The Hepatic Protection Effects of Hepassocin in Hyperglycemic Crisis

Horng-Yih Ou,1 Hung-Tsung Wu,2,3 Ching-Han Lin,1 Ye-Fong Du,1 Che-Yuan Hu,4 Hao-Chang Hung,1 Pansee Wu,5 Hung-Yuan Li,6 Shu-Huei Wang,7 and Chih-Jen Chang2

1Division of Endocrinology and Metabolism, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan; 2Department of Family Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan; 3Research Center of Clinical Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan; 4Department of Urology, National Cheng Kung University Hospital, Tainan 70403, Taiwan; 5Institute for Science and Technology in Medicine, Keele University, Keele ST5 5BG, United Kingdom; 6Department of Internal Medicine, National Taiwan University Hospital, Taipei 10048, Taiwan; and 7Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan

Context: High glucose generates reactive oxygen species (ROS) and contributes to glucotoxicity in hepatocytes, and hyperglycemia causes structural and functional damage to the liver. However, only a mild hepatic dysfunction was observed in subjects with hyperglycemic crisis, implying a factor exists to exert a hepatic protective effect. Hepassocin is a hepatokine that modulates the proliferation and metabolism of hepatocytes and also exerts protective activity in liver injury. However, its role in hyperglycemic crisis-induced hepatic dysfunction remains unknown.

Objective: To investigate the possible hepatic protection effects of hepassocin in hyperglycemic crisis.

Design, Setting, and Patients: Plasma hepassocin concentrations and routine biochemistry were measured in 21 patients with hyperglycemic crisis before and after standard treatments. The effects of hepassocin on hepatic functions were evaluated in streptozotocin-induced hyperglycemic mice (STZ mice). HepG2 cells were used to clarify the possible mechanisms regulating hepassocin expression.

Results: Plasma hepassocin concentrations decreased significantly in subjects with hyperglycemic crisis after standard treatment accompanied by improved hepatic functions. Correction of hyperglycemia in STZ mice also decreased the hepatic hepassocin expression. Injection of recombinant hepassocin improved hepatic functions and histologic changes and increased the expression of antioxidative stress proteins, including superoxide dismutase 1 (SOD1). In HepG2 cells, high glucose increased hepassocin expression through signal transducer and activator of transcription 3 and hepatocyte nuclear factor-related pathways. We also demonstrated that hepassocin increased SOD1 expression through an extracellular signal-regulated kinase 1/2 nuclear factor erythroid-2-related factor 2 pathway, decreasing ethyl acetate-induced ROS production and improving cell viability.

Conclusions: Increased hepassocin secretion in hyperglycemic crisis might offset the deleterious effects of hyperglycemia on hepatocytes. (J Clin Endocrinol Metab 102: 2407–2415, 2017)
The liver, in association with adipose tissue and skeletal muscle, plays a pivotal role in the regulation of whole-body energy homeostasis. The liver secretes glucose through both breakdown of glycogen (glycogenolysis) and de novo glucose synthesis (gluconeogenesis) to maintain normoglycemia (1). In addition, the liver also releases humoral factors, called hepatokines, to regulate insulin sensitivity in insulin-responsive tissue (2–5).

Despite its role in the regulation of glucose homeostasis, the liver is vulnerable to glucotoxicity. It is known that hyperglycemia causes structural and functional changes of the liver in diabetic animal models (6). In addition, the liver is characterized by free glucose uptake because insulin-independent glucose transporter 2 enables high-capacity, facilitated diffusion of glucose into the hepatocyte and thus is directly proportional to blood glucose concentrations (7). Moreover, strict glycemic control with intensive insulin therapy has been shown to reverse ultrastructural and functional abnormalities of hepatocytes in critically ill patients (8). It is thus plausible that in conditions of severe hyperglycemia, there will be severe hepatic damage. However, in metabolic emergencies, like hyperglycemic crisis, only a mild increase in aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) level is observed, and the exact mechanisms remain obscure.

Hepassocin, also called fibrinogen-like protein 1 and hepatocyte-derived fibrinogen-related protein 1, is a hepatokine that has been reported to be a mitogen for hepatocytes and exerts a hepatic protection action against chemical-induced liver injury (9, 10). In addition, hepatocyte nuclear factor 1 (HNF1) is critical to the liver-specific expression of hepassocin, and downregulation of HNF1 causes the decreased expression of hepassocin in hepatocellular carcinoma (11). Recently, our group found that hepassocin is increased in diabetic patients with or without hepatic steatosis (12) and further contributes to hepatic lipid accumulation and insulin resistance in human subjects and an animal model (13, 14). In addition, high glucose regulates the expression of hepassocin, and the fasting plasma glucose level is an independently associated factor for the plasma hepassocin concentrations (14). Although the hepatic protection effects of hepassocin have been explored, its role in hyperglycemic crisis is still unknown.

Therefore, in the current study, we hypothesized that increased hepassocin secretion in hyperglycemic crisis might offset the deleterious effects of hyperglycemia on hepatocytes. We also investigated the regulation of hepassocin expression in hyperglycemia and explored the possible mechanisms by which hepassocin ameliorated hyperglycemia-induced hepatic injury.

**Materials and Methods**

**Human subjects**

The study protocol was approved by the Human Experiment and Ethics Committee of the National Cheng Kung University Medical Center (B-ER-102-418), and all eligible subjects gave written informed consent prior to participation. A total of 30 patients who were admitted to the endocrine ward of the National Cheng Kung University Hospital for hyperglycemic crisis were screened for this study. Subjects with the following conditions or diseases were excluded: (1) type 1 diabetes mellitus; (2) alcohol consumption ≥20 g/d in the last year; (3) a positive test for hepatitis B surface antigen, hepatitis C antibody, and other parenchymal liver diseases (such as autoimmune hepatitis, Wilson disease, and hemochromatosis); (4) serum creatinine >1.5 mg/dL; (5) acute ischemic heart event, cerebrovascular accident, or pancreatitis; (6) any acute infection, such as pneumonia, urinary tract infection, soft tissue infection or cellulitis, or sepsis; (7) pregnancy; (8) current usage of drugs that affect the carbohydrate metabolism, such as corticosteroids, thiazides, sympathomimetic agents, and atypical antipsychotic drugs; and (9) any other major diseases, including generalized inflammation or advanced malignant diseases contraindicating this study. Finally, a total of 21 participants were included in the analysis.

In the current study, all of the patients had an emergency department visit due to hyperglycemic crisis before hospitalization. After triage and initial treatment, they were admitted to the endocrine/metabolism ward for (1) correction of dehydration (with fluid therapy), hyperglycemia (with continuous intravenous insulin until the hyperglycemic crisis was resolved), and electrolyte imbalances; (2) identification of any comorbid precipitating events (and then treated accordingly); and (3) frequent patient monitoring. Each subject was treated with the standard care recommended by the American Diabetes Association (15). When the patients’ condition stabilized, a transition to subcutaneous insulin was planned for each before discharge. After discharge, they were treated with insulin injection until a scheduled outpatient clinic visit 3 weeks later, when the antidiabetic regimens were reevaluated and adjusted accordingly. Blood samples were collected on admission and 1 month after discharge from the hospital. In addition, data of complete blood count, biochemistry (including plasma glucose, AST and ALT, creatinine, blood urea nitrogen, electrolytes, arterial blood gases, and lipid profile), hemoglobin A1C, and hepassocin were measured at the same time.

Blood glucose was measured by a hexokinase method (Roche Diagnostic GmbH, Mannheim, Germany). Serum total cholesterol, triglycerides, and high-density lipoprotein cholesterol levels were determined in the central laboratory of the National Cheng Kung University Hospital with an autoanalyzer (Hitachi 747E; Hitachi, Tokyo, Japan). A1C was measured with a high-performance liquid chromatographic method (Tosoh Automated Glycohemoglobin Analyzer HLC-723 GHBV A1c 2.2, Tokyo, Japan; intra-assay coefficient of variation (CV) of 0.5%, interassay CV of 2.0%). Plasma hepassocin (Cusabio, Wuhan, China; intra-assay CV <10%, interassay CV <12%) was measured by enzyme-linked immunosorbent assay. The estimated glomerular filtration rate (mL/min/1.73 m²) was calculated by the Modification of Diet in Renal Disease equation.

The anion gap was calculated as $\left[\text{Na}^{+}\right] - \left[\text{Cl}^{-} + \text{HCO}_{3}\right]$ (mEq/L), and the free-water deficit (L) was calculated as $\left[\text{Na}^{+} - 140\right] / 140 \times 0.6 \times \text{body weight (kg)}$. 
**Animals**

C57BL/6J male mice were purchased from the Animal Center of the National Cheng Kung University Medical College, and the animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health as well as the guidelines of the Animal Welfare Act. Hyperglycemia was induced by intraperitoneal injection of 65 mg/kg streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO) after starvation for 4 hours over 5 consecutive days (16). Successful induction was confirmed by the presence of hyperglycemia (>300 mg/dL), polyuria, and weight loss in the animals. Insulin (Humulin®; Eli Lilly Pharmaceuticals, Melbourne, VIC, Australia) was intraperitoneally injected at a dose of 1 µg/kg once a day for 2 weeks. Phlorizin (Sigma-Aldrich) was dissolved in a solution containing 10% ethanol, 15% dimethylsulfoxide, and 75% saline, and this was injected subcutaneously at a dose of 0.4 g/kg twice daily for 2 weeks. Heparasin recombinant protein (Novus Biologicals, Littleton, CO) was intraperitoneally injected at a dose of 1 µg/kg once a day for 2 weeks. Control mice were injected with the same volume of vehicle solution.

At the end of the experiments, each group of the mice was euthanized, and the liver was removed. The tissue samples were fixed in 10% formaldehyde at 4°C, and the fixed specimens were then dehydrated and embedded in paraffin. The specimens were cut into 5-µm-thick sections at 50-µm intervals and stained with hematoxylin and eosin (Muto Pure Chemicals, Tokyo, Japan) or Picro-Sirius red stain (Abcam, Cambridge, United Kingdom), and the sections were observed with a microscope (100×). The data were obtained from at least 8 mice from each group.

**Blood biochemistry**

Whole blood from the retro-orbital venous sinus was collected using sodium-heparinized microhematocrit capillary tubes (Marienfeld-Superior, Lauda-Königshofen, Germany) and then centrifuged at 12,000 g for 5 minutes. Blood glucose concentrations were measured with a glucose kit (Biosystems SA, Barcelona, Spain) by an automatic blood glucose meter (Biosystem BTS-330; Biochemistry Analyzer, Barcelona, Spain). Enzyme-linked immunosorbent assay kits (Mercodia AB, Uppsala, Sweden) were used to determine serum insulin levels. Finally, ALT and AST levels were determined using reagents obtained from the instrument manufacturer (Teco Diagnostics, Anaheim, CA).

**Cell culture**

HepG2 cell line was purchased from the Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). The short tandem repeat profiling of the HepG2 cell line was recently performed at the Center for Genetic Medicine of the National Cheng Kung University, and the use of the authenticated cell line was verified. Cells were maintained (5% CO2, 37°C) in Dulbecco’s modified Eagle medium (HyClone, South Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum. The cells were cultured in low-glucose Dulbecco’s modified Eagle medium without serum overnight for different experiments and then harvested at the times indicated in the figure legends.

**Small-interfering RNA transfection**

HepG2 cells were transfected with duplexed RNA oligonucleotides (Stealth RNAi; Invitrogen, Carlsbad, CA) of human HNF1 or scrambled small-interfering RNA (as a negative control) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were used for the following experiments at 48 hours posttransfection.

**Western blot analyses**

The samples were lysed with a buffer (pH 7.5) containing 450 mm NaCl, 3% Nonidet P-40, 1.5% sodium deoxycholate, 0.3% sodium dodecyl sulfate, 150 mm tris(hydroxymethyl)-aminomethane (Tris)-HCl (pH 8.0), and 3 mm EDTA, with a proteinase and phosphatase inhibitor cocktail (Sigma-Aldrich). The supernatant was collected, and the protein levels were quantified with a bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL). The cell lysates were then boiled in sample buffer for 5 minutes. Protein (30 µg) from the lysates was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gels) and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). After blocking with 10 mm Tris (pH 7.6), 150 mm NaCl, and 0.05% Tween 20 containing 10% skim milk at room temperature for 1 hour, the blots were probed with primary antibodies such as phospho-signal transducer and activator of transcription 3 (STAT3), protein phosphatase 2A (PP2A), type 1 collagen (Abcam), superoxide dismutase 1 (SOD1), glutathione peroxidase, HNF1 (Novus Biologicals, Littleton, CO), nuclear factor erythroid-2-related factor 2 (NRF2; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-extracellular signal-regulated kinase 1/2 (ERK1/2; Signaling Technology, Beverly, MA), heparasin (Proteintech Group, Wuhan, China), methyl-PP2A, and actin (Millipore) at 4°C overnight. After the membranes had been washed with 10 mm Tris (pH 7.6), 150 mm NaCl, and 0.05% Tween 20, the blots were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour. The protein bands were detected using Immobilon (Millipore). The optical density of the protein levels was determined using VisionWorks LS software (Upland, CA).

**Flow cytometry**

The intracellular reactive oxygen species (ROS) level was detected by flow cytometry using 2′,7′-dichlorodihydrofluorescein-diacetate (DCF-DA; Invitrogen) as the probe, as previously described (17). HepG2 cells were treated with 20 µmol/L acetoacetate (AA; Sigma-Aldrich) for 6 hours (18), followed by staining with 100 µmol/L DCF-DA for an additional 30 minutes in the dark. The cells were washed in phosphate-buffered saline, trypsinized, and resuspended in phosphate-buffered saline. The fluorescence in cells under different treatments was examined with flow cytometry analysis (BD Biosciences, San Jose, CA).

**Real-time quantitative polymerase chain reaction**

Total RNA was extracted from the liver with TRIzol reagent (Roche Molecular Systems, Alameda, CA), and complementary DNA synthesis was performed with a Transcriptor First Strand cDNA Synthesis Kit (Roche Molecular Systems) using 2 µg total RNA. The real-time polymerase chain reaction was monitored online using a LightCycler 2.0 thermocycler (Roche Molecular Systems) according to the manufacturer’s instructions. The primer sequences are available upon request.
**Statistical analysis**

SPSS software (version 17.0; Chicago, IL) was used for the statistical analysis. All normally distributed continuous variables were expressed as means ± standard deviations or means ± standard errors. Analysis of variance was used to compare the continuous variables among the subjects. Student *t* tests or paired *t* tests were used for comparison of variables between groups. Regression analyses were used to evaluate the relationship between the levels and changes of hepassocin and ALT concentrations and indexes of dehydration. The relationship between hepassocin and ALT levels and changes in these were examined with Pearson correlation analysis. A *P* value of <0.05 was considered statistically significant.

**Results**

**Plasma hepassocin concentrations in patients with hyperglycemic crisis decreased significantly after treatment of hyperglycemia**

Tables 1 and 2 shows the clinical characteristics of the study subjects. After standard treatment of hyperglycemia, the blood glucose concentrations (728 ± 439 mg/dL, *P < 0.001*) and A1C levels (13.20% ± 2.29% to 9.74% ± 1.66%, *P < 0.001*) were significantly decreased after correction of the hyperglycemia. In addition, we also found that the elevated ALT decreased significantly (40 ± 30 to 29 ± 20 U/L, *P = 0.04*), in parallel with the decrease in plasma hepassocin concentrations (6.80 ± 5.74 and 2.70 ± 1.99 mg/mL, *P = 0.003*) (Fig. 1). As dehydration contributes to the pathogenesis of hyperglycemic crisis, regression analysis was conducted to examine the effects of surrogate markers of volume depletion/hemoconcentration (“free-water deficit,” osmolality, Na, blood urea nitrogen/creatinine ratio, creatinine, estimated glomerular filtration rate, and hemoglobin) on the levels and changes of hepassocin and ALT. The results show that the levels and changes of hepassocin and ALT were not associated with these markers (Supplemental Tables 1 and 2), thus excluding the possibility that dehydration led to the increases in hepassocin and ALT. In addition, using correlation analysis, we found there was no correlation between concentrations (*r = −0.054, *P = 0.815*) and changes (*r = 0.241, *P = 0.351*) of hepassocin and ALT.

**High glucose levels regulated the expression of hepassocin**

An STZ-induced hyperglycemia mice (STZ mice) model was used to investigate the causal relationship of hepassocin in hyperglycemia. In this, we found that the expression of hepatic hepassocin was significantly increased in hyperglycemia. After correction of the hyperglycemia by insulin or phlorizin, the increased hepassocin expression was reversed, indicating that it was regulated by glucose [Fig. 2(a)]. To further test this hypothesis, we treated hepatoma cells with different doses of glucose and found that the expressions of hepassocin were dose-dependently increased in HepG2 cells [Fig. 2(b)].

**High glucose–induced hepassocin expression through STAT3 and PP2A-HNF1 pathways**

It is known that interleukin-6 (IL-6) induces promoter activity of hepassocin, depending on STAT3 and HNF1 binding sites in the hepassocin promoter (11). In addition, high glucose activates STAT3 signaling and promotes cell proliferation (19), and HNF1 is a major regulator of glucose homeostasis, regulating the expression of genes that are expressed in the liver (20). We thus investigated the role of STAT3 and HNF1 in the regulation of hepassocin expression by high glucose. As shown in Fig. 3(a), the phosphorylation of STAT3 was significantly increased within 15 minutes in HepG2 cells treated with 25 mM high glucose. The expression of HNF1 in the nucleus of hepatocytes was significantly elevated at 1 hour after high glucose treatment, whereas a significant rise in hepassocin expression was observed at 6 hours. Moreover, glucose dose-dependently increased STAT3 activity and HNF1 expression [Fig. 3(b)]. Pretreatment with STAT3 inhibitor (Stattic) [Fig. 3(c)] or deletion of HNF1 [Fig. 3(d)] in HepG2 cells reversed the high glucose–induced hepassocin increase. Because the activity of HNF1 isoforms is regulated by PP2A (21), and high glucose exposure promotes activation of PP2A (22), we thus investigated the role of

**Table 1. Baseline Characteristics of the Study Subjects**

<table>
<thead>
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<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>n</td>
<td>21</td>
</tr>
<tr>
<td>Age (y)</td>
<td>48 ± 17</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>13/8</td>
</tr>
<tr>
<td>Diabetes duration (y)</td>
<td>0 (0–11)*</td>
</tr>
<tr>
<td>Coronary artery disease (%)</td>
<td>9.5</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>33</td>
</tr>
<tr>
<td>Retinopathy (%)</td>
<td>19</td>
</tr>
<tr>
<td>Proteinuria (%)</td>
<td>39</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>4.8</td>
</tr>
<tr>
<td>Drinking (%)</td>
<td>9.5</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>15.6 ± 2.0</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dL)</td>
<td>27 ± 15</td>
</tr>
<tr>
<td>Corrected Na (mEq/L)</td>
<td>145 ± 10</td>
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<tr>
<td>Measured osmolality (mOsmo/kg)</td>
<td>332 ± 44</td>
</tr>
<tr>
<td>Total osmolality (mOsmo/kg)</td>
<td>349 ± 47</td>
</tr>
<tr>
<td>Effective osmolality (mOsmo/kg)</td>
<td>309 ± 29</td>
</tr>
<tr>
<td>Anion gap (mEq/L)</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>PH</td>
<td>7.37 ± 0.65</td>
</tr>
<tr>
<td>Free-water deficit (L)</td>
<td>1.55 ± 3.93</td>
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</tbody>
</table>

Data are expressed as mean ± standard deviation. Corrected Na = measured Na + 0.016 × (glucose – 100). Effective osmolality = 2[m(2Na+ (mEq/L)) + glucose (mg/dL)/18. Total osmolality = 2(Na+ + K+) + urea (mmol/L) + glucose (mmol/L). Anion gap = (Na+ − [(Cl− + HCO3−) (mEq/L)]. Free-water deficit = [(Na+ − 140)/140] × 0.6 × body weight (kg).

*Median (range).*
PP2A in the regulation of hepassocin expression. Consistent with previous studies, we found that high glucose increased the methylation of PP2A, implying the activation of this protein [Fig. 3(e)]. In addition, inhibition of PP2A activity by okadaic acid reversed the effects of high glucose on hepassocin expression [Fig. 3(f)].

Hepassocin treatment improved liver functions in STZ mice

As shown in Fig. 4(a), the blood glucose level was significantly increased in STZ mice by the decreased plasma insulin level. After hepassocin treatment of 7 days, the plasma insulin levels were slightly increased but without significant differences as compared with the STZ group. Following the slightly increased insulin levels, the elevated blood glucose levels were slightly decreased [Fig. 4(a)]. On the other hand, the elevated hepatic transaminase (ALT and AST) levels were significantly decreased after treatment with hepassocin [Fig. 4(b)]. Increased immune cell infiltration [Fig. 4(c)] and fibrosis [Fig. 4(d)] were observed in STZ mice. After treatment with hepassocin, the distortion of liver architecture, inflammation and necrotic change, and collagen fiber accumulation improved [Fig. 4(c) and 4(d)]. As shown in

Table 2. Clinical Characteristics of the Study Subjects on Admission and 1 Month After Treatment

<table>
<thead>
<tr>
<th></th>
<th>On Admission</th>
<th>1 Month</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>70.4 ± 23.0</td>
<td>72.3 ± 21.1</td>
<td>0.036</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.8 ± 6.8</td>
<td>26.6 ± 6.3</td>
<td>0.019</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>141 ± 25</td>
<td>135 ± 17</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>86 ± 17</td>
<td>86 ± 17</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma glucose (mg/dL)</td>
<td>728 ± 439</td>
<td>115 ± 24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemoglobin A1c (%)</td>
<td>13.20 ± 2.29</td>
<td>9.74 ± 1.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.30 ± 0.79</td>
<td>0.87 ± 0.44</td>
<td>0.016</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>63 ± 25</td>
<td>80 ± 19</td>
<td>0.005</td>
</tr>
<tr>
<td>Hepassocin (µg/mL)</td>
<td>6.80 ± 5.74</td>
<td>2.70 ± 1.99</td>
<td>0.003</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>40 ± 30</td>
<td>29 ± 20</td>
<td>0.04</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>173 ± 112</td>
<td>136 ± 65</td>
<td>NS</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol (mg/dL)</td>
<td>43 ± 13</td>
<td>52 ± 19</td>
<td>0.003</td>
</tr>
<tr>
<td>Low-density lipoprotein cholesterol (mg/dL)</td>
<td>133 ± 47</td>
<td>115 ± 34</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation.

Abbreviations: eGFR, estimated glomerular filtration rate; NS, not significant.

Figure 2. The expressions of hepassocin in STZ mice. Eight-week-old C57Bl/6 mice were intraperitoneally injected with 65 mg/kg STZ (STZ mice) for 5 days to induce hyperglycemic crisis. After the establishment of the hyperglycemic animal model, two groups of mice (n = 8 for each group) were injected with 1 IU/kg regular insulin or 1 mg/kg phlorizin three times a day for 7 days to correct hyperglycemia (a, left panel). After that, each group of mice was euthanized, and the liver samples were collected for the detection of hepassocin (HPS) expression by Western blots (a, right panel). (b) HepG2 hepatoma cells were serum-starved overnight and treated with different concentrations of glucose for 24 hours for the detection of hepassocin expression by Western blots. *P < 0.05 and **P < 0.01 as compared with control or indicated groups. Glu, glucose; I, insulin-treated STZ mice; Man, mannitol; P, phlorizin-treated STZ mice; S, STZ mice; W, wild-type.

Figure 1. The changes in plasma hepassocin concentrations and ALT in patients with hyperglycemic crisis before and after the treatment. A total of 21 patients with hyperglycemic crisis were enrolled, and the blood samples were collected before and after hyperglycemia treatment of the determination of (a) plasma hepassocin concentrations by enzyme-linked immunosorbent assay as well as (b) ALT levels. Closed circles indicate before the standard treatment; empty circles indicate after the standard treatment.
Fig. 4(e), we found the expressions of inflammation-related genes were significantly increased in STZ mice, and treatment with hepassocin significantly improved the hepatic inflammation in these. Moreover, the type 1 collagen was significantly decreased as compared with the STZ group [Fig. 4(f)]. Furthermore, we found that the expression of antioxidative stress proteins, including glutathione peroxidase and SOD1, was significantly increased after hepassocin treatment in STZ mice [Fig. 4(g)].

Hepassocin decreased ketone body–induced intracellular ROS through the increase in SOD1 expression

As shown in Fig. 5(a) and 5(b), we found that treatment with hepassocin decreased the AA-induced ROS production. After the fall in ROS levels, the AA-induced hepatocyte death was reversed, suggesting the hepatoprotection effect of hepassocin in hyperglycemic crisis [Fig. 5(c)]. In addition, the AA-induced decrease in SOD1 was reversed after hepassocin treatment in a dose-dependent manner [Fig. 5(d)]. We further investigated the detailed mechanisms in hepassocin-induced SOD1 expression. As shown in Fig. 5(e), the phosphorylation of ERK1/2 was significantly increased within 30 minutes in HepG2 cells treated with hepassocin. The expression of NRF2 was significantly elevated at 1 hour, whereas a significant rise in SOD1 expression was observed at 6 hours. Pretreatment with ERK1/2 inhibitor (U0126) in HepG2 cells reversed hepassocin-induced NRF2 and SOD1 expression [Fig. 5(f)].

Discussion

Hyperglycemic crisis is a metabolic emergency associated with uncontrolled diabetes that may result in various morbidities. Although it is known that ROS production by hyperglycemia leads to hepatic dysfunction (23),
only a mild increase in AST and ALT levels was observed in the patients with hyperglycemic crisis. To the best of our knowledge, this is the first study to explore the hepatic protection effect of hepassocin in hyperglycemia. We found that hyperglycemia induced hepassocin expression to exert a protective effect on the liver by increasing antioxidative stress, based on the results of human, cell, and animal studies.

It is known that elevated hepassocin levels are related to the development of nonalcoholic fatty liver disease, and these also activate ERK1/2 signaling to facilitate lipogenesis in hepatocytes (13). In addition, hepassocin serves as a link between nonalcoholic fatty liver disease and insulin resistance. Accumulation of the fatty acids in hepatocytes increases the expression of hepassocin, and the increased hepassocin in the circulation then disrupts insulin signaling in peripheral tissues and thus contributes to the development of type 2 diabetes (14). In this study, we found a significantly increased concentration of hepassocin in response to high plasma glucose levels among patients with hyperglycemic crisis. The increased hepassocin levels then protect against high glucose-induced glucotoxicity in the liver. Interestingly, although we found the levels of hepassocin and ALT changed in the same directions, they were not statistically significantly related. This is because although extreme hyperglycemia caused severe hepatic damage and induced hepassocin expression from hepatocytes, the elevated hepassocin then offset the deleterious effects of hyperglycemia. Clearly, the mildly increased AST levels in the hyperglycemic crisis indicate the hepatic protection effect of hepassocin had started well before the emergency visit. Therefore, the observed change in serum ALT levels depends on the net effects of hyperglycemia toxicity and hepassocin protection on hepatocytes, as well as the time point measured considering the half life of ALT.

Hepassocin is an acute-phase reactant that responds to IL-6 in a dose-dependent manner (24). IL-6 depends on STAT3 and HNF-1 binding sites in the hepassocin promoter to induce the promoter activity of hepassocin (11). In addition, fasting glucose concentrations are positively associated with the circulating hepassocin levels in human studies (12, 14), and correction of hyperglycemia decreases the expression of hepassocin in the liver (14). Furthermore, it is known that inhibition of PP2A decreases the DNA-binding activity of the HNF1, due to a net decrease in their total cellular and nuclear amounts (21). In the current study, we found that high glucose...
regulates hepassocin expression in an HNF1- and STAT3-dependent manner, which is consistent with previous studies. Moreover, high glucose increases the activity of PP2A by methylation (22, 25) and raises the activity of HNF1, and these effects further increase the expression of hepassocin.

It is known that transient but marked insulin resistance is present in virtually all patients with hyperglycemic crisis (26–29), and this severe insulin resistance is shared by both hepatic and peripheral, primarily muscle, tissues (27, 28). Interestingly, the comparable treatment efficacy of low- and high-dose insulin regimens indicates the presence of a state of extreme insulin resistance, which cannot be overcome by even very high insulin concentration (26). Therefore, the marked elevation in insulin-antagonistic hormones (such as catecholamines, growth hormones, cortisol, and glucagon) and severe metabolic derangements have been proposed to contribute to this insulin resistance (28). In the current study, we found that high glucose increased hepassocin expression to protect hepatocytes from glucotoxicity; however, the increased hepassocin levels might possibly induce extreme insulin resistance in patients with hyperglycemic crisis.

There are some limitations in this work. First, in the human study, owing to the relatively small number of participants with unprovoked hyperglycemic crisis and the lack of accurate assessment of dehydration clinically, this study may not have been able to completely exclude the effects of volume depletion on hepassocin levels. Although we demonstrated that hyperglycemia induced hepassocin expression to exert a protective effect on the liver in both cell and animal studies, future human studies with larger sample sizes are needed to more confidently support the effects of hyperglycemia on hepassocin. Second, due to the lack of studies investigating the metabolism and excretion of hepassocin in humans, we were not able to exclude with certainty the possibility that volume depletion may alter plasma hepassocin levels, even though none of the patients were diagnosed as having acute kidney injury according to the RIFLE (30) or AKIN criteria (31). Future studies exploring the effects of renal function on hepassocin may be needed.

Taken together, we provide evidence to clarify the pathophysiological role of hepassocin in hyperglycemic crisis. Hepassocin increases antioxidative stress proteins to protect hepatocytes from high glucose–induced glucotoxicity. Moreover, the increased hepassocin levels might be involved in the development of extreme insulin resistance in hyperglycemic crisis.

**Acknowledgments**

Address all correspondence and requests for reprints to: Horng-Yih Ou, MD, PhD, Division of Endocrinology and Metabolism, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung
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