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Research paper

Glucose regulated protein 78 is potentially an important player in the development of nonalcoholic steatohepatitis

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ABSTRACT

Endoplasmic reticulum stress (ERS) plays an important role in metabolic diseases. Glucose regulated protein 78 (GRP78) is a molecular chaperone in the ER where it is a marker for ERS activation. This study investigates the role of GRP78 in the pathogenesis of nonalcoholic steatohepatitis (NASH) in rats. Our rat model of NASH was induced by both a high sucrose and a high fat diet. The expression levels of LPS, ALT, FFA, and TG in the serum and FFA, TG, MDA, and TNFα in the liver were assessed. H & E, TUNEL and IHC staining were performed to examine histological changes, apoptosis and macrophage infiltration in the NASH liver tissue, respectively. The expression level of GRP78 in the liver was evaluated by Western blot and RT-PCR. The plasma levels of LPS, ALT, FFA, and TG in and the contents of FFA, TG, TNFα, and MDA in the liver were gradually increased. Macrophage infiltration and hepatocytic apoptosis was significantly increased in the livers of the rats from the NASH group compared to the control group. The protein and mRNA levels of GRP78 in the liver of rats from the NASH group were also upregulated. In addition, GRP78 expression levels were positively correlated with the levels of ALT, TNFα, CD68 and hepatocytic apoptosis. Thus, our results suggest that GRP78 may be an important player in the pathogenesis of NASH.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by an excessive deposition of triglycerides in the liver, which can lead to chronic liver disease without history of excessive alcohol consumption. The NAFLD spectrum starts from fatty liver, progressing through nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, liver failure and finally leads to end-stage liver disease and liver cancer (Chalasani et al., 2012; Onyekwere et al., 2015; Liu et al., 2016). NASH is a critical intermediate stage in the NAFLD spectrum and the pathogenesis of NASH may follow the classic "two-hit theory" (Day and James, 1998) in which the first hit refers to an accumulation of fatty acids and triglycerides within the liver that sensitizes it to additional proinflammatory insults and the second hit is chronic stress, such as enhanced lipid peroxidation (Sanyal et al., 2001) and endoplasmic reticulum stress (ERS) (Zhang et al., 2014b). Induction of ERS can enable liver cells to mount a resistant and adaptive response to prevent damage using the unfolded protein response pathways. However, if the ERS is sustained and serious, it will induce apoptosis, inflammation and hepatocyte injury (Hotamisligil, 2010; Gentile et al., 2011). Glucose regulated protein 78 (GRP78) is known to be a marker for ERS (Ji et al., 2011). GRP78 upregulation has been observed in various diseases, such as metabolic syndrome, alcoholic liver disease and CCL4-induced liver cirrhosis (Cheng, 2008; Sage et al., 2012; Galligan et al., 2014; Zhang et al., 2014a). Clinical observations and experimental studies show that patients with NASH often concomitantly present with intestinal endotoxemia (IETM) (Brun et al., 2007; Harte et al., 2010), which is interesting as endotoxin is a potential inducer of ERS (Hiramatsu et al., 2006).

In this study, we have successfully established an animal model of NASH, which was induced by high fat and high sucrose (Zhou et al., 2014). The GRP78 expression at both RNA transcription and protein levels were examined in the development of NASH in this murine model. Based on our results, we propose that GRP78 may play an important role in the pathogenesis of NASH. Additionally, our study attempts to establish a relationship between GRP78 expression and IETM.

Abbreviations: GRP78, glucose regulated protein 78; NASH, nonalcoholic steatohepatitis; ERS, endoplasmic reticulum stress; IETM, intestinal endotoxemia; ALT, alanine transferase; TG, triglyceride; FFA, free fatty acid; TNFα, tumor necrosis Factor-α; MDA, malondialdehyde

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2. Material and methods

2.1. Animal model

30 SD male rats, weighing 220–250 g, were obtained from the Experimental Animal Center of Shanxi Medical University. Experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Shanxi Medical University. After a week of adaptive feeding, the rats were randomly divided into two groups: the normal control group and the experimental group which was treated with sucrose and fat-enriched diet (70% normal fodder + 20% lard + 10% sucrose + 1% cholesterol + 0.25% Cholic acid) for 12 weeks. Blood and liver tissue was collected at the end of the 4th, 8th, and 12th weeks.

2.2. Cell culture and treatments

HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Pen/Strep (100 u/l) and incubated at 37 °C. To identify the ER stress signaling pathways related to GRP78 upregulation, HepG2 cells were treated with 2 mM 4-phenylbutyric acid (PBA) when treated with either 33 mM glucose or 200 μM Palmitic acid or with both. To establish the relationship between GRP78 upregulation and the endpoint readouts (FFA, ALT, TNFα and apoptosis), the cells were treated with PBA in the presence of thapsigargin (TG). The serum levels of FFA, ALT, TNFα and the liver expression levels of C/EBP homologous proteins (CHOP) were assayed.

2.3. Determination of biochemical and inflammatory indicators

The levels of ALT (Alanine transferase kit, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), TG (Triglyceride kit, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), FFAs (Free fatty acid kit, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and TNFα (Tumor necrosis Factor-α, TNF-α radioimmunoassay kit, Radioimmunity Institute of PLA General Hospital, Beijing, China) in the plasma and TG, FFA and MDA (Malondialdehyde kit, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in the liver were measured according to the manufacturer's instructions.

2.4. Measurement of serum endotoxin levels in the abdomen aorta

Blood samples were collected from abdominal aorta of the rats which were anaesthetized with pentobarbital sodium at the concentration of 0.3 ml/100 g. The endotoxin level in the plasma was determined by using the Limulus kit (Clinical Sciences, Xiamen) according to the manufacturer's instructions (UV-2102C, Shanghai).

2.5. Histological sectioning of the liver

Liver samples were harvested and then fixed in 10% formalin, embedded in paraffin, cut into 4 μm-thick sections, and stained with hematoxylin and eosin ((HE); Hematoxylin and eosin staining kit, Junruishengwu Technology Corporation, Shanghai, China). The sections were examined under a light microscope (Olympus BX51 microscope) to evaluate the inflammation, steatosis and fibrosis in the liver.

2.6. Immunohistochemistry

Paraffin-embedded sections were stained with CD68 specific antibody (1:100 dilution; Zhong Shan-Golden Bridge Biological Technology Co., Beijing, China) to examine the expression level of CD68 and quantified using a computerized image analysis system (IPP6.0 software, Media Cybernetics Inc., USA).

2.7. TUNEL assay

Paraffin sections were dewaxed and washed in 0.01 M PBS (pH 7.0) twice for 5 min, flooded with 3% hydrogen peroxide for 20 min, washed with PBS twice for 5 min and then dried. 50 μl of reaction solution (TUNEL kit, Roche Diagnostics GmbH, Germany) was added to each sample and incubated at 37 °C for 60 min in dark humid conditions. The samples were then washed twice for 5 min in PBS. 50 μl of Converter-POD (TUNEL kits, Roche Diagnostics GmbH, Germany) was added to each sample and incubated at 37 °C for 30 min, washed twice in PBS and stained with DAB Horseradish Peroxidase (DAB Horseradish Peroxidase Color Development Kit, Beyotime Institute of Biotechnology, Shanghai, China). The samples were subsequently stained with hematoxylin, conventionally dehydrated and then mounted with glycerol. The specimens were analyzed by using a computerized image analysis system (IPP6.0 software, Media Cybernetics Inc., USA) and the resultant data were expressed as the number of TUNEL-positive cells per field (400 ×).

2.8. Reverse transcriptase polymerase chain reaction (RT-PCR)

The total RNA was extracted from liver and the RT-PCR was performed using the following primers: GRP78 forward (5′-TAATCAGCCCACCGTAACAATC-3′); GRP78 reverse (5′-ACCTCCCAGC TTCTCTTTATCT-3′); GAPDH forward (5′-ACCACAGTCCATGCCATCAC-3′); GAPDH reverse (5′-TCCACCACCCTGTTGCTGTA-3′). The expected band sizes were 385 bp and 450 bp, respectively. The PCR reaction was comprised of 2 μl of cDNA, 10 μl of MixTaq, 1 μl of primer for each, and ddH_2O was added to a total volume of 20 μl. GAPDH was used as an internal control. PCR protocol: 94 °C for 2 min, 94 °C for 30 s, 56 °C for 30 s, 72 °C for 40 s for 30 cycles, 72 °C for 5 min. PCR products were electrophoresed in 2% of agarose gel and analyzed by Quantity One gel analysis system (Bio-Rad Corporation). The relative transcript level of GRP78 was normalized by GAPDH.

2.9. Western blot

Total protein was extracted from frozen cerebral cortex homogenate by using PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM EDTA, 50 mM NaF and 2 mM Na_3VPO_4 (NaF and Na_3VPO_4 were only used for phosphorylated proteins). After 30 min of incubation on ice, the lysate was centrifuged at 15,000 rpm for 10 min. 40 μg of total protein was loaded for each sample into the lane and separated on a 7.5% SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% (w/v) non-fat dried milk for 1 h at room temperature. After 3 TBST washes, the membrane was incubated with GRP78 antibody (dilution 1:1000, Cell Signaling Technology Inc. Danvers, USA) or other antibodies overnight at 4 °C. The membranes were then incubated with the peroxidase-conjugated goat anti-rabbit secondary antibody (dilutions 1:2000, Zhong Shan-Golden Bridge Biological Technology CO. Beijing, China) and then developed with enhanced chemiluminescence method. The intensity of the target band was analyzed by Quantity One (Bio-Rad Laboratories, Inc. Hercules, USA).

2.10. Statistical analysis

SPSS13.0 software (Statistical Product and Service Solutions, USA) was used to perform statistical analyses. To assess the mean comparison of multiple samples, the pairwise comparison and the correlation analysis of the data were analyzed by one-way analysis of variance (ANOVA), LSD-*t*-test and the linear correlation method. All values were expressed as means ± standard error (SE). $p < 0.05$ was considered as statistically significant.