Expression of 78 kD glucose-regulated protein is increased in cirrhotic cardiomyopathy induced by intestinal endotoxin

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Abstract: Aims: This study is to investigate the role of 78 kD glucose-regulated protein (GRP78) in cardiomyopathy induced by intestinal endotoxin in a rat model of liver cirrhosis. Methods: The liver cirrhosis model was established by challenging rats with carbon tetrachloride, lard and alcohol. A total of 51 male SD rats were randomly divided into liver cirrhosis group (n = 33) and control group (n = 18). Measurements were conducted at week 4, week 6 and week 8 of the liver cirrhosis modeling. At each time point, 11 rats of the liver cirrhosis group and 6 rats of the control group were used. The cardiac functions were determined at week 8. Levels of tumor necrosis factor-α (TNF-α) and malondialdehyde (MDA) were detected by radioimmunoassay and thiobarbituric acid assay, respectively. The numbers of cardiomyocytes were detected by toluidine blue staining. The contents of collagen were determined by Van Giesan staining. Expression of GRP78 and hypoxia-inducible factor-1α (HIF-1α) was detected by immunohistochemistry. Results: The systolic and diastolic function of the heart was significantly decreased in the liver cirrhosis group at week 8 compared with that of the control group (P < 0.05). The levels of TNF-α, MDA, collagen, GRP78, and HIF-1α expression in myocardial tissues were significantly increased in the liver cirrhosis group compared with those in the control group (P < 0.05). The numbers of cardiomyocytes were decreased in the progression of liver cirrhosis (P < 0.05). The plasma endotoxin levels were positively correlated with MDA and GRP78 expression levels in myocardial tissues (P < 0.05). The expression levels of GRP78 were positively correlated with levels of homocysteine, alanine aminotransferase, MDA, and HIF-1α expression (P < 0.05). Conclusion: Expression of 78 kD glucose-regulated protein is increased during myocardial remodeling induced by intestinal endotoxin in the rat model of liver cirrhosis.

Keywords: Liver cirrhosis, endotoxin, 78 kD glucose-regulated protein, myocardial remodeling

Introduction

The 78 kD glucose-regulated protein (GRP78) is a chaperone molecule that is located in the endoplasmic reticulum [1]. In the event of endoplasmic reticulum stress (ER stress), GRP78 plays an important role in the unfolded protein response, reestablishing the balance of endoplasmic reticulum functions, and attenuating the ER stress-induced damages to cells [2]. However, when the sustained and serious ER stress occurs, GRP78 may lead to apoptosis and tissue damages through induction and activation of caspase-12, CHOP, and other pro-apoptotic factors [3] (Xu et al.).
Role of GRP78 in cirrhotic cardiomyopathy

Occurrence and development of various complications of liver cirrhosis [9]. Cirrhotic cardiomyopathy is likely to be induced by intestinal endotoxemia. Cardiomyocytes contain abundant sarcoplasmic reticulum/ER system, in which ER stress can be caused by endotoxin [10, 11]. ER contains a large amount of lipids, which is susceptible to peroxidation. Peroxidized lipid further stimulates ER stress and leads to serious damages of cellular functions [12].

In patients with impaired hepatic functions, methionine metabolism is affected, which results in occurrence of hyperhomocysteine. Excessive alcohol consumption can induce liver fat accumulation, liver damages and hyperhomocysteine. The ER stress response genes are also highly expressed, suggesting that ER stress can be induced by high levels of homocysteine [13]. Malondialdehyde (MDA) is the intermediate product of lipid peroxidation, which can reflect the degree of lipid peroxidation and cell damages.

Liver disease is often complicated by hepatopulmonary syndrome at an earlier time. Hypoxia is an important pathological change of the hepatopulmonary syndrome. Hypoxia-inducible factor-1α (HIF-1α) is an important regulatory factor to maintenance of cellular and systemic oxygen homeostasis [14-16]. Previous studies suggested that ER stress played an important role in liver cirrhosis and hepatopulmonary syndrome induced by multiple pathogenic factors. The expression levels of GRP78 were significantly increased in the occurrence of ER stress [10, 17]. The evidence suggests that GRP78 may play a role in cirrhotic cardiomyopathy induced by intestinal endotoxin. In this study, we investigated the effect of GRP78 on cirrhotic cardiomyopathy induced by intestinal endotoxin.

Materials and methods

Reagents

Rabbit anti-rat GRP78 polyclonal antibodies, rabbit anti-HIF-1α polyclonal antibodies and Histostain-Plus Kit (HRP, Broad Spectrum) were purchased from Beijing Biosynthesis Biotechnology Co. Ltd (Beijing, China). Alanine aminotransferase activity assay kit and thiobarbituric acid assay kit for MDA were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Limulus Amebocyte Lysate Endotoxin Detection kit was purchased from Xiamen LAL Experimental Factory Co., Ltd (Xiamen, Fujian, China). The radioimmunoassay kit for tumor necrosis factor-α (TNF-α) was purchased from PurevalleyBiotech (Beijing, China). CCl₄ (analytical pure) was purchased from Fuyu Fine Chemical Co., Ltd (Tianjin, China).

Establishment of the liver cirrhosis model

A total of 51 male SD rats, weighed 200 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 = 33) and the control group (n = 18). Liver cirrhosis was established in rat as previously described [18]. Briefly, animals in the liver cirrhosis group were fed with a mixture of maize flour, lard, cholesterol, and alcohol plus subcutaneously injection of 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 fourth 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 appended at 0.5% of the feed for the whole experiment. Alcohol was used in the drinking water exclusively (300 ml/l). For control, the animals of the control group had free access to the standard food and water. At week 8 of liver cirrhosis modeling, the cardiac functions were measured. At week 4, week 6 and week 8 of liver cirrhosis modeling, rats were sacrificed by deep anaesthesia and heart tissues were collected for further analysis. At each time point, 11 rats of the liver cirrhosis group and 6 rats of the control group were sacrificed. All animal experiments were conducted according to the ethical guidelines of Changzhi Medical College.

Cardiac function analysis

At week 8 of liver cirrhosis modeling, the cardiac functions were measured. Briefly, a catheter was inserted into the left ventricle via the right common carotid artery. The parameters of left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), and the maximum rate of change in left ventricular pressure in the isovolumic contraction or relax-
Role of GRP78 in cirrhotic cardiomyopathy

Table 1. Cardiac function of rats in the liver cirrhosis group and the control group (mean ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>LVSP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>+ dp/dt max (mmHg/ms)</th>
<th>- dp/dt max (mmHg/ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver cirrhosis</td>
<td>11</td>
<td>99.13 ± 8.01</td>
<td>18.09 ± 2.81</td>
<td>5.21 ± 1.07</td>
<td>2.02 ± 2.30</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>108.44 ± 7.16</td>
<td>29.71 ± 5.1</td>
<td>8.09 ± 1.81</td>
<td>3.75 ± 1.44</td>
</tr>
</tbody>
</table>

Note: LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; + dp/dt max, the maximum rate of change in left ventricular pressure in the isovolumic contraction period; - dp/dt max, the maximum rate of change in left ventricular pressure in isovolumic relaxation period. *P < 0.05 vs. control group.

Immunohistochemical staining

The sections of heart tissues were prepared as mentioned above. To inactivate endogenous peroxidase, 3% fresh prepared hydrogen peroxide was added and incubated at room temperature for 15 min. After washing with distilled water, the antigen was repaired by citrate buffer (0.01 M, pH 6.0) with microwave heating. The sections were washed with PBS and blocked with serum blocking solution for 20 min. Then the primary rabbit anti-rat GRP78 polyclonal antibodies (dilution, 1:100) or rabbit anti-HIF-1α polyclonal antibodies (dilution, 1:300) were added and incubated at 4°C in the dark overnight. The secondary biotined goat anti rabbit IgG was added for a 20 min incubation at room temperature. After incubation with streptavidin-biotin complex, the sections were developed with 3, 3'-diaminobenzidine tetrahydrochlorid edihydrate chromogenic reagent. Sections were counterstained with haematoxylin. After hydrochloric acid differentiation and dimethylbenzene transparency, he sections were mounted withneutral gum. Cells with brown staining were defined as GRP78-positive or HIF-1α-positive. Ten fields at high-magnification (× 400) were randomly taken from every section and the average numbers of cardiomyocytes were calculated.

Statistical analysis

All results were expressed as mean ± standard deviation (SD). All statistical analyses were performed with SPSS version 10.0 for Windows (SPSS Inc., Chicago, IL, USA). LSD-t test was used to analyze comparisons between groups. ANOVA was used to analyze paired data. Pearson correlation analysis was used to analyze the association between GRP78 expression and other main indicators. P value less than 0.05 was considered to be significantly different.
Role of GRP78 in cirrhotic cardiomyopathy

Table 2. Levels of TNF-α and MDA in myocardial tissues of rats (mean ± SD)

<table>
<thead>
<tr>
<th>Time point</th>
<th>TNF-α (μg/l)</th>
<th>MDA (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver cirrhosis group (n = 11)</td>
<td>Control group (n = 6)</td>
</tr>
<tr>
<td>Week 4</td>
<td>0.37 ± 0.45</td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td>Week 6</td>
<td>0.71 ± 0.16*</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>Week 8</td>
<td>1.23 ± 0.25*</td>
<td>0.14 ± 0.08</td>
</tr>
</tbody>
</table>

Note: *P < 0.05 vs. control group; **P < 0.05 vs. those at week 4; †P < 0.05 vs. those at week 6.

Results

The systolic and diastolic function of the heart is affected by liver cirrhosis

To investigate the cardiac functions in rats with liver cirrhosis, cardiac function analysis was performed at week 8 of liver cirrhosis modeling to measure the parameters of LVSP, LVEDP, and ± dp/dt max. As shown in Table 1, LVEDP and ± dp/dt max were significantly decreased in the liver cirrhosis group compared with those in the control group (P < 0.05). Also, LVSP showed a downward trend in rats with liver cirrhosis compared with control. However, the difference in LVSP between these two groups was not significant. These results indicate that both systolic and diastolic function of the heart can be affected in the liver cirrhosis group.

Levels of TNF-α and MDA in myocardial tissues are significantly increased during the progression of liver cirrhosis

To detect the levels of TNF-α and MDA in myocardial tissues, the radioimmunoassay and thiobarbituric acid assay were performed, respectively. The results were shown in Table 2. For levels of TNF-α, at week 4 of liver cirrhosis modeling, there was no significant difference in TNF-α level between liver cirrhosis group and control group. At week 6 and week 8 of liver cirrhosis modeling, TNF-α levels in the liver cirrhosis group were significantly higher than those in the control group (P < 0.05). Compared with the corresponding control group, the liver cirrhosis group had significantly higher levels of MDA at week 4, week 6 and week 8 (P < 0.05). MDA levels in the liver cirrhosis group at week 6 and week 8 were significantly higher than that at week 4 (P < 0.05). MDA level in the liver cirrhosis group at week 8 was significantly higher than that at week 6 (P < 0.05). These results indicate that levels of TNF-α and MDA in myocardial tissues are significantly increased during the progression of liver cirrhosis.

The numbers of cardiomyocytes are decreased during the progression of liver cirrhosis

To investigate the effects of liver cirrhosis on the myocardial structures, toluidine blue staining was performed to observe the morphology and numbers of myocardial cells. In the control group, cardiomyocytes showed normal morphology (Figure 1A). At week 4 in the liver cirrhosis group, there were spotty pathological changes, mild edema, smaller intercellular spaces, and slightly blurred boundaries in cardiomyocytes (Figure 1B). At week 6 in the liver cirrhosis group, cardiomyocytes showed severe edemas and a range of different cell sizes (Figure 1C). At week 8 in the liver cirrhosis group, a large area of dissolved necrosis cells, obvious muscle fiber fractures and blurred boundaries were observed in myocardial tissue. In addition, varying degrees of inflammatory cell infiltration were observed from lesion area (Figure 1D).

The numbers of cardiomyocytes were counted by BI-2000 Medical image analysis software. The number of cardiomyocytes was 816.76 ± 7.17 in the control group (n = 18). The number of cardiomyocytes in the liver cirrhosis group was 245.41 ± 3.1 at week 4, 113.84 ± 2.77 at week 6, and 40.17 ± 2.67 at week 8, significantly lower than that of the control group (P < 0.05). The number of cardiomyocytes in liver cirrhosis group at week 8 was significantly decreased compared with those at week 6 (P < 0.05). These results indicate that the number

Table 1. Levels of TNF-α and MDA in liver tissues of rats (mean ± SD)

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<th>Time point</th>
<th>TNF-α (μg/l)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Liver cirrhosis group (n = 11)</td>
<td>Control group (n = 6)</td>
</tr>
<tr>
<td>Week 4</td>
<td>0.42 ± 0.08</td>
<td>0.60 ± 0.06</td>
</tr>
<tr>
<td>Week 6</td>
<td>1.23 ± 0.25</td>
<td>0.68 ± 0.09</td>
</tr>
</tbody>
</table>
| Week 8     | 4.92 ± 2.67 | 2.67 μM, respectively. The MDA levels in the control group at week 4, week 6 and week 8 were 0.42 ± 0.08 μM, 0.60 ± 0.06 μM, and 0.68 ± 0.09 μM, respectively. Compared with the corresponding control group, the liver cirrhosis group had significantly higher levels of MDA at week 4, week 6 and week 8 (P < 0.05). MDA levels in the liver cirrhosis group at week 6 and week 8 were significantly higher than that at week 4 (P < 0.05). MDA level in the liver cirrhosis group at week 8 was significantly higher than that at week 6 (P < 0.05). These results indicate that levels of TNF-α and MDA in myocardial tissues are significantly increased during the progression of liver cirrhosis.

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Role of GRP78 in cirrhotic cardiomyopathy

of cardiomyocytes is decreased during the progression of liver cirrhosis.

The myocardial interstitial collagen is significantly increased in the liver cirrhosis group

To investigate the distribution of myocardial interstitial collagen, Van Giesan staining was performed. As shown in Figure 2A, myocardial interstitial collagen in the control group was rarely detectable. For the liver cirrhosis group, small amounts of collagen fibers were stained red at week 4 (Figure 2B). The content of interstitial collagen was significantly increased, and the necrotic myocardial cells were surrounded by a large number of disordered crisscross of collagen fibers in the liver cirrhosis group at week 6 (Figure 2C). The myocardial collagen fibers were radially distributed in the myocardial necrosis around the foci of myocardial necrosis in the cirrhosis group at week 8 (Figure 2D).

The CVF value in the control group was 2.11 ± 0.52 (n = 18). CVF values in the liver cirrhosis group at week 4, week 6 and week 8 were 5.83 ± 0.927, 7.47 ± 1.04, and 12.56 ± 2.88, respectively. The CVF values in the liver cirrhosis group at week 4, week 6 and week 8 were significantly increased compared with those in the control group, respectively (P < 0.05). Compared with that at week 6, the CVF value in the liver cirrhosis group at week 8 was significantly increased (P < 0.05). These results indicate that the myocardial fibrosis occurs in the liver cirrhosis progress.

Expression levels of GRP78 are increased during the progression of liver cirrhosis

To detect the expression of GRP78 in heart tissues, immunohistochemical staining was per-
Role of GRP78 in cirrhotic cardiomyopathy

Expression levels of HIF-1α are increased during the progression of liver cirrhosis

To determine the expression level of HIF-1α in the myocardial tissues, immunohistochemical staining was performed. Representative immunohistochemical staining results were shown. Cells with brown granules were positive. As shown in Figure 4A, in the control group, a small amount of brown granules were evenly distributed in cytoplasm of myocardial cells. However, in the liver cirrhosis group, the brown granules were mainly distributed in the nuclei of myocardial cells, with some surrounding the nuclei. The numbers of dark brown granules were increased in the liver cirrhosis group at week 4, week 6 and week 8 (Figure 4B-D). Positive rate of HIF-1α expression in the control group was 0.929 ± 0.171. Positive rates of HIF-1α expression in the liver cirrhosis group at week 4, week 6 and week 8 were 2.087 ± 0.597, 5.840 ± 0.803,
1555

and 12.31 ± 0.994, respectively. Positive rates of HIF-1α expression in the liver cirrhosis group at week 4, week 6 and week 8 were significantly increased compared with those in the control group (P < 0.05). The positive rate of HIF-1α expression in the liver cirrhosis group at week 8 was significantly increased compared with those at week 6 (P < 0.05). These results indicate that the expression levels of HIF-1α are increased in the progression of liver cirrhosis.

Expression of GRP78 is positively correlated with other main indicators induced by intestinal endotoxin

To investigate the association between GRP78 expression and other main indicators induced by intestinal endotoxin, Pearson correlation analysis was performed. The plasma endotoxin levels were positively correlated with MDA (r = 0.768, P < 0.05) and GRP78 expression levels (r = 0.861, P < 0.01) in myocardial tissues. The expression levels of GRP78 were positively correlated with levels of homocysteine (r = 0.649, P < 0.05), alanine aminotransferase (r = 0.691, P < 0.05), MDA (r = 0.794, P < 0.01), CVF (r = 0.826, P < 0.01) and HIF-1α expression (r = 0.618, P < 0.05). These results suggest that GRP78 is an important molecule in the pathogenesis of cardiopathy induced by intestinal endotoxin.

Discussion

In this study, a rat model of liver cirrhosis induced by multiple pathogenic factors was established. Systolic and diastolic dysfunction was observed. Myocardial edema, fatty degeneration, spotty necrosis, decreased cardiomyocytes and obvious myocardial fibrosis were associated with the liver cirrhosis, which suggests that liver cirrhosis is complicated by the alterations of myocardium.

Intestinal endotoxemia plays an important role in the development of various complications of liver cirrhosis [19-21]. During intestinal endotoxemia, levels of intestinal endotoxin are increased, further stimulating ER stress. Our results showed that the levels of myocardial MDA were significantly increased in the liver cirrhosis group compared with those in the control group, which indicates that lipid peroxidation occurs in myocardial tissues. ER stress caused by oxidative stress or intestinal endotoxin is likely to play an important role in cirrhotic cardiomyopathy. There was hyperhomocysteinemia in the rat model of liver cirrhosis, which may be a cause for the ER stress response associated with cirrhotic cardiomyopathy [22-24].

Hypoxia causes accumulation of lactic acid and acidosis in myocardial tissue, which further induces expression of HIF-1α [25, 26]. In this study, we found that the numbers of myocardial cells were decreased but the content of interstitial collagen was increased in the progression of liver cirrhosis. The expression levels of GRP78 were positively correlated with the expression levels of HIF-1α. These results indicate that hypoxia caused by hepatopulmonary syndrome induces the expression of HIF-1α, which stimulates GRP78 expression to protect cardiomyocytes. However, excessive and prolonged hypoxia may lead to severe ER stress responses with high expression of HIF-1α, which increases apoptosis and damages to cardiomyocytes. In addition, myocardial fibrosis affects blood supply, which also induces the expression of HIF-1α. Thus, hypoxia may be another important factor leading to cirrhotic cardiomyopathy.

Multiple factors are involved in the cirrhotic cardiomyopathy. The decreased cardiomyocytes, interstitial fibrosis, myocardial remodeling, and the gradual declining of cardiac function may be resulted from the liver cirrhosis. Furthermore, liver cirrhosis may induce heart failure and threaten the patient’s lives. The expression of GRP78 during the ER stress response plays an important role in myocardial remodeling in the cirrhotic cardiomyopathy.

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Role of GRP78 in cirrhotic cardiomyopathy


Disclosure of conflict of interest

None.

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Role of GRP78 in cirrhotic cardiomyopathy


