Protective Effects of Reduced Beta 2 Glycoprotein I on Liver Injury in Streptozotocin (STZ)-Diabetic Rats by Activation of AMP-Activated Protein Kinase

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Background: Protective effects of reduced beta 2 glycoprotein I (Rβ2GPI) against vascular injury of diabetes mellitus have been extensively investigated. However, the effects of Rβ2GPI on liver injury in diabetic animals have not been reported.

Material/Methods: A diabetic rat model of was produced by systemic injection of streptozotocin (STZ). Rats were divided into a normal control group, a model group, and an Rβ2GPI treatment group (N=6 in each group). After treatments, blood serum and liver tissue were collected to test the protection of Rβ2GPI. AMP-activated protein kinase (AMPK) was detected by immunohistochemistry and Western blotting.

Results: Our results revealed that Rβ2GPI reduced blood glucose, serum creatinine, and urea nitrogen levels, as well as serum inflammation cytokines, including interleukin (IL)-6, tumor necrosis factor (TNF)-α and C-reactive protein in the diabetic rats. Importantly, Rβ2GPI prevented liver injury in the diabetic rats as confirmed by hematoxylin-eosin (H&E) staining, alanine transaminase, aspartate transaminase, and gamma-glutamyl transferase. Reactive oxygen species (ROS) were promoted by diabetic modeling and were attenuated by Rβ2GPI administration. Moreover, Rβ2GPI significantly reduced liver catalase, malondialdehyde, and superoxide dismutase levels in the diabetic rats. Rβ2GPI reduced liver glycolipid storage in STZ diabetic rats. Both immunohistochemistry and Western blotting demonstrated that Rβ2GPI promoted AMPK phosphorylation in the diabetic rats.

Conclusions: Our data proved that Rβ2GPI prevented liver injury in diabetic rats, likely through activating the AMPK signaling pathway.

MeSH Keywords: AMP-Activated Protein Kinases • Diabetic Ketoacidosis • Pregnancy-Specific beta 1-Glycoproteins

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/909598
Background

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia, affecting approximately 422 million people worldwide [1]. Liver injury is a major complication in DM patients, caused by activation of hepatic stellate cells and liver fibrosis [2]. Liver injury in diabetes mellitus has turned out to be a central issue for liver metabolic diseases. The mechanisms underlying liver injury are extremely interesting for disclosing the pathogenesis and finding therapeutic approaches for DM.

Beta 2 glycoprotein I (β2GPI) is predominantly synthesized in hepatocytes and has now been identified as the most prominent antigen in antiphospholipid syndrome [3,4]. Interestingly, Rβ2GPI has been found to protect against cellular injury status. Accumulating evidence reveals that AMPK regulates a wide variety of gene expression and metabolic signaling pathways [9,10]. Moreover, AMPK plays a pathophysiological role in lipatrophic diabetes [11]. The present study investigated the effect of Rβ2GPI on liver injury in a streptozotocin (STZ)-induced diabetic model and assessed the underlying mechanisms.

Material and Methods

Purification of β2GPI and preparation of reduced β2GPI

β2GPI and Rβ2GPI were prepared following a method detailed in a previous publication [12]. Purified β2GPI (2 μM) was reduced using TRX-1 (3.5 μM) activated with DTT (70 μM). Reduced glutathione was used to protect the thiol of Rβ2GPI. The levels and structure of Rβ2GPI were confirmed, as previously described [13–15].

Animal model and groups

Wistar rats (male, 6 weeks old, body weight 160–180 g) were kept in animal cages with free access to food and water with 12/12-h light/dark cycle and temperature (22±2°C). Diabetes was induced by a single intraperitoneal injection of STZ at a dose of 50 mg/kg bw as previously described [16]. We randomly divided 18 rats into 3 groups (n=6 for each group): control rats, STZ-diabetic rats, and STZ-diabetic rats treated with Rβ2GPI. Rats were administered Rβ2GPI once daily by an intravenous injection of 20 μg Rβ2GPI once a day for 3 weeks starting on the third day after injection of STZ, as previously described [17]. A blood glucose level above 11 mmol/L was considered indicative of diabetes. All the protocols were approved by the Ethics Committee of Tianjin Medical University Metabolic Disease Hospital (Ethics protocol number: DXBYHMEC2017-11).

Blood parameters were tested immediately after blood sampling from animals. The rats were decapitated under sodium pentobarbital anesthesia (60 mg/kg bw) 3 weeks after Rβ2GPI treatments. The livers were rapidly removed, rinsed with 0.9% NaCl, frozen in liquid nitrogen, and stored at −80°C for biochemical analysis and immunohistochemistry.

Serum biochemical parameters

We collected 2-mL blood samples and placed them in pre-cooled tubes, centrifuged at 987 g × for 10 min at 4°C. Serum levels of activities of glucose, creatinine, urea nitrogen, alanine transaminase (ALT), aspartate transaminase (AST), and gamma-glutamyl transferase (GGT) were measured using an automated biochemical analyzer (BioTek, USA). Serum IL-6 (RA80311, SIGMA, USA) and TNF-α (RA80479, SIGMA, USA) were detected followed the instructions of the assay kits.

Histologic evaluation

Liver tissue was collected for hematoxylin-eosin (H&E) staining. The tissues were fixed in 4% paraformaldehyde for ~1 week at 4°C, cryoprotected in 30% sucrose for 1 h at 4°C, and sectioned into 5-µm-thick sections with a frozen microtome. After that, the slides were stained by hematoxylin and eosin for 5 min. The images were taken under light microscopy.

Liver reactive oxygen species (ROS)

Liver ROS level was measured by means of chemiluminescence (CL) in the Fenton reaction (2 mM Fe(II), 8.8 mM hydrogen peroxide, pH 7.4), as previously described [16]. Briefly, liver tissue was homogenized in Hanks’ balanced salt solution (Sigma-Aldrich, USA) on ice. Then, CL was monitored for 30 s (Luminometer EA-1, Ukraine) at 37°C and continuous mixing. The sum of light signals at a sampling frequency of 0.25 Hz was calculated and is expressed as relative light units.

Oxidative stress markers

The level of oxidative stress was determined in the liver by assessing the levels of malondialdehyde (MDA) (S0131, Beyotime, Shanghai, China), catalase (CAT100, SIGMA, USA), and superoxide dismutase (SOD) (ab65354, ABCAM, USA), as previously described [16].
Table 1. Rβ2GPI treatment attenuated signs of type 2 diabetes.

| Groups          | Control       | Diabetic      | Rβ2GPI        |
|-----------------|---------------|---------------|---------------|
| Body weight (g) | 213.4±12.9    | 207.0±12.5    | 211.4±14      |
| Blood glucose (mM) | 9.7±1.1      | 21.9±1.1**   | 13.4±1.2*     |
| Serum creatinine (μM) | 9.7±0.8      | 22.9±1.1**   | 13.2±1.2*     |
| Blood urea nitrogen (mM) | 9.4±0.5      | 16.9±0.7*   | 11.3±1.1*     |
| IL-6 (pg/L)     | 96.4±4.2      | 129.3±2.3*   | 105.2±5.3*    |
| TNF-α (pg/L)    | 0.78±0.07     | 1.64±0.07**  | 1.15±0.06*    |
| CRP (mg/L)      | 4.6±0.5       | 9.3±0.4**    | 5.1±0.3*      |

* p<0.05, ** p<0.01 compared with control; * p<0.05 compared with diabetic.

Liver glycolipid storage

The liver tissues were weighed, digested with 1 mol/L NaOH (1: 9 wt/vol) at 80°C for 10 min, and neutralized with 1 mol/L HC. The resulting solution was incubated at 85°C for 2 h and neutralized again with 5 mol/L NaOH. After that, liver FFA was detected using a fatty acid kit (MAK044, Sigma, USA) following the manufacturer’s instructions. The liver TG was determined using a triglyceride determination kit (T2449, Sigma, USA) following the manufacturer’s instructions.

Immunohistochemistry

After fixation in 4% paraformaldehyde for 1 h, liver tissues were cryoprotected in 30% sucrose for 1 h at 4°C and sectioned on a freezing microtome at 20 μm. Sections were blocked in 0.1 M phosphate-buffered saline (PBS) containing 10% goat serum, but without Triton X-100, and then incubated with primary antibody (mouse anti-p-AMPK, 1: 200, CST) in 0.1 M PBS containing 5% goat serum overnight at 4°C. Sections were washed 3 times (15 min each) in PBS and incubated with goat anti-mouse horseradish peroxidase (HRP) (IgG H&L) (Life Technologies, Carlsbad, CA, USA) for 2 h at room temperature. DAB was used to color the staining. The imaging and density analysis were following previous description [18] by using ImageJ software. At least 4 fields in each image were analyzed. The expression of p-AMPK was normalized to control.

Western blotting

Liver tissue was used in Western blotting, and cytoplasmic protein was isolated using a Cytoplasmic Protein Extraction kit (Beyotime, Jiangsu, China). Protein concentration was measured using the bicinchoninic acid method (Beyotime, China), and an amount of protein (25 μg) was loaded into 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The membranes were incubated with anti-AMPK, anti-p-AMPK, and anti-GAPDH at 4°C overnight. After washing, the membranes were incubated with a secondary antibody (1: 5000; CST, USA). The signal was detected using an enhanced chemiluminescence detection kit (Amersham ECL RPN 2106 Kit, Amersham Pharmacia Biotech, QC, Canada).

Statistical analysis

Results are presented as mean ± standard deviation (mean ±SD). Statistical analysis was performed using GraphPad Prism 5. Differences between groups were identified using one-way ANOVA, where p-values <0.05 were considered as statistically significant.

Results

Rβ2GPI reduces the liver injury in STZ diabetic rats

In the experiment, diabetic rats were confirmed by the increased blood glucose levels (more than 3 times compared to control) 3 days after the STZ injection. As shown in Table 1, STZ-diabetic rats showed signs of diabetes, such as hyperglycemia and higher levels of creatinine and urea nitrogen, but without bodyweight increase compared with control rats. By contrast, Rβ2GPI significantly reduced those signs compared with the STZ-diabetic group. We also detected the levels of inflammation cytokines. As shown in Table 1, diabetic modeling increased serum IL-6 and TNF-α levels, which were reduced by Rβ2GPI administration.

In this study, we focused on the liver injury in diabetic rats. Pathologically, liver histological structure was normal in the healthy control group, but diabetic rats showed fatty changes in centrilobular portions of the livers. Rβ2GPI prevented the pathologic changes and no fatty change was observed in the Rβ2GPI treatment group (Figure 1). The liver injury was also confirmed by biochemical indices. Alanine transaminase, aspartate transaminase, and gamma-glutamyl transferase levels were
significantly elevated in diabetic rats, which were down-regulated by Rβ2GPI administration (Figure 2). These data suggest that Rβ2GPI protects against liver injury in diabetic model rats.

**Rβ2GPI decreases oxidative stress in STZ diabetic rats**

An intensification (by about 2-fold) of free radical oxidation was found in livers of diabetic rats compared to the control, as shown by chemiluminescence test (Figure 3A). ROS generation can lead to increased peroxide process and cause cellular oxidative stress. As shown in Figure 3C, the malondialdehyde level was significantly increased in the liver tissue in diabetic rats, which indicated an intensification of lipid peroxidation processes. Meanwhile, a depletion of the antioxidant system was shown in diabetic rats. The activities of catalase (Figure 3B) and SOD (Figure 3D) were reduced significantly in the livers of diabetic rats compared to the control rats. Critically, Rβ2GPI reduced the ROS and malondialdehyde levels, and significantly increased the activities of catalase and SOD compared with diabetic rats. These results suggest that Rβ2GPI prevents the ROS process in diabetic rats.

**Rβ2GPI reduces liver glycolipid storage in STZ diabetic rats**

We also assessed glycolipid storage in liver tissue. As shown in Figure 4, liver glycolipid level was reduced, while TG and FFA were accumulated in liver tissue from diabetic rats. Liver glycolipid was elevated, while TG and FFA were reduced by Rβ2GPI administration.

**Rβ2GPI promotes AMPK phosphorylation in STZ diabetic rats**

The signaling pathway was also investigated in the protection of Rβ2GPI. As shown in Figure 5, p-AMPK level was obviously down-regulated in liver tissue from diabetic rats, which was promoted by Rβ2GPI administration. Western blotting further confirmed that the phosphorylation of AMPK, but not total AMPK level, was reduced in liver tissue from diabetic rats (Figure 6). Rβ2GPI administration promoted the phosphorylation of AMPK.
β2GPI is associated with antiphospholipid syndrome [19–21] as well as DM [22]. By contrast, we revealed that Rβ2GPI prevented liver injury in diabetic rats. Moreover, Rβ2GPI reduced liver oxidative stress and glycolipid storage in diabetic rats. As a mechanism, Rβ2GPI promoted AMPK phosphorylation in diabetic rats, which might explain the protection of Rβ2GPI against liver injury in diabetic model rats.

Rβ2GPI level is down-regulated in antiphospholipid syndrome [23,24]. Moreover, Rβ2GPI, but not non-Rβ2GPI, was reported to protect human umbilical vein cell line from oxidative stress-induced endothelial cell damage [14]. We have also reported that Rβ2GPI protects against high glucose-induced cell death in HUVECs through the miR-21/PTEN pathway [5,6]. Rβ2GPI is a candidate treatment for DM, especially the vascular complications. In our present study, we demonstrated that Rβ2GPI prevented liver injury in DM rats. On the one hand, DM signs were attenuated by Rβ2GPI treatment, including reduction of blood glucose, creatinine, and urea nitrogen levels.

**Discussion**

β2GPI is associated with antiphospholipid syndrome [19–21] as well as DM [22]. By contrast, we revealed that Rβ2GPI prevented liver injury in diabetic rats. Moreover, Rβ2GPI reduced liver oxidative stress and glycolipid storage in diabetic rats. As a mechanism, Rβ2GPI promoted AMPK phosphorylation in diabetic rats, which might explain the protection of Rβ2GPI against liver injury in diabetic model rats.
The increase of serum inflammation factors is an important indicator of DM [25]. We also found that diabetic modeling upregulated IL-6 and TNF-α levels. On the other hand, liver injury, another feature of DM, was also prevented by Rβ2GPI injury. Both morphological observation and biochemical indices indicated that Rβ2GPI prevented liver injury in diabetic rats. Typical fatty changes were observed in the diabetic model group but not in the control and Rβ2GPI treatment groups. Our data and results of others suggest that Rβ2GPI may have value in treatment of DM complications.
Reactive oxygen species (ROS) are reactive chemical species, including peroxides, superoxide, hydroxyl radical, and singlet oxygen. ROS are produced as a natural byproduct during normal metabolism of oxygen. As reported, ROS have important roles in cell signaling and homeostasis [26,27]. In DM, hepatic cells are widely recognized to be damaged by ROS [28]. In our study, we demonstrated that ROS level was elevated in the liver tissue of DM rats and Rβ2GPI obviously reduced ROS level. These data further confirmed the hepatic protection of Rβ2GPI in DM. Catalase and superoxide dismutase are effective regulators of hydrogen peroxide and superoxide, respectively, by converting these compounds into oxygen and hydrogen peroxide [29]. Therefore, we also detected liver catalase and superoxide dismutase. Our data showed that catalase and superoxide dismutase were reduced in DM rats and were promoted by Rβ2GPI treatment. Malondialdehyde (MDA) is also a marker for oxidative stress [30]. In our study, MDA levels in DM rats were elevated, which was reduced by Rβ2GPI treatment.

Hepatic glucose and lipid metabolic disorder are also features of liver injury in DM [31]. In our study, hepatic G was reduced, while TG and FFA were promoted in DM. Rβ2GPI treatment could promote hepatic G level and decrease TG and FFA levels. These data further show that Rβ2GPI can repair lipid metabolic disorder in DM.

AMPK is a key molecule in the regulation of bioenergy metabolism and is the core of the study of diabetes and other metabolic related diseases [32]. It is expressed in many metabolic-related organs, and can be activated by various stimuli, including cell pressure, exercise, and many hormones and substances that can affect cell metabolism [33]. Genetic and pharmacological studies have shown that AMPK is essential for the body to maintain the balance of glucose [34]. In our study, we revealed that AMPK phosphorylation was down-regulated in liver tissue in DM rats. Consistent with the biochemical or morphological alterations, Rβ2GPI treatment also promoted p-AMPK level but did not affect total AMPK level. AMPK activation is elicited by phosphorylation at Thr172 catalyzed by liver kinase B1 in response to an increase of AMP-to-ATP ratio, or by the calmodulin-dependent protein kinase β (CaMKKβ) in response to elevated Ca2+ levels [35]. Other possible mechanisms may involve liver kinase B1 or protein phosphatase-2C, a phosphatase that dephosphorylates and inactivates AMPK [35]. We plan to perform further research to elucidate the underlying mechanisms involved.

**Conclusions**

We demonstrated the hepatoprotection of Rβ2GPI in diabetic rats for the first time. The potential mechanism is related to the regulation of ROS and the AMPK signaling pathway. Our study may provide important evidence for the protection of Rβ2GPI in liver complications of DM, although further experimental and clinical trials are needed.

**Conflict of interest**

None.
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