RESEARCH ARTICLE

Diabetes Management

Serum hepassocin concentrations in diabetic patients with or without nonalcoholic fatty liver disease

Feng-Hwa Lu[1], Horng-Yih Ou[2], Hung-Tsung Wu[1,3], Hao-Chang Hung[2], Jin-Shang Wu[1], Yi-Ching Yang[1] & Chih-Jen Chang*[1,3]

Practice points

- Serum hepassocin concentrations are significantly increased in patients with diabetes and nonalcoholic fatty liver disease (NAFLD).
- Serum hepassocin concentrations are gradually increased in diabetic patients with NAFLD.
- Serum triglyceridelevel, fasting plasma glucose and NAFLD were significantly independently associated with hepassocin concentrations.
- Diabetes mellitus (DM) versus non-DM, NAFLD versus non-DM and DM + NAFLD versus non-DM were independently related to hepassocin concentrations.
- Increased hepassocin levels might have clinical implications and play a role as a biomarker for diabetes and NAFLD.

SUMMARY: **Aims:** Hepassocin is a hepatokine that is related to nonalcoholic fatty liver disease (NAFLD), and NAFLD is associated with increased risk of diabetes. However, the relationship between hepassocin, NAFLD and diabetes is thus far unknown. **Patients & methods:** A total of 200 age- and sex-matched nondiabetic (DM[-]), and newly diagnosed-diabetes subjects (DM[+]) with or without NAFLD were recruited. **Results:** Serum hepassocin concentrations were elevated in DM(+), NAFLD and DM(+) groups compared with the DM(-) group. Of the variables tested in the multivariate linear regression model, fasting plasma glucose, NAFLD and triglycerides were associated with hepassocin concentrations. **Conclusion:** Increased hepassocin levels might have clinical implications and play a role as a biomarker for diabetes and NAFLD.

KEYWORDS
• biomarkers • diabetes
• hepassocin • lipid profile
• nonalcoholic fatty liver disease

Hepassocin is a hepatokine that plays an important role in the regulation of hepatocyte proliferation [1]. It is expressed and secreted into the extracellular medium in cultured hepatocytes and further induces proliferation of the hepatocytes. Deletion of hepassocin results in hepatocyte growth inhibition and ERK1/2 inhibitor blocks hepatic cell proliferation induced by hepassocin [1]. Moreover, administration of hepassocin to rats protects against chemical-induced liver injury through a MAPK-dependent manner [2]. On the other hand, downregulated expression of hepassocin has been observed in heptocellular carcinoma cell lines and human tissues [3].

IL-6 is highly specific in confirming the absence of nonalcoholic steatohepatitis at normal values, as well as normal values of spleen

[1]Department of Family Medicine, National Cheng Kung University & Hospital, Tainan, Taiwan
[2]Division of Endocrinology & Metabolism, Department of Internal Medicine, National Cheng Kung University & Hospital, Tainan, Taiwan
[3]Research Center of Herbal Medicine, New Drugs & Nutritional Supplements, National Cheng Kung University, Tainan, Taiwan
*Author for correspondence: Tel.: +886 6 2353535 ext. 5210; Fax: +886 6 2754243; changcj.ncku@gmail.com

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longitudinal diameter. IL-6 is also strongly associated with NAFLD [4]. In addition, IL-6 plays an important role in the development of insulin resistance [5]. Hepassocin expression is upregulated by IL-6 in HepG2 cells through a hepatocyte nuclear factor (HNF)-dependent pathway [3], and HNF regulates the expression of gluconeogenesis-related enzymes. A recent study has demonstrated that the serum hepassocin concentration is significantly increased in subjects with nonalcoholic fatty liver disease (NAFLD). In addition, increase of hepatic hepassocin expression induces hepatic steatosis, and deletion of hepatic hepassocin ameliorates high-fat diet-induced hepatic steatosis in mice [6]. However, the relationship between hepassocin and diabetes was still unknown.

A previous study has shown that NAFLD is highly associated with Type 2 diabetes [7]. Since insulin resistance is the main pathogenic determinant of both NAFLD and diabetes, and it can facilitate triglyceride accumulation in the liver, Type 2 diabetes has the potential to promote the progression of NAFLD. Long-term storage of triglycerides in NAFLD increases hepatic oxidative stress and further aggravates insulin resistance [8,9]. Although the link between NAFLD and diabetes is well-established and increased hepassocin levels induce the development of NAFLD, so far there is no study exploring the association between NAFLD, Type 2 diabetes and serum hepassocin concentrations.

In this study, a total of 200 age- and sex-matched nondiabetic subjects (DM [-]), newly diagnosed diabetic patients (DM [+]), subjects with NAFLD, and DM(+) with NAFLD (DM[+] + NAFLD; n = 50 in each group) were included, and the aim of our study was to investigate the relationship between hepassocin and diabetic patients with or without NAFLD.

Patients & methods

• Study subjects

The Human Experiment and Ethics Committee of National Cheng Kung University (NCKU) Medical Center (Taiwan) approved the study protocol, and all eligible subjects gave written informed consent. From June 2007 to July 2008, all subjects who had been admitted for a physical checkup at the Preventive Health Center of NCKU Hospital in Taiwan were screened.

Subjects:

- Alcohol consumption ≥20 g/day in the last year;
- Serum aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels more than two-times the normal upper limit;
- A positive test for hepatitis B surface antigen, hepatitis C antibody and other causes of liver disease;
- Serum creatinine >133 μmol/l;
- Any acute or chronic inflammatory disease as determined by a leukocyte count >10,000/mm^3 or clinical signs of infection;
- Any other major diseases, including generalized inflammation or advanced malignant diseases;
- The enrolled subjects were taking any medication;
- The enrolled women were pregnant when tested.

• Biochemical analysis

The body height and weight of the study subjects wearing light indoor clothes were measured, and the BMI (in kg/m^2) was calculated. The blood pressure of each subject was measured by DINAMAP vital sign monitor (Critikon, Inc., CA, USA). Each subject was assessed by abdominal ultrasound to diagnose the presence or absence of NAFLD by an experienced radiologist with high-resolution ultrasonography (Xario SSA-660A; Toshiba, Nasu, Japan) using a 3.5-MHz linear transducer. After an overnight 12-h fast, all subjects received a blood test, including routine biochemistry. Blood glucose was measured by a hexokinase method (Roche Diagnostic GmbH, Mannheim, Germany). Serum insulin (intra-assay coefficient of variation [CV] <4%, inter-assay CV <3%; Mercodia AB, Uppsala, Sweden), hepassocin (intra-assay CV <10%, inter-assay CV <10%; Uscn Life Science Inc, Wuhan, China) and highly sensitive-C reactive protein (hs-CRP; intra-assay CV <3%, inter-assay CV <5%; Immunology Consultants Laboratory, OR, USA) were measured by ELISA kits. Insulin resistance was defined by the homeostasis model assessment-insulin resistance (HOMA-IR) index [10]. HbA_{1c} was measured with a high-performance liquid chromatographic method (Tosoh Automated Glycohemoglobin Analyzer HLC-723GHbVA1c 2.2; intra-assay CV of 0.5%, interassay CV of 2.0%; Tokyo, Japan). Serum total cholesterol, triglycerides and high-density lipoprotein

(HDL)-cholesterol levels were determined by an autoanalyzer (Hitachi 747E; Hitachi, Tokyo, Japan).

• Study design

The NAFLD diagnostic criteria included characteristic echo patterns of hepatorenal echo contrast, bright liver, deep (posterior beam) attenuation and vascular blurring [11]. Diabetes was defined according to American Diabetes Association criteria [12]: nondiabetes (normal glucose tolerance) defined if fasting plasma glucose was less than 100 mg/dl and 2-h post-load glucose was less than 140 mg/dl without a history of diabetes; diabetes defined if fasting plasma glucose was more than or equal to 126 mg/dl or 2-h postload glucose more than or equal to 200 mg/dl. None of the diabetic patients had been diagnosed as having diabetes or had been treated with insulin or an anti-diabetic agent before. Subjects with a systolic blood pressure (SBP) ≥140 mmHg or diastolic blood pressure (DBP) ≥90 mmHg were defined as suffering form hypertension. Smoking habit was defined as at least one pack/month for the past 6 months. Alcohol drinking habit was defined by at least one drink/week for the past 6 months. Habitual exercise was defined as vigorous exercise >three-times/week when the subjects engaged in activities sufficient to work up a sweat [13].

To avoid the confounding effects of age and sex, we selected subjects using the following algorithm of the previous study [14]: the study subjects were classified into four groups: DM(-), NAFLD, DM(+), and DM(+) with NAFLD, in the order of their admission to the study. Each consecutive index of DM(+) patient was matched to the other three groups or the closest age of the index subject (within ±1 year). Using this method, we selected 50 subjects for each group.

• Statistical analysis

SPSS software (version 17.0; SPSS, IL, USA) was used for statistical analysis. All normally distributed continuous variables are expressed as means ± standard deviation. The continuous variables among groups were compared using analysis of variance or a Kruskal–Wallis test when the distribution of the variable was not normal. Chi-square tests were used to analyze the differences in categorical variables among groups. Serum hepassocin concentration was set as a continuous dependent variable, and the associations between serum hepassocin and individual variables were examined using multivariate linear regression analysis. The independent variables included age, sex, BMI, fasting plasma glucose, insulin, HOMA-IR, systolic blood pressure, ALT, creatinine, log triglyceride, HDL-cholesterol, hs-CRP, NAFLD, hypertension, DM(+) versus DM(-), NAFLD versus DM(-), DM(+) + NAFLD versus DM(-), smoking habit, habitual exercise, and alcohol consumption. $p < 0.05$ was considered statistically significant.

Results

A total of 200 age- and sex-matched subjects with DM(-), DM(+), NAFLD and DM(+) + NAFLD (n = 50 for each group) were included. Serum hepassocin concentrations gradually increased from DM(-), DM(+), NAFLD to DM(+) + NAFLD groups (test for trend; $p < 0.001$) **(Figure 1)**. *Post hoc* tests showed that subjects with DM(-), NAFLD and DM (+) + NAFLD (7193.2 ± 2072.5, 7423.3 ± 2106.7 and 7864.5 ± 1573.5 μg/ml, respectively; $p < 0.001$) had significantly higher serum hepassocin levels than those without DM (4843.6 ± 1176.8 μg/ml; $p < 0.001$), but there were no significant differences among DM(+), NAFLD and DM(+) + NAFLD groups.

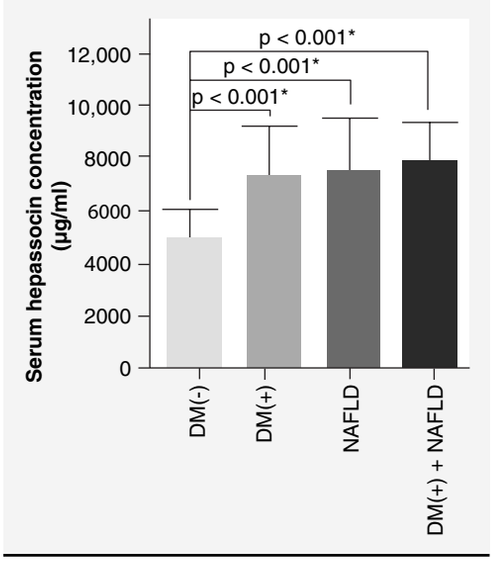

Figure 1. Comparison of serum hepassocin concentration among nondiabetic subjects, newly diagnosed diabetes patients, subjects with nonalcoholic fatty liver disease, and DM[+] with nonalcoholic fatty liver disease.
**Post-hoc* test.
DM(-): Nondiabetic subjects; DM(+): Newly diagnosed diabetic subjects; NAFLD: Nonalcoholic fatty liver disease.

Table 1. Clinical characteristics of the study subjects.

Characteristic	DM(-)	DM(+)	NAFLD	DM(+) + NAFLD	p-value
n	50	50	50	50	--
Age (years)	61.72 ± 11.46	61.98 ± 11.49	61.46 ± 10.31	61.61 ± 10.82	NS
Sex (F/M)	20/30	20/30	20/30	20/30	NS
BMI (kg/m^2)	22.85 ± 2.64	23.60 ± 3.00	26.23 ± 3.14	27.34 ± 2.78	<0.001
Systolic blood pressure (mmHg)	123.45 ± 17.34	127.80 ± 18.63	125.34 ± 14.40	136.66 ± 17.23	<0.01
Diastolic blood pressure (mmHg)	71.86 ± 9.58	74.67 ± 10.40	74.05 ± 8.29	80.89 ± 12.04	<0.001
Fasting plasma glucose (mmol/l)	4.78 ± 0.40	7.27 ± 3.38	4.88 ± 0.34	8.14 ± 2.58	<0.001
Insulin (μU/ml)	2.48 ± 2.63	2.66 ± 2.48	4.91 ± 2.38	7.95 ± 4.45	<0.001
HbA_{1c} (%)	5.65 ± 0.29	7.35 ± 2.05	5.84 ± 0.34	8.04 ± 2.19	<0.001
ALT (U/l)	23.22 ± 9.32	28.76 ± 21.88	31.30 ± 11.81	45.70 ± 33.02	<0.001
AST (U/l)	25.68 ± 6.41	27.00 ± 13.43	28.28 ± 7.75	35.40 ± 16.51	<0.001
Creatinine (mmol/l)	75.37 ± 15.11	74.96 ± 21.09	76.87 ± 15.11	78.50 ± 18.62	NS
hs-CRP (mg/l)	2.83 ± 4.38	6.72 ± 9.69	4.72 ± 8.23	6.35 ± 7.52	<0.001
Total cholesterol (mmol/l)	5.34 ± 1.04	5.39 ± 1.22	5.54 ± 1.08	5.31 ± 0.99	NS
Triglyceride (mmol/l)†	1.13 ± 0.45	1.63 ± 1.23	1.81 ± 0.82	2.11 ± 1.68	<0.001
HDL cholesterol (mmol/l)	1.55 ± 0.44	1.29 ± 0.29	1.24 ± 0.24	1.19 ± 0.25	<0.001
HOMA-IR	0.52 ± 0.52	0.90 ± 0.88	1.06 ± 0.52	2.96 ± 2.67	<0.001
Hypertension (%)	24	18	12	34	NS
Habitual exercise (%)	2	4	2	0	NS
Smoking habit (%)	6	8	8	2	NS
Alcohol consumption (%)	2	0	2	0	NS

A total of 200 age- and sex-matched subjects were enrolled in this study, and divided into DM(-), DM(+), NAFLD, and DM (+) + NAFLD groups (n = 50 for each group) to determine the serum concentrations of hepassocin. All values are given as % or means ± standard deviation.†Kruskal–Wallis test.†

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; F: Female; HDL: High-density lipoprotein; HOMA-IR: Homeostasis Model Assessment-Insulin Resistance; hs-CRP: Highly sensitive C-reactive protein; M: Male; NAFLD: Nonalcoholic fatty liver disease; NS: Not significant.

In addition, there were significant differences in BMI, SBP, DBP, FPG, insulin, ALT, AST, hs-CRP, triglyceride, HDL-cholesterol and HOMA-IR among tested groups **(Table 1)**.

To further determine the independent factors associated with serum hepassocin levels, we performed multiple linear regression analysis. Both stepwise and backward selection strategies showed a consistent result, which is summarized in **Table 2**. The results of multivariate linear regression analysis showed that FPG (β = 9.65; 95% CI: 3.20–16.11; p = 0.004), NAFLD (β = 1022.12; 95% CI: 339.80–1704.43; p = 0.004) and triglyceride (β = 1596.83; 95% CI: 25.64–3168.01; p = 0.046) were positively related to serum hepassocin concentrations after adjustment for the confounders (model 1). We then used DM(-) as a reference group, and found that DM(+) (β = 2045.68; 95% CI: 1317.7–2773.63; p < 0.001), NAFLD (β = 2037.74; 95% CI: 1240.46–2835.02; p < 0.001), DM(+) + NAFLD (β = 2204.54; 95% CI: 1251.90–3157.18; p < 0.001) and triglyceride (β = 1794.25; 95% CI: 344.52–3243.98; p = 0.016) were independently associated factors of serum hepassocin concentrations after adjustment for the confounders (model 2).

Discussion

To the best of our knowledge, this is the first study addressing the relationship between serum hepassocin, diabetes and NAFLD. We found that both NAFLD and NDD are independently associated factors of serum hepassocin

concentration after adjusting for other metabolic risk factors.

Recently, we demonstrated the causal relationship between hepassocin and NAFLD [6]. We found that hepassocin affects the phosphorylation of ERK1/2 to increase the activity of sterol regulatory element-binding protein, and further induce lipogenesis in the liver of experimental animals. Furthermore, the steatogenic reagent, oleic acid, not only increased triglyceride accumulation in hepatocytes, but also increased hepassocin expression in a dose-dependent manner. Consistent with these findings, we found in the present study that both triglyceride and NAFLD are independently associated factors of serum hepassocin concentrations.

Previous studies have shown that HNF-1 plays an important role in the regulation of hepassocin expression [3], and HNF-1 can regulate several hepatic metabolic genes involved in gluconeogenesis, such as glucose 6-phosphatase [15] and phosphoenolpyruvatecarboxykinase [16]. However, the relationship between hepassocin and glucose metabolism is still unknown. In this study, we found that both FPG and diabetes were independently associated with hepassocin, implying that hepassocin might play a role in the regulation of hepatic glucose output and gluconeogenesis. Although the causal relationship between hepassocin and diabetes is still unknown, activation of ERK1/2 by hepassocin might possibly play a role in the development of insulin resistance [17]. Further studies are required to investigate the detailed metabolic effects of hepassocin in the regulation of lipogenesis, as well as hepatic insulin sensitivity and gluconeogenesis.

There are some limitations in this work. First, the cross-sectional design of our study does not allow for causal inference between serum hepassocin concentrations and diabetes. Second, the diagnosis of NAFLD in this work was made by ultrasound, but not gold-standard liver biopsy. Although it is liver histology that dictates the natural course of hepatic and extrahepatic disease, several factors might be helpful for the

Table 2. Regression analysis between serum hepassocin concentrations and clinical variables.

Characteristic	Model 1		Model 2	
	β (95% CI)	*p-value*	*β (95% CI)*	*p-value*
Age	17.74 (-10.45–45.93)	NS	14.07 (-11.05–39.20)	NS
Sex	-256.35 (-1055.47–542.77)	NS	-141.75 (-892.93–609.43)	NS
BMI	55.14 (-42.13–152.41)	NS	64.57 (-25.85–155.00)	NS
Fasting plasma glucose	9.65 (3.20–16.11)	0.004	–	
Insulin	28.15 (-61.30–117.61)	NS	–	
HOMA-IR	–		112.16 (-94.22–318.53)	NS
Systolic blood pressure	-0.68 (-17.67–16.32)	NS	–	
ALT	2.55 (-10.25–15.34)	NS	1.21 (-11.12–13.54)	NS
Creatinine	3.93 (-1887.11–1894.97)	NS	117.40 (-1668.25–1903.05)	NS
Log triglyceride	1596.82 (25.64–3168.01)	0.046	1794.25 (344.52–3243.98)	0.016
HDL-cholesterol	-1.24 (-25.28–22.81)	NS	11.20 (-12.16–34.57)	NS
hs-CRP	0.21 (-36.46–36.88)	NS	-5.10 (-39.94–29.73)	NS
NAFLD (yes vs no)	1022.12 (339.81–1704.43)	0.004	–	
Hypertension (yes vs no)	–		96.73 (-522.85–716.31)	NS
DM(+) vs DM(-)	–		2045.68 (1317.72–2773.63)	<0.001
NAFLD vs DM(-)	–		2037.74 (1240.46–2835.02)	<0.001
DM(+) + NAFLD vs DM(-)	–		2204.54 (1251.90–3157.18)	<0.001
Smoking habit (yes vs no)	997.06 (-237.14–2231.25)	NS	884.94 (-278.43–2048.31)	NS
Habitual exercise (yes vs no)	652.51 (-1193.16–2498.17)	NS	396.94 (-1351.80–2145.67)	NS
Alcohol consumption (yes vs no)	-565.50 (-3499.49–2368.50)	NS	-703.08 (-3476.25–2070.09)	NS

Multivariate linear regression analysis was performed to identify independent predictors of the serum concentration of hepassocin. Dependent variable: serum hepassocin concentration.

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HDL: High-density lipoprotein; HOMA-IR: Homeostasis Model Assessment-Insulin Resistance; hs-CRP: Highly sensitive-C-reactive protein; NAFLD: Nonalcoholic fatty liver disease; NS: Not significant.

diagnosis of NAFLD [18,19]. Abdominal ultrasonography with a scoring system could provide accurate information about hepatic steatosis, visceral obesity and the metabolic syndrome [20,21]. Thus, ultrasound is an established noninvasive tool used as a screening modality with acceptable sensitivity and specificity [22]. In addition, to minimize the interobserver variability, a single experienced radiologist performed the ultrasound examinations in this work.

Conclusion & future perspective

Although the prevalence of diabetes is highly correlated with NALFD, and the development of NAFLD is a risk factor to insulin resistance [8], the causal relationship between diabetes and NAFLD is still obscure [23]. In this study, we found that serum hepassocin concentrations were significantly increased in NAFLD subjects and diabetic patients, and we provided a new concept that both diabetes and NAFLD are important independent factors that are associated with serum concentrations of hepassocin. According to the role of hepassocin in the development of NAFLD [6], we speculate that hepassocin may promote steatogenesis in patients with diabetes. On the other hand, the results from a previous study imply that hepassocin might play a role in diabetes [3], but the mechanistic role of hepassocin in the development of diabetes on those with NAFLD is still unknown. Thus, hepassoin might be a missing link between NAFLD and diabetes, and future studies are needed to explore the possible mechanisms involved in elevating hepassocin levels and its clinical implications in the pathogenesis of diabetes.

Author contributions

F-H Lu and C-J Chang were responsible for designing and conducting the study, interpreting the data, and writing the manuscript. H-Y Ou, H-T Wu, and H-C Hung contributed to data collection, analysis, and interpretation. J-S Wu, and Y-C Yang contributed to data interpretation.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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