Plasma levels of PBEF/Nampt/visfatin are decreased in patients with liver cirrhosis

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Plasma levels of PBEF/Nampt/visfatin are decreased in patients with liver cirrhosis. Am J Physiol Gastrointest Liver Physiol 296: G196–G201, 2009. First published December 12, 2008; doi:10.1152/ajpgi.00029.2008.—Liver cirrhosis is a catabolic disease associated with a higher arterial ketone body ratio (KBR) and higher hepatic glucose production (P = 0.01). Consistent with these data, hepatic visfatin mRNA expression was significantly lower in cirrhotic livers (P < 0.05). Circulating visfatin in cirrhosis was correlated with body cell mass (r = 0.72, P < 0.01) as well as with body fat mass (r = 0.53, P < 0.05) but not with plasma glucose, insulin, the degree of insulin resistance, or whole body glucose oxidation rates. Higher visfatin levels were associated with a higher hepatic glucose production (r = 0.53, P < 0.05) and also with a higher arterial ketone body ratio (KBR) (r = 0.48, P < 0.05), an indicator of increased hepatic NAD generation. In conclusion, circulating visfatin is a potential indicator of increased hepatic NAD generation. Visfatin is a 52-kDa protein that has been cloned already several years ago as pre-B cell colony-enhancing factor (PBEF), and interestingly, liver and muscle have been reported to be the tissues with the highest expression levels of this protein (23).

MATERIALS AND METHODS

Patients. We studied 19 patients with histologically proven liver cirrhosis in different clinical stages of the disease (Child A = 5, Child B = 7, Child C = 7) and attributable to different etiologies (post-

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hepatic flow (HPF) was calculated as: HPF = IICG/(ICGa-ChVa) \times 60, with IICG representing the individual ICG infusion rate (mg/h), and ICGa-ChVa representing the hepatic venous ICG concentration (mg/l).

Laboratory analyses. Blood samples were immediately placed on crushed ice and processed without delay, and aliquots were stored at −80°C until analysis. Glucose, free fatty acids, acetacetate, and 3-hydroxybutyrate levels were measured enzymatically using commercially available assay kits (Boehringer Mannheim, Mannheim, Germany). Plasma alanine levels were determined by ion-exchange chromatography on an automated AA analyzer (Liquimat 5001; Biotronic, Maintal, Germany) essentially as described (29). Percent hepatic extraction/production was calculated by dividing the arterial hepatic venous/hepatic venous arterial concentration differences by the respective arterial concentration values. Hepatic extraction rates per minute were calculated by multiplying the respective arteriohepatic venous concentration differences with the HPF as follows: (C1a − C0a) × HPF; hepatic production rates were calculated as follows: (C1b − C0a) × HPF (31).

A commercially available radioimmunoassay was used to determine plasma concentrations of insulin (Pharmacl Insulin RIA 100; Pharmacia Diagnostics, Uppsala, Sweden). Visfatin was measured with a commercially available ELISA according to the manufacturer’s instructions (Phoenix Pharmaceuticals, Burlingame, CA). Concentrations of TNF-α, IL-6, and IL-1β were assessed with commercially available ELISAs (Medgenix Diagnostics, Brussels, Belgium).

Quantitative insulin sensitivity check index and homeostasis model assessment of insulin resistance as indices of insulin resistance were calculated essentially as published (16).

Indirect calorimetry and body composition analysis. Resting energy expenditure was measured by indirect calorimetry with a ventilated open hood (Deltatrac metabolic monitor; Datex Instruments, Helsinki, Finland) as published previously (3). Substrate oxidation rates for carbohydrates, fat, and protein were calculated using previously published formulas (29). Body composition was determined by bioelectrical impedance analysis (BIA 101; RJL Systems, Detroit, MI) to assess body cell mass (BCM) and body fat mass exactly as described (31).

Immunohistochemistry. Deparaffinized slides of formalin-fixed material were blocked with 4% normal goat serum (Dako, Glostrup, Denmark) and incubated with a rabbit anti-human visfatin antibody (H-003–84, Phoenix Pharmaceuticals) at a dilution of 1:300 in PBS + 0.1% BSA for 1.5 h. After being washed, the slides were incubated with a peroxidase-conjugated goat anti-rabbit antibody (Dako) at a dilution of 1:100 for 30 min and washed again. Antibody binding was visualized using diaminobenzidine as a peroxidase substrate.

Analysis of gene expression by real-time quantitative PCR. RNA was extracted from liver samples using Tri-reagent (Sigma St. Louis, MO, USA) and quantified with a NanoDrop ND-100 UV-Vis spectrophotometer. A sample (1 μg) of RNA was reverse transcripted using M-MLV reverse transcriptase (Sigma) according to the manufacturer’s instructions. Real-time qPCR analysis was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany) using multiexon-spanning primer/probe sets designed to exclude multiplication of genomic DNA. Visfatin mRNA was amplified using the following oligonucleotides: 5’-CTCCGCCTCCTGATATGATTGGTTTATAGTGAGTAACCTTG-3’ (forward primer), 5’-TGTTTATAGTGAGTAACCTTG-TGTTTATAGTGAGTAACCTTG-3’ (reverse primer), and 5’-CGGCCAGAGGC-CAATTCACAGCTTTCCAGCAGGTTGATTGTTAAT-3’ (probe). Gene expression was normalized to cyclophilin, which was amplified using the following oligonucleotides: 5’-CTCCGCCTCCTGATATGATTGGTTTATAGTGAGTAACCTTG-3’ (forward primer), 5’-TGTTTATAGTGAGTAACCTTG-TGTTTATAGTGAGTAACCTTG-3’ (reverse primer), and 5’-CAACGCTCCGTCTTTTTTGGATACCAGTTTAC-3’ (probe); it was further normalized to the expression level of the controls.

Statistics. Statistical analyses were performed using the statistical package for social sciences (SPSS, SPSS, Chicago, IL). Data are expressed as means ± SE. Kruskal-Wallis analysis of variance was used to compare values of three or more different groups. Either the Mann-Whitney’s U-test or the Student’s t-test was used for unpaired

Table 1. Clinical data of the patients with liver cirrhosis and controls

| Clinical data of the patients with liver cirrhosis and controls                                                                 |
|---|---|
| Cirrhosis (all) | Controls |
| n=19 | n=19 |
| Age, years | 47.4±2.5 | 46.7±1.4 |
| BMI, kg/m² | 23.0±0.6 | 22.8±0.6 |
| Male/female | 13/6 | 12/7 |
| Bilirubin, μmol/l | 41±6 | 121±1 * |
| Albumin, g/l | 32±2 | 45±2 * |
| Prothrombin time, % | 70±3 | 99±2 * |
| AST, U/l | 34±5 | 24±2 * |
| ALT, U/l | 31±4 | 22±2 |
| γ-GT, U/l | 80±13 | 17±2 * |
| Glucose, mmol/l | 5.9±0.3 | 5.3±0.1 * |
| Insulin, mU/l | 18±3 | 5±1 * |
| HOMA-IR | 4.67±0.67 | 1.27±0.16 * |
| QUICKI | 0.315±0.006 | 0.419±0.008 * |

Data are given as means ± SE. Results significantly different from cirrhosis: *P < 0.05 or less. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostasis model assessment of insulin resistance; QUICKI, quantitative insulin sensitivity check index.
data. Spearman’s rank correlation coefficient was used to assess possible associations between different parameters. Stepwise regression analysis was performed to determine the association of different parameters with visfatin as the dependent variable. P values < 0.05 were considered statistically significant.

RESULTS

Circulating visfatin levels are decreased in liver cirrhosis attributable to decreased hepatic expression and secretion. Patients with liver cirrhosis exhibited drastically reduced circulating visfatin levels compared with age-, sex-, and BMI-matched healthy control subjects (16.0 ± 1.8 vs. 80.4 ± 8.6 ng/ml, P < 0.001, Fig. 1A). Patients in early clinical stages of cirrhosis had already decreased plasma visfatin levels (Child A: 23.9 ± 3.2 ng/ml) that were, however, significantly higher compared with Child B or Child C patients (14.0 ± 2.0, 12.3 ± 2.6 ng/ml, respectively, each P < 0.05 to Child A, Fig. 1A). One reason for decreased circulating visfatin with worsening of the clinical stage could be reduced hepatic production since Child A patients had significantly higher hepatic venous visfatin levels (69.2 ± 25.8% increased compared with arterial, Fig. 1B) as well as per minute splanchnic visfatin production rates (4.86 ± 2.04 µg/min, Fig. 1C) compared with Child B and C patients (−5.6 ± 9.2 vs. −8.6 ± 5.4% increased compared with arterial, respectively, each P < 0.05 to Child A, Fig. 1B; 0.01 ± 0.21 vs. −1.17 ± 0.81 µg/min, respectively, each P < 0.05 to Child A, Fig. 1C). The different underlying etiologies of liver cirrhosis had no significant impact on plasma visfatin levels or on hepatic visfatin production. In addition, no significant differences were found comparing male and female patients.

Circulating visfatin correlated well with the half-life time of ICG, a sensitive test compound to assess liver function (r = −0.57, P = 0.01, Fig. 2) providing additional support for the impact of liver function on plasma visfatin levels. On the other hand, plasma visfatin levels were not associated with portal pressure as assessed by the HVPG (r = 0.35, not significant, ns). To further investigate the basis of decreased plasma visfatin levels in cirrhosis, hepatic visfatin expression was determined. First, we performed immunohistochemistry on healthy control livers and found substantial visfatin protein expression in hepatocytes that was reduced in livers from patients with liver cirrhosis (Fig. 3A). Given methodological difficulties to quantitate changes in expression levels with immunohistochemistry, we next used quantitative real-time PCR to determine hepatic visfatin mRNA expression. In control livers visfatin expression with Ct values between 20 and 21 on healthy control livers and found substantial visfatin protein expression in hepatocytes that was reduced in livers from patients with liver cirrhosis (Fig. 3A). Given methodological difficulties to quantitate changes in expression levels with immunohistochemistry, we next used quantitative real-time PCR to determine hepatic visfatin mRNA expression. In control livers visfatin expression with Ct values between 20 and 21 indicated a relatively high abundance of the visfatin transcript within liver. Interestingly, in cirrhotic livers mRNA expression of visfatin was significantly decreased (1.00 ± 0.07 vs. 0.68 ± 0.11, P < 0.05, Fig. 3B), pointing toward decreased hepatic expression as the basis for the decreased hepatic production rates.

Plasma visfatin levels correlate with parameters of body composition but not with proinflammatory cytokines or energy metabolism. Since plasma visfatin levels have been reported to correlate with fat mass in patients without liver disease (5), we assessed a possible association of visfatin with parameters of body composition in patients with cirrhosis. Body fat mass (r = 0.53, P < 0.05, Fig. 4A) and BCM representing a reflection of muscle mass (r = 0.72, P < 0.01, Fig. 4B) were both positively correlated with plasma visfatin levels.
To assess a potential contribution of the proinflammatory state of cirrhosis to circulating visfatin levels, plasma visfatin was correlated with several proinflammatory cytokines. However, neither plasma levels of TNF-\(\alpha\) (\(r = 0.19\), ns), IL-6 (\(r = 0.11\), ns), nor IL-1\(\beta\) (\(r = 0.08\), ns) showed a significant association with plasma visfatin levels.

In addition, no correlations were observed between circulating visfatin levels and BMI (\(r = 0.23\), ns), energy expenditure (\(r = 0.21\), ns), and the total body oxidation rates of carbohydrates (\(r = 0.03\), ns), fat (\(r = 0.31\), ns), and protein (\(r = 0.29\), ns) in patients with cirrhosis.

Circulating visfatin levels in cirrhosis are not related to insulin resistance but rather to hepatic glucose production. In patients with cirrhosis, circulating visfatin levels did not correlate with plasma glucose (\(r = 0.33\), ns), insulin (\(r = -0.14\), ns), and free fatty acids (\(r = 0.03\), ns) or two established indices for the assessment of insulin resistance (16), quantitative insulin sensitivity check index (\(r = -0.36\), ns) and homeostasis model assessment of insulin resistance (\(r = -0.05\), ns).

However, circulating visfatin levels were positively correlated with hepatic glucose production (\(r = 0.53\), \(P < 0.05\), Fig. 5A) and with the extraction of alanine, the major gluconeogenic amino acid (\(r = 0.62\), \(P < 0.01\), Fig. 5B), indicating that visfatin might impact on hepatic metabolism. The correlations of plasma visfatin with hepatic glucose production and alanine extraction were both not dependent on Child class.

*Plasma visfatin levels in cirrhosis are associated with the arterial ketone body ratio.* The arterial ketone body ratio (KBR) has been established as an index reflecting the hepatic NAD\(^+/\)NADH ratio (28, 35). Visfatin has been demonstrated to participate in NAD\(^+\) generation (18, 34). Interestingly, the arterial KBR in patients with cirrhosis was significantly positively correlated with circulating visfatin levels (\(r = 0.48\), \(P < 0.05\), Fig. 6).

To delineate the interactions between the different parameters significantly associated with plasma visfatin levels in patients with liver cirrhosis, a multiple regression analysis with a stepwise model was used. Including ICG half-life, body fat mass, BCM, and the arterial KBR, this analysis revealed that BCM and ICG half-life were independently associated with circulating visfatin levels [visfatin (ng/ml) = 0.760 \times BCM (kg) − 0.666 \times ICG half-life (min) + 5.482, \(r = 0.913\), \(P = 0.001\)].
DISCUSSION

The results of this study demonstrate that 1) plasma visfatin levels are dramatically decreased in patients with liver cirrhosis, presumably attributable to decreased hepatic expression and secretion, 2) visfatin in cirrhosis is associated with hepatic glucose production, and 3) visfatin in cirrhosis correlates with the arterial KBR, an index reflecting the hepatic NAD\(^+/\)NADH ratio.

The patients with liver cirrhosis investigated in this study showed a dramatic reduction in circulating visfatin levels compared with age-, sex-, and BMI-matched controls. Visfatin is expressed at highest levels in liver, muscle, and bone marrow but also in adipose tissue (8, 23). Our data suggest that one contributing factor to decreased visfatin levels in cirrhosis is decreased hepatic production. After showing visfatin protein expression in hepatocytes within human liver by immunohistochemistry, we demonstrated that, on the mRNA level, hepatic visfatin expression is decreased in cirrhotic livers compared with controls. In line with these results was the finding that Child A patients had significant hepatic visfatin secretion along with higher circulating levels, whereas worsening of the clinical stage was associated with no discernable hepatic visfatin production along with even lower circulating levels. The relationship between plasma visfatin and liver function is further underscored by the negative correlation of circulating visfatin with the ICG half-life time. ICG is a sensitive test substance for liver function assessing decreased functional hepatocellular capacity as well as capillarization of the hepatic sinusoids (22, 30). However, we cannot exclude that also the contribution of extrahepatic tissues to plasma visfatin levels is lower in cirrhosis. The positive correlation of visfatin with BCM, a surrogate measure for muscle mass, and with body fat mass might indicate a decreased contribution of muscle and adipose tissue, respectively, to circulating visfatin levels. The observed correlation between plasma visfatin and fat mass is in line with some studies conducted in patients without liver disease (8), whereas others could not detect such an association (5). Weight loss, though, has been associated with a concomitant decrease in plasma visfatin levels (9, 10).

In addition, our data demonstrate for the first time that circulating visfatin levels are not correlated with insulin levels or insulin resistance in cirrhosis. Data in the literature regarding the relationship between plasma visfatin and insulin resistance in patients without liver disease are somewhat conflicting (1, 25). There are studies reporting higher visfatin levels in patients with type 2 DM (7, 8), but it has also been reported that visfatin is not linked to insulin levels or insulin resistance (2, 5, 27, 33). An interesting finding of our study is the positive correlation of plasma visfatin levels with hepatic glucose production, indicating that visfatin might have some impact on glucose metabolism. Increased hepatic glucose production occurs in classical DM and is thought to be a major contributor to hyperglycemia (4, 20). Further in vivo studies are required in experimental animals as well as humans to clarify the possible impact of visfatin on hepatic glucose metabolism, an aspect that has not been explored thus far.

Another potentially important result of our study is the positive correlation of plasma visfatin with the arterial KBR. The arterial KBR has been established as an index reflecting the hepatic NAD\(^+/\)NADH ratio (28, 35), and visfatin has been demonstrated to participate in NAD+ generation (18, 19, 34). To our knowledge this is the first indication of a possible link between visfatin and NAD generation in vivo in humans. Visfatin-mediated NAD biosynthesis might connect this adipokine to metabolism via altering the activity of NAD-dependent factors (19). Among these, Sirt1, a member of the NAD-dependent Sir2 family, has been proposed as an interesting and intriguing candidate (19). Sirt1 regulates insulin secretion in pancreatic \(\beta\) cells (6, 13). Even more important might be the effect of Sirt1 on hepatic glucose production since it has been demonstrated that induction of Sirt1 results in increased glu-

Fig. 5. Correlation between circulating visfatin levels and hepatic glucose production (A) and hepatic alanine extraction (B) in patients with liver cirrhosis.

Fig. 6. Correlation between circulating visfatin levels and the arterial ketone body ratio as an index reflecting the hepatic NAD\(^+/\)NADH ratio in patients with liver cirrhosis.
coseogenesis and increased hepatic glucose output (21). Although this concept would require extensive validation including the use of experimental animal models, it is tempting to speculate that visfatin-mediated NAD generation modulates the activity of Sir2. Such a scenario might provide an explanation for the positive correlation of visfatin and hepatic glucose production observed in our study.

In summary, we established that in liver cirrhosis circulating visfatin levels are significantly decreased, presumably attributable to decreased hepatic expression and secretion. Plasma visfatin in cirrhosis is not associated with insulin resistance but correlates with hepatic glucose production and the arterial KBR, potentially indicating a link between the NAD-generating properties of visfatin and metabolism. However, alterations in circulating visfatin are not suitable to explain IGT and DM in patients with cirrhosis.

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REFERENCES