellular apoptosis, upregulated the expression of caspase-9 and cytochrome c, and inhibited the gene and protein expression of proliferating cell nuclear antigen (PCNA). The gene expression of ER stress–related markers, including glucose-regulated protein 78 (GRP78), protein kinase R–like ER kinase (PERK), eukaryotic initiation factor 2α (eIF2α), inositol requiring enzyme-1α (IRE-1α) and activating transcription factor 6 (ATF6) was upregulated by ANIT in mice. ANIT also upregulated the protein expression of GRP78 and activated the phosphorylation of IRE1. These results suggested that ANIT induced liver injury with cholestasis partly due to its ability to activate the ER stress pathway.

1. Introduction

Cholestasis is a common clinical syndrome and is reflected in low bile flow from the hepatocytes to the duodenum, which is induced mainly by infections, drugs, and autoimmune, metabolic or genetic disorders [1,2]. Without appropriate treatments, cholestasis ultimately leads to hypercholesterolemia and jaundice, and later into aggravated outcomes including cholestatic hepatitis, hepatic fibrosis, cirrhosis or even liver failure [3,4]. Therefore, it is important to further study the pathogenesis of cholestasis, and look for new targets of cholestasis treatment, which will provide crucial clues for drug development.

Previous studies showed that cholestasis appeared in various dysfunctions, but current studies mainly concentrate on dysregulation of bile acid transporters, oxidative stress, and inflammation in hepatocytes, which directly induces the apoptosis of hepatocytes [5,6]. Sustained or massive endoplasmic reticulum (ER) stress leads to apoptosis. Several apoptosis mediators are implicated in ER stress–associated cell death in liver disease [7–9]. However, whether hepatic ER stress is involved in hepatocellular apoptosis induced by cholestasis is still unknown. The mechanisms of α-naphthyl isothiocyanate (ANIT)-induced liver injury with cholestasis have been proposed but have not been entirely clarified yet. Therefore, in-depth investigations on related mechanisms of cholestasis are needed.

Abbreviations: ANIT, α-naphthyl isothiocyanate; AST, aspartate aminotransferase; TBil, total bilirubin; ALT, alanine aminotransferase; ER, endoplasmic reticulum; PKR, protein kinase dependent on RNA; PERK, PKR-like ER kinase; GRP78, glucose-regulated protein 78; eIF2α, eukaryotic initiation factor 2α; ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; IRE-1α, inositol requiring enzyme-1α; XIAP, X-linked inhibitor of apoptosis; Cyt-c, cytochrome c; PCNA, proliferation cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PVDF, polyvinylidene difluoride; TBS, tris-buffered saline; TBST, Triton X-100-TBS.

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In the present study, cholestasis in the liver of mice was modeled by the administrating ANIT, a well-characterized cholestatic agent [10]. The study concluded that the induction of ER stress is one of the underlying mechanisms of ANIT-induced liver injury with cholestasis.

2. Materials and methods

2.1. Reagents

ANIT was purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TBil) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (China). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) was purchased from Wuhan Boster Biological Engineering Co., Ltd. (China). The PrimeScriptTM RT Reagent kit was purchased from TAKARA Bio Inc. (Japan). UltraSYBR Mixture was purchased from Beijing ComWin Biotech Co.,Ltd. (China). Trizol was purchased from BioDev Tech Co., Ltd. (China). BCA protein assay kit was obtained from TianGen Biotech (BeiJing) Co., Ltd. (China). Radioimmunoprecipitation (RIPA) buffer (P0013) was purchased from Beyotime Institute of Biotechnology (China). All primers were synthesized by Beijing AuGCT Biological Technology Co., Ltd. (China). Glucose-regulated protein 78 (GRP78), protein kinase R–like ER kinase (PERK), phospho-PERK, eukaryotic initiation factor 2α (eIF2α), phospho-eIF2α, inositol requiring enzyme-1α (IRE1α), phospho-IRE1α, cytochrome c (cyt c), caspase-9, proliferating cell nuclear antigen (PCNA) and β-actin antibodies were purchased from Abcam Co. (UK), Cell Signaling Technology (USA), R&D systems Inc. (USA) and Santa Cruz Biotechnology (USA). Other chemicals were purchased from the local market.

2.2. Animals

Male ICR mice weighing 22–24 g were obtained from Beijing Vital River Experimental Animal Co., Ltd. (China). The animal study protocol was in compliance with the guidelines of China for animal care, which conform to the internationally accepted principles in the care and use of experimental animals.

All animals were randomly divided into 3 groups with 10 mice in each group. In the ANIT-intoxicated group, mice were intragastrically given 100 mg/kg ANIT (dissolved in corn oil), while mice in the control group were given an equal volume of vehicle as control. After 24 and 48 h of ANIT administration, blood samples of all animals were collected by harvesting eyeball and liver tissues of all animals after 12-h food deprivation for further analysis. Liver tissues were rapidly dissected, and two pieces of tissues from the same lobe of liver from each animal were fixed properly in formaldehyde saline (10%) solution for one week in 4 °C for histopathological examinations. The rest of the liver tissues were snap frozen in liquid nitrogen for biochemical assays, RNA, and protein isolation.

2.3. Serum biochemistry

Blood samples for biochemical analysis were obtained after 24 and 48 h of ANIT administration. Serum ALT, AST and TBil levels were determined by biochemical analyzer (PUZS-300, Beijing Prolong New Technology Co., Ltd., China) using commercial assay kits according to the standard procedures.

2.4. Histopathology and TUNEL assay

Formalin-fixed liver samples from all mice were embedded in paraffin, and 5 μm-thick sections were cut and stained with hematoxylin and eosin (HE) for pathological morphological examination.

For the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, paraffin sections of liver were examined by an in situ cell apoptosis detection kit. The sections were treated with proteinase K for 15 min, rinsed with Tris-buffered saline (TBS; pH7.4, 0.01 mol/L) for 3 × 2 min, and then incubated for 2 h at 37 °C in terminal deoxynucleotidyl transferase and digoxigenin-11-dUTP labeling buffer in a humid atmosphere. The sections were rinsed for 2 min with TBS (pH 7.4, 0.01 mol/L) three times and then blocked in a blocking buffer for 30 min at room temperature. The sections were covered with biotinylated anti-digoxin antibodies (1:100 dilution) for 30 min at 37 °C and rinsed for 2 min with TBS (pH 7.4, 0.01 mol/L) three times. The sections were then stained with streptavidin–fluorescein isothiocyanate and rinsed for 5 min with TBS (pH 7.4, 0.01 mol/L) four times. At last, the sections were mounted in antifade solution and analyzed by confocal laser scanning microscopy.

2.5. RNA isolation and real-time polymerase chain reaction analysis

Total RNA was extracted from the liver tissue using a TRIzol reagent according to the manufacturer's protocol. Using the Prime-

Table 1
Polymerase chain reaction primer sets in real-time PCR.

Gene	Primer sequences	Size (bp)	GenBank™ accession no.
GRP78	Forward GTTTGCTGAGGAAGACAAAAAGCTC Reverse CACTTCCATAGAGTTTGCTGATAATTG	271	NM_001163434
CHOP	Forward CAGCGACAGAGCCAGAATAAC Reverse ACCGTCTCCAAGGTGAAAGG	147	NM_007837.4
IRE1α	Forward CGCATCACCAAGTGGAAGTA Reverse CCTTCCAGCAAAGGAAGAGT	170	NM_023913
ATF6	Forward TGATGGCTGTCCAGTACACA Reverse GCAGATGATCCCTTCGAAAT	151	NM_001081304
eIF2α	Forward GTTCAGATGGAGCCCAAAGT Reverse CTGCATCATCATCTCCATCC	108	NM_026114.3
PERK	Forward GACCTCAAGCCTTCCAACAT Reverse TTTCCATGAATCTGCTCTGG	188	NM_010121
Cyt-c	Forward ATCTCCACGGTCTGTTCGG Reverse GCCCTTTCTCCCTTCTTCTTA	183	XM_975140.1
Caspase-9	Forward CTAGTGAGCGAGCTGCAAGT Reverse CAGATCCTGCCTGCTGAATA	100	NM_015733.5
PCNA	Forward CGAAGCACCAAATCAAGAGA Reverse CGGCATATACGTGCAAATTC	138	NM_011045.2
β-actin	Forward CAGGCATTGCTGACAGGATG Reverse TGCTGATCCACATCTGCTGG	155	NM_007393