Original Article

Increased expression of 78 kD glucose-regulated protein promotes cardiomyocyte apoptosis in a rat model of liver cirrhosis

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Abstract: Aims: This study was to investigate the role and underlying mechanism of 78 kD glucose-regulated protein (GRP78) in cardiomyocyte apoptosis in a rat model of liver cirrhosis. Methods: A rat model of liver cirrhosis was established with multiple pathogenic factors. A total of 42 male SD rats were randomly divided into the liver cirrhosis group and control group. Cardiac structure analysis was performed to assess alterations in cardiac structure. Cardiomyocytes apoptosis was detected by TdT-mediated dUTP nick end labeling method. Expression of GRP78, CCAAT/enhancer-binding protein homologous protein (CHOP), caspase-12, nuclear factor kappa-light-chain-enhancer of activated B cells p65 subunit (NF-κB p65) and B cell lymphoma-2 (Bcl-2) was detected by immunohistochemical staining. Results: The ratios of left ventricular wall thickness to heart weight and heart weight to body weight were significantly increased with the progression of liver cirrhosis (P < 0.05). Apoptosis index of cardiomyocytes was significantly increased with the progression of liver cirrhosis (P < 0.05). The expression levels of GRP78, CHOP and caspase-12 were significantly increased in the progression of liver cirrhosis (P < 0.05). The expression levels of NF-κB p65 and Bcl-2 were highest in the 4-wk liver cirrhosis, and they were decreased in the 6-wk and 8-wk in the progression of liver cirrhosis. GRP78 expression levels were positively correlated with apoptosis index, CHOP and caspase-12 expression levels (P < 0.05). CHOP expression levels were negatively correlated with NF-κB p65 and Bcl-2 expression levels (P < 0.05). Conclusion: Increased expression of GRP78 promotes cardiomyocyte apoptosis in rats with cirrhotic cardiomyopathy.

Keywords: Endotoxin, 78 kD glucose-regulated protein, cardiomyocytes apoptosis, endoplasmic reticulum stress, cirrhotic cardiomyopathy

Introduction

Endoplasmic reticulum has multiple cellular functions, including protein folding and the transport of synthesized proteins to the Golgi apparatus. In case of endoplasmic reticulum stress (ER stress), the unfolded protein response cascade is activated to reduce unfolded proteins and restore the normal function of endoplasmic reticulum. However, excessive and prolonged ER stress triggers apoptosis to eliminate dysfunctional cells [1]. Apoptosis induced by ER stress is an important pathogenic factor in a number of widespread diseases, including diabetes, neurodegenerative diseases, atherosclerosis, and renal disease [2-4].

78 kD glucose-regulated protein (GRP78) is a molecular chaperone involved in ER stress. Previous study showed that the expression of GRP78 plays an important role in the alteration of myocardium and decreased numbers of cardiomyocytes in a rat model of liver cirrhosis [5, 6]. Cardiomyocytes are terminally differentiated cells and the basic unit of myocardial tissues. Heart dysfunction, such as heart failure, may be resulted from the reduction in cardiomyo-
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cyte number [7]. However, the pathogenetic mechanism of cardiomyocyte apoptosis in cirrhotic cardiomyopathy and whether GRP78 plays a role in cardiomyocyte apoptosis has not been fully elucidated in recent studies.

Caspase-12 is an important factor involved in apoptosis. Caspase-12 forms a complex with GRP78 in an inactive state, which locates in endoplasmic reticulum membrane. When excessive stress occurs such as ER stress, the complex dissociates and generates activated caspase-12 [8]. Activated caspase-12 will activate caspase-9 and caspase-3, which leads to apoptosis [9].

CCAAT/enhancer-binding protein homologous protein (CHOP) is another important signaling molecule of ER stress, which play an important role in promoting apoptosis through the regulation of B cell lymphoma-2 (Bcl-2) [10, 11]. In the occurrence of ER stress, CHOP protein can directly inhibit the expression of Bcl-2 and thus weaken its anti-apoptotic function [11]. Early ER stress can activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [12], which positively regulates Bcl-2 protein, thereby activating cell pro-survival pathways [13, 14]. In this study, cardiac alterations in a rat model of liver cirrhosis were assessed. The effect of GRP78 on the cardiomyocytes apoptosis in the development of cirrhotic cardiomyopathy was investigated.

**Methods and materials**

**Reagents**

In situ apoptosis detection kit for TdT-mediated dUTP nick end labeling was purchased from KeyGEN Biotech Co. Ltd. (Nanjing, China). Rabbit anti-rat GRP78 polyclonal antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-rat CHOP polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-rat NF-kB p65 subunit (NF-kB p65) polyclonal antibodies, rabbit anti-rat caspase-12 polyclonal antibodies, rabbit anti-rat Bcl-2 polyclonal antibodies and Histostain-Plus Kit was purchased from Beijing Biosynthesis Biotechnology Co. Ltd (Beijing, China). Biotined goat anti rabbit IgG antibodies were purchased from Abcam (Cambridge, MA, USA).

**Establishment of liver cirrhosis model and grouping**

A total of 42 male SD rats, 200 g to 240 g, were provided by the Experimental Animal Center of Shanxi Medical School (Taiyuan, Shanxi, China). Experimental animals were randomly divided into the liver cirrhosis group (n = 24) and control group (n = 18). Animals in the liver cirrhosis group were fed with a mixture of maize flour, lard, cholesterol, and alcohol plus subcutaneously injection with CCl₄ oil solution for 8 weeks. The CCl₄ oil solution (400 g/l) was injected at 5 ml/kg body weight at the first day of experiment and at 3 ml/kg body weight from the third day on at an interval of three days. Lard was used only in the first two weeks accounting for 20% of the feed. Cholesterol was supplied at 0.5% of feed for the whole experiment. Alcohol was used in the drinking water exclusively (300 ml/l) during the whole experiment [15]. The animals of the control group had free access to the standard food and water. Myocardial tissues were taken at the end of 4-wk, 6-wk and 8-wk. At each time point, 8 rats of the liver cirrhosis group and 6 rats of the control group were used. All animal experiments were conducted according to the ethical guidelines of Changzhi Medical College.

**Cardiac structure analysis**

For the rats in the liver cirrhosis group and the control group, the hearts of these rats were removed after anesthesia and weighted at the end of 4-wk, 6-wk and 8-wk. The hearts were transversely cut at 1/3 from the aortic arch to the apical. The thickness of left ventricular wall (maximum distance between endocardial and epicardial) was measured by HPIAS-2000 microscope camera system (Chengdu Taimeng Tech. Co., Chengdu, China). The ratio of left ventricular wall thickness to heart weight (mm/mg) and heart weight to body weight (mg/g) were statistically analyzed, respectively.

**Cardiomyocytes apoptosis assay**

Cardiomyocytes apoptosis was detected by TdT-mediated dUTP nick end labeling method according to the instructions of the in situ apoptosis detection kit.

Ten fields at high-magnification (×400) were randomly taken from each section. Apoptosis
index referred to the ratio of the apoptotic cell number relative to the total myocardial cell number. At least 50 cells were counted.

**Immunohistochemical staining**

After being fixed in formaldehyde and embedded in paraffin, the tissues were cut into 4 μm sections. Then the sections were dewaxed and rehydrated in graded alcohols. To inactivate endogenous peroxidase, 3% fresh prepared hydrogen peroxide was added and incubated at room temperature for 15 min. After antigen retrieval, primary antibodies (rabbit anti-rat GRP78 polyclonal antibodies, rabbit anti-rat CHOP polyclonal antibodies, rabbit anti-rat NF-κB p65 polyclonal antibodies, rabbit anti-rats caspase-12 polyclonal antibodies or rabbit anti-rats Bcl-2 polyclonal antibodies) were added and incubated in dark overnight. After washing by PBS, biotined goat anti rabbit IgG antibodies were added and incubated for 20 min at room temperature. The sections were developed with DAB chromogenic reagent. Finally, sections were counterstained with hae-matoxylin. After hydrochloric acid differentiation and dimethylbenzene transparency, sections were mounted with neutral gum. Cells were observed under a microscope (Olympus BX51, Olympus, Tokyo, Japan).

Cells with brown staining were defined as GRP78-positive, CHOP-positive, NF-κB p65-positive, caspase-12-positive or Bcl-2-positive. These proteins were mainly expressed in the cytoplasm. Ten fields at high-magnification (× 400) were randomly taken and positive cells were counted. Positive rate referred to the ratio of the positive cell number relative to the total cell number. At least 50 cells were counted.

### Table 1. Ratios of left ventricular wall thickness to heart weight and ratios of heart weight to body weight (mean ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ratio of left ventricular wall thickness to heart weight (mm/mg)</th>
<th>Ratio of heart weight to body weight (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 18)</td>
<td>Liver cirrhosis (n = 24)</td>
</tr>
<tr>
<td>4-wk</td>
<td>2.817 ± 0.423</td>
<td>2.860 ± 0.27</td>
</tr>
<tr>
<td>6-wk</td>
<td>3.020 ± 1.431</td>
<td>3.526 ± 0.745</td>
</tr>
<tr>
<td>8-wk</td>
<td>3.289 ± 0.912</td>
<td>4.487 ± 1.325*</td>
</tr>
</tbody>
</table>

*Note:* "P < 0.05 vs. control group.

### Results

**The ratio of left ventricular wall thickness to heart weight and heart weight to body weight are increased in the progression of liver cirrhosis**

To determine cardiac alterations in a rat model of liver cirrhosis, cardiac structure analysis was performed. Left ventricular wall thickness and heart weight of rats in the liver cirrhosis group and the control group were measured at 4-wk, 6-wk, and 8-wk. As shown in Table 1, there were no significant differences in the ratio of left ventricular wall thickness to heart weight between the 4-wk liver cirrhosis group and the 4-wk control group (P > 0.05). The ratio of left ventricular wall thickness to heart weight in the liver cirrhosis group was increased in the progression of liver cirrhosis. The ratio in the 8-wk liver cirrhosis group (4.487 ± 1.325 mm/mg) was significantly increased when compared with that in the 8-wk control group (3.289 ± 0.912 mm/mg) (P < 0.05). The ratio of heart weight to body weight was similar to the ratio of left ventricular wall thickness to heart weight. The ratio of heart weight to body weight in the 8-wk liver cirrhosis group (3.777 ± 0.302 mg/g) was significantly increased when compared with that in the 8-wk control group (3.029 ± 0.641 mg/g) (P < 0.05). These results indicate that the ratio of left ventricular wall thickness to heart weight and heart weight to body weight

### Statistical analysis

All results were expressed as mean ± standard deviation. All statistical analyses were performed with SPSS version 10.0 for Windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used to determine whether there are any significant differences between groups. Paired t-test was used to analyze comparisons between groups and analysis of paired data. Pearson correlation coefficient was used to analyze the association between the expression levels of two proteins. P value less than 0.05 was considered to be significantly different.
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<table>
<thead>
<tr>
<th>Control</th>
<th>4-wk</th>
<th>6-wk</th>
<th>8-wk</th>
</tr>
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</table>

Figure 1. Numbers of apoptotic cardiomyocytes in the liver cirrhosis group and the control group. Cardiomyocyte apoptosis assay was performed to measure the number of apoptotic cardiomyocytes by TdT-mediated dUTP nick end labeling method between the liver cirrhosis group and the control group. Cell nucleus with brown staining indicated apoptosis of cardiomyocytes. Apoptotic cardiomyocytes in the control group, the 4-wk liver cirrhosis group, the 6-wk liver cirrhosis group, and the 8-wk liver cirrhosis group was visualized by an optical microscope at high magnification (× 400).

Figure 2. Expression of GRP78 in myocardial tissues of the liver cirrhosis group and the control group. Immunohistochemical staining was performed to detect GRP78 expression in myocardial tissues. Cells with brown staining indicated GRP78-positive. The expression of GRP78 in cardiomyocytes of the control group, the 4-wk liver cirrhosis group, the 6-wk liver cirrhosis group, and the 8-wk liver cirrhosis group was visualized by an optical microscope at high magnification (× 400).

are increased in the progression of liver cirrhosis.

The number of apoptotic cardiomyocytes is increased in the progression of liver cirrhosis.

To investigate the effect of liver cirrhosis on cardiomyocytes, cardiomyocytes apoptosis assay was performed in the liver cirrhosis group and the control group. Cell nucleus with brown staining indicated apoptosis of cardiomyocytes. Representative staining results of control group at week 4 and liver cirrhosis group at week 4, week 6, and week 8 were shown.

As shown in Figure 1, the number of cardiomyocytes apoptotic cells was increased in the 4-wk, 6-wk, and 8-wk liver cirrhosis group when compared with that in the control group. The number of apoptotic cells could be presented in the form of apoptosis index. The apoptosis index in the control group was $3.12 \pm 0.86\%$ at week 4, $3.74 \pm 1.26\%$ at week 6 and $3.21 \pm 0.69\%$ at week 8. In the 4-wk, 6-wk, and 8-wk liver cirrhosis group, the apoptosis index was $5.82 \pm 1.27\%$, $7.96 \pm 0.94\%$, and $16.52 \pm 1.46\%$, respectively. The apoptosis index was increased in the progression of liver cirrhosis. The apoptosis index in the 8-wk liver cirrhosis group was significantly increased when compared with that in the control group ($P < 0.05$), in the 4-wk liver cirrhosis group ($P < 0.05$), and in the 6-wk liver cirrhosis group ($P < 0.05$). These results...
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The number of apoptotic cardiomyocytes is increased in the progression of liver cirrhosis. Expression of GRP78 is increased in the progression of liver cirrhosis.

To investigate the relationship between GRP78 expression and cardiac alterations induced by liver cirrhosis, expression of GRP78 was detected by immunohistochemical staining in myocardial tissues. Cells with brown staining indicated GRP78-positive. As shown in Figure 2, in the control group (week 4), a small amount of light brown granules evenly distributed in the cytoplasm of cardiomyocytes. However, visible dense dark brown granules in the cytoplasm of cardiomyocytes were increased in the 4-wk, 6-wk, and 8-wk liver cirrhosis group. The positive rate of GRP78 expression in the control group was 0.08 ± 0.06% at week 4, 1.02 ± 0.14% at week 6 and 1.0 ± 0.07% at week 8. The positive rate was significantly increased in the progression of liver cirrhosis. The positive rate of GRP78 expression in the 8-wk liver cirrhosis group (1.48 ± 0.21%) was significantly increased when compared with that in the control group (P < 0.05), in the 4-wk liver cirrhosis group (0.29 ± 0.09%) (P < 0.05), and in the 6-wk liver cirrhosis group (0.75 ± 0.20%) (P < 0.05). These results indicate that the expression of GRP78 is increased in the progression of liver cirrhosis.

Figure 3. Expression of CHOP, caspase-12, NF-κB p65, and Bcl-2 in myocardial tissues in the liver cirrhosis group and the control group. Immunohistochemical staining was performed to detect the expression of CHOP, caspase-12, NF-κB p65, and Bcl-2 in myocardial tissues. Cells with brown staining indicated CHOP-positive, NF-κB p65-positive, caspase-12-positive, or Bcl-2-positive. The expression of CHOP (A), caspase-12 (B), NF-κB p65 (C), and Bcl-2 (D) in cardiomyocytes of the 4-wk control group, the 4-wk liver cirrhosis group, the 6-wk liver cirrhosis group, and the 8-wk liver cirrhosis group was visualized by an optical microscope at high magnification (× 400).
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Expression of apoptosis-related proteins is related to the progression of liver cirrhosis

To investigate the expression of apoptosis-related protein in myocardial tissues between the liver cirrhosis group and the control group, immunohistochemical staining of CHOP, caspase-12, NF-κB p65, and Bcl-2 expression was performed. CHOP and caspase-12 were expressed in the cytoplasm, and the staining intensities increased in the progression of liver cirrhosis (Figure 3A and 3B). As shown in Table 2, expression of CHOP and caspase-12 were increased in the liver cirrhosis group, which exhibited an upward trend in the progression of liver cirrhosis. Expression of CHOP and caspase-12 was significantly increased in the 8-wk liver cirrhosis group when compared with that in the control group (P < 0.05), in the 4-wk liver cirrhosis group (P < 0.05), and in the 6-wk liver cirrhosis group (P < 0.05). In contrast, the staining intensities of Bcl-2 and NF-κB p65 were increased in the 4-wk liver cirrhosis group. Then staining intensities of Bcl-2 and NF-κB p65 were decreased in the progression of liver cirrhosis (Figure 3C and 3D). As shown in Table 2, expression of NF-κB p65 and Bcl-2 in the 4-wk liver cirrhosis group was highest when compared with that in the control group (P < 0.05), in the 6-wk liver cirrhosis group (P < 0.05), and in the 8-wk liver cirrhosis group (P < 0.05). NF-κB p65 and Bcl-2 expression exhibited a significant down-regulation in the progression of liver cirrhosis. NF-κB p65 expression in the 8-wk liver cirrhosis group was higher than that in the control group (P < 0.05). However, Bcl-2 expression in the 8-wk liver cirrhosis group was low than that in the control group (P < 0.05).

Expression of GRP78 is positively correlated to pro-apoptosis proteins and negatively correlated to anti-apoptosis proteins

To investigate the possible mechanism through which GRP78 may promote apoptosis, the relationship between the expression of GRP78 and the apoptosis-related proteins was analyzed. Correlation analysis indicated that the expression levels of GRP78 were positively correlated with the apoptosis index (r = 0.769, P < 0.05), the expression levels of CHOP (r = 0.613, P < 0.05) and caspase-12 (r = 0.704, P < 0.05), respectively. These results indicate that GRP78 expression is positively correlated to the apoptosis and pro-apoptosis proteins.

Discussion

Cardiac remodeling is alterations in cardiac structure caused by self-repair after myocardial injury, which may be effected by tumor necrosis factor-alpha through cardiomyocytes apoptosis [16]. Cardiac alteration in the patients with cirrhotic cardiomyopathy typically occur in the left
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Caspase-12 is an important apoptotic signaling molecule during ER stress induced by apoptosis. The expression levels of caspase-12 were increased in the progression of liver cirrhosis, which is positively correlated to the expression levels of GRP78. These results indicate that activated caspase-12 play an important role of cardiomyocytes apoptosis in the rats with cirrhotic cardiomyopathy.

CHOP is an importance factor of ER stress in apoptotic signaling pathway. Excessive and prolonged ER stress triggers apoptosis through CHOP protein [10]. In this study, we found that CHOP was weakly expressed in normal myocardial tissues. The expression levels of CHOP were increased in the progression of liver cirrhosis, and highest in rats with liver cirrhosis at 8-wk. The trend of CHOP expression was corresponded with that of GRP78 expression. CHOP expression was positively correlated with GRP78 expression. These results indicate that in the apoptotic pathway induced by ER stress, expression of CHOP promotes the apoptosis of cardiomyocytes.

Apoptosis index of cardiomyocytes in rats with liver cirrhosis was increased in the progression of liver cirrhosis, which indicates that the decrease of cardiomyocytes is mainly attributed to the apoptosis. In addition, alteration in cardiac structure and function is affected by cardiomyocytes apoptosis during the cirrhotic cardiomyopathy.

Transcription factor NF-κB positively regulates Bcl-2 protein, which plays an important role in apoptosis induced by ER stress [13, 14]. In this study, we found that the expression levels of NF-κB p65 and Bcl-2 is negatively correlated to CHOP in the 8-wk liver cirrhosis group. These results indicate the activation of NF-κB may play a protective role to the cardiomyocytes at early stage of liver cirrhosis. With the increased expression of CHOP, the anti-apoptotic role of NF-κB is weakened. The cell pro-survival pathways may be inhibited through the expression of GRP78 and CHOP in the cardiomyocytes apoptosis induced by the excessive and prolonged ER stress.

Acknowledgements

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Disclosure of conflict of interest

None.

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