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Nonalcoholic Fatty Liver Disease in Humans Is Associated with Increased Plasma Endotoxin and Plasminogen Activator Inhibitor 1 Concentrations and with Fructose Intake[1]

Sabine Thuy,[2] Ruth Ladurner,[3] Valentina Volynets,[2] Silvia Wagner,[3] Stefan Strahl,[4] Alfred Königsrainer,[3] Klaus-Peter Maier,[4] Stephan C. Bischoff,[2] and Ina Bergheim[1]*

[2]Department of Nutritional Medicine (180a), University of Hohenheim, 70599 Stuttgart, Germany; [3]Department of General, Visceral and Transplant Surgery, Tuebingen University Hospital, 72076 Tuebingen, Germany; and [4]Liver Center, City Hospital Esslingen, 73730 Esslingen, Germany

Abstract

Results of animal experiments suggest that consumption of refined carbohydrates (e.g. fructose) can result in small intestinal bacterial overgrowth and increased intestinal permeability, thereby contributing to the development of nonalcoholic fatty liver disease (NAFLD). Furthermore, increased plasminogen activator inhibitor (PAI)-1 has been linked to liver damage of various etiologies (e.g. alcohol, endotoxin, nonalcoholic). The aim of the present pilot study was to compare dietary factors, endotoxin, and PAI-1 concentrations between NAFLD patients and controls. We assessed the dietary intake of 12 patients with NAFLD and 6 control subjects. Plasma endotoxin and PAI-1 concentrations as well as hepatic expression of PAI-1 and toll-like receptor (TLR) 4 mRNA were determined. Despite similar total energy, fat, protein, and carbohydrate intakes, patients with NAFLD consumed significantly more fructose than controls. Endotoxin and PAI-1 plasma concentrations as well as hepatic TLR4 and PAI-1 mRNA expression of NAFLD patients were significantly higher than in controls. The plasma PAI-1 concentration was positively correlated with the plasma endotoxin concentration (Spearman $r = 0.83$; $P < 0.005$) and hepatic TLR4 mRNA expression (Spearman $r = 0.54$; $P < 0.05$). Hepatic mRNA expression of PAI-1 was positively associated with dietary intakes of carbohydrates (Spearman $r = 0.67$; $P < 0.01$), glucose (Spearman $r = 0.58$; $P < 0.01$), fructose (Spearman $r = 0.58$; $P < 0.01$), and sucrose (Spearman $r = 0.70$; $P < 0.01$). In conclusion, our results suggest that dietary fructose intake, increased intestinal translocation of bacterial endotoxin, and PAI-1 may contribute to the development of NAFLD in humans. J. Nutr. 138: 1452–1455, 2008.

Introduction

Nonalcoholic fatty liver disease (NAFLD)[5] usually develops in the setting of obesity and insulin resistance (1) and ranges from simple steatosis to steatohepatitis and cirrhosis. As the mechanisms involved in the development of NAFLD are not yet fully clarified, therapeutic options are still limited. Therefore, a better understanding of the biochemical and pathological changes associated with the development of NAFLD in humans is desirable to improve intervention strategies.

High dietary carbohydrate intake has been claimed to be a key factor in the development of NAFLD. Indeed, results of recent human studies suggest that a diet rich in carbohydrates may be a major cause of NAFLD, increasing the odds of later stages of the disease (2,3). In animal studies, an increased consumption of fructose (e.g. up to 60% of daily energy derived from fructose) may result in increased lipid accumulation in the liver accompanied by insulin resistance, elevated plasma triglyceride concentration, and oxidative stress (4–7). Furthermore, we recently found that moderate fructose consumption can lead to increased intestinal translocation of bacterial endotoxin, induction of hepatic tumor necrosis factor (TNF) α, and subsequently liver steatosis in mice (5). In these studies, the concomitant treatment with antibiotics almost completely blocked the effect of fructose on mouse liver (5).

Increased plasminogen activator inhibitor 1 (PAI-1) concentrations have been linked to not only liver fibrosis but also to earlier stages (e.g. steatosis) of alcoholic liver disease and NAFLD (8–10). For instance, it has been shown that in morbidly obese patients and genetically obese mice (*ob/ob* mice), PAI-1 concentration is related to liver steatosis (8). Furthermore, results of studies performed in animal models of alcohol liver disease suggest that in the early stage of the disease, PAI-1 is a key modulator of hepatic lipid transport, whereas in later stages

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[5] Abbreviations used: NAFLD, nonalcoholic fatty liver disease; PAI-1, plasminogen activator inhibitor 1; TLR4; toll-like receptor 4; TNF, tumor necrosis factor.
* To whom correspondence should be addressed. E-mail: bergheim@uni-hohenheim.de.

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of the disease (e.g. steatohepatitis), PAI-1 contributes to inflammation (9). Starting from this background, the aim of this study was to assess dietary intake, endotoxin, and PAI-1 concentration of NAFLD patients and controls to further investigate the mechanisms involved in the development of NAFLD in humans.

Patients and Methods

Patients. The study protocol was approved by the ethics committee of the Tuebingen University Hospital (Tuebingen, Germany). Written informed consent was obtained from all subjects. Subjects included had no: *1*) history of taking lipid-lowering drugs or drugs affecting lipid metabolism; *2*) known medical conditions affecting lipid and glucose metabolism (e.g. diabetes); *3*) medical records of alcohol abuse and alcohol intake < 15 g/d ethanol; *4*) drug-induced hepatotoxicity; *5*) infection with hepatitis B or C virus; and *6*) clinical indication of impaired nutritional status. A total of 18 subjects, all of whom were undergoing partial liver resection (e.g. because of liver metastasis) or liver biopsies for medical reasons were included in the study. Characteristics of study participants are summarized in **Table 1**. Blood samples were drawn from fasting patients and controls before surgical intervention or liver biopsy. Liver tissue was immediately placed in RNAlater (Ambion) and stored at −80°C or fixed in 10% buffered formalin until later analysis.

Dietary intake, alcohol consumption, physical activity, and anthropometrics. Dietary intake and alcohol consumption, as well as anthropometric variables and physical activity during leisure time, of cases and controls was assessed by an experienced nutritionist using the computer software EBISpro (Germany).

Clinical chemistry and pathologic evaluation. Plasma transaminase concentration was measured by a routine clinical chemistry laboratory using a MODULAR analyzer (Hitachi/ Roche). An experienced pathologist assessed liver histology by using the nonalcoholic steatohepatitis clinical network scoring system by Kleiner and Brunt et al. (11).

PAI-1 ELISA. The concentration of functionally active PAI-1 in plasma was assessed using an ELISA kit purchased from LOXO.

Endotoxin assay. Plasma samples were heated at 75°C for 20 min. Endotoxin plasma concentration was then measured using a commercially available endpoint limulus amebocyte lysate assay (Charles River) for a concentration range of 0–1200 endotoxin units/L following the instructions of the manufacturer.

RNA isolation and real-time RT-PCR. One microgram total RNA was reverse transcribed followed by a DNase digestion step (Fermentas). Primer sequences used for the detection of toll-like receptor (TLR) 4, PAI-1, and 18S ribosomal (r) RNA were as follows: 18S rRNA (forward) 5′-TCT GCC CTA TCA ACT TTC GAT GGT A-3′, 18S rRNA (reverse) 5′- GGC CTC GAA AGA GTC CTG TAT TGT T-3′, PAI-1 (forward) 5′-AGG CAG CTC GGA TTC AAC TAC CTT-3′, PAI-1 (reverse) 5′-TAA AGA GAC GGG GGT CTT GGT ATG T-3′, TLR4 (forward) 5′-AGC CCT GGG AGC CTT TTC TG-3′, and TLR4 (reverse) 5′-GAA CCC GCA AGT CTG TGC AA-3′. Using SybrGreen Universal PCR Master mix (Applied Biosystems), the PCR amplification reactions were carried out in an iCycler (Bio-Rad Laboratories) with an initial hold step (95°C for 2 min) and 45 cycles of a 3-step PCR (95°C for 15 s, 60°C for 15 s, 72°C for 30 s). The comparative threshold cycle method was used to determine the amount of target, normalized to an endogenous reference (18 S rRNA) and relative to a calibrator ($2^{-\Delta\Delta Ct}$). The purity of PCR products were verified by melting curves and gel electrophoresis.

Statistical analyses. Results are reported as means ± SEM. The Mann-Whitney U test was used compare the means of the 2 groups. Chi-square or Fisher's exact test was used to compare categorical factors. Spearman rank correlation was performed to test associations between variables. $P \leq 0.05$ was selected before the study as the level of significance.

TABLE 1 Characteristics of patients with NAFLD and controls[1]

	Controls	NAFLD patients
n	6	12
Sex, *% female*	66	25
Age, *y*	47 ± 7	55 ± 4
BMI, *kg/m²*	22.5 ± 1.2	27.8 ± 0.7*
Leisure physical activity, *yes/no*	6/0	6/6*
Plasma ALT, *U/L* (0–40 U/L)[2]	40 ± 10	59 ± 12
Plasma AST, *U/L* (0–38 U/L)[2]	39 ± 10	39 ± 5
Plasma γ-GT, *U/L* (0–40 U/L)[2]	57 ± 19	173 ± 50
Liver resection, *n*	6	7
Liver biopsy,[3] *n*	0	5
Basic illness[4]		
Malignancy, *n*	5	6
Other, *n*	1	1
Liver pathology		
Without NAFLD, *n*	6	—
Steatosis, *n*	—	2
Steatohepatitis, *n*	—	3
Steatohepatitis with fibrosis, *n*	—	7

[1] Values are means ± SEM. *Different from controls, $P < 0.05$.
[2] Normal range (27).
[3] Biopsy taken for staging of NAFLD.
[4] Basic illness of subjects undergoing liver resection.

Results

Dietary intake. Despite a significant difference in BMI (Table 1), total energy intake, intake of carbohydrate, fat, and protein did not differ between NAFLD patients and controls (**Table 2**). However, regular leisurely physical activity was significantly less frequent in the group of NAFLD patients (50%) than in controls (100%). Total glucose and sucrose consumption did not differ between cases and controls. In contrast, total fructose intake, derived from free fructose and sucrose, was significantly higher by ~10 g/d in patients with NAFLD than in controls (Table 2).

Plasma endotoxin concentration and hepatic TLR4 mRNA expression. In peripheral plasma samples, endotoxin was detected in only 1 of 6 controls. In contrast, in patients with NAFLD, endotoxin was detected significantly more frequently (10 of 12 subjects) with concentrations ranging from 0 to 9600 EU/L. The plasma endotoxin concentration in NAFLD patients (279.5 ± 90.8 EU/L) was greater than in controls (9.7 ± 9.7 EU/L) ($P < 0.05$) and hepatic TLR4 mRNA expression, normalized to 18S rRNA, was ~4.3 times that of controls ($P < 0.05$). Furthermore, TLR4 mRNA expression in liver and plasma endotoxin concentration was correlated in cases and controls (Spearman $r = 0.61$; $P = 0.007$).

PAI-1 plasma concentration and hepatic PAI-1 mRNA expression. The plasma concentration of PAI-1 in NAFLD patients (29,030 ± 7401 U/L) was greater than in controls (2742 ± 1463 U/L) as was hepatic hepatic PAI-1 mRNA expression, which was ~12.5 times that of controls ($P < 0.05$).

Correlations of plasma endotoxin and hepatic TLR4 mRNA expression with PAI-1 mRNA expression in liver and plasma PAI-1 concentration. Plasma endotoxin and PAI-1 concentrations were positively associated (Spearman $r = 0.83$; $P < 0.005$), as was hepatic TLR4 mRNA expression and PAI-1 concentration in plasma (Spearman $r = 0.54$; $P < 0.05$).

TABLE 2 Nutritional intake of patients with NAFLD and controls[1]

	Controls	NAFLD patients
n	6	12
Energy, *kJ/d*	9106 ± 670	9387 ± 461
Protein, *g*	79 ± 6	88 ± 3
% Energy	15 ± 0.4	16 ± 0.7
Fat, *g*	87 ± 10	89 ± 7
% Energy	36 ± 2	35 ± 2
Carbohydrate, *g*	255 ± 15	253 ± 14
% Energy	49 ± 3	49 ± 1
Glucose,[2] *g/d*	37.0 ± 4.0	42.8 ± 4.3
Sucrose, *g/d*	48.3 ± 7.2	57.3 ± 6.3
Fructose,[3] *g/d*	41.0 ± 3.2	51.5 ± 5.2*
Alcohol, *g/d*	6.5 ± 2.3	4.0 ± 1.2

[1] Values are means ± SEM. *Different from controls, $P < 0.05$.
[2] Glucose intake derived from free glucose and sucrose.
[3] Fructose intake derived from free fructose and sucrose.

However, neither the plasma endotoxin concentration nor hepatic TLR4 mRNA expression were associated with PAI-1 expression in the subjects.

Correlation of carbohydrate intake with plasma endotoxin concentration, hepatic TLR4, PAI-1 expression, and plasma PAI-1 concentration. Hepatic PAI-1 mRNA expression was positively correlated with intakes of total carbohydrate (Spearman $r = 0.67$; $P < 0.01$), total glucose (Spearman $r = 0.58$; $P < 0.01$), total fructose (Spearman $r = 0.58$; $P < 0.01$), and total sucrose (Spearman $r = 0.70$; $P < 0.01$). However, neither plasma endotoxin concentration nor hepatic TLR4 mRNA expression or plasma PAI-1 concentration were associated with the total carbohydrate or mono- or disaccharide intakes of the subjects.

Discussion

Fructose intake, endotoxin, and PAI-1 concentration increased in patients with NAFLD. Obesity and insulin resistance have been shown to be key risk factors for the development of NAFLD (1); however, mechanisms involved in the pathogenesis of NAFLD have not yet been clarified. Results of epidemiological and animal studies suggest that a diet rich in carbohydrates increases the risk to develop later stages of the disease (2,3) and that the kind of carbohydrate (e.g. fructose vs. glucose) may affect the development of the disease (5). Besides nutritional intake, alterations of the intestinal motility and bacterial flora as well as an increased intestinal permeability and PAI-1 concentration have been shown to be associated with the development of NAFLD (8,12–14,15). In animal studies, it was further shown that dietary carbohydrates can be fermented to ethanol when intestinal stasis permits bacterial overgrowth in the upper parts of the gastrointestinal tract (16). In the present study, we assessed the nutritional intakes and markers of intestinal permeability (e.g. plasma endotoxin concentration and TLR4 expression) as well as PAI-1 in patients with NAFLD and controls. In accordance with the results of other groups (17), total intake of carbohydrates was also similar between NAFLD patients and controls in the present study; however, we found that consumption of fructose was significantly higher in NAFLD patients compared with controls. Plasma endotoxin concentration, hepatic expression of the endotoxin receptor TLR4, and PAI-1 concentration were also higher in NAFLD patients than in controls. Taken together, these data suggest that fructose intake, intestinal translocation, and PAI-1 are associated with the pathogenesis of NAFLD in humans, thereby lending further support to the hypothesis that not only over-nutrition but also dietary pattern (e.g. fructose intake) and intestinal permeability and PAI-1 might play a role in the development of NAFLD.

Endotoxin and PAI-1 concentration are related. It has been suggested that endotoxin by itself, but also through cytokines such as TNFα and interleukin-1, is a key regulator of PAI-1 gene expression in the liver (18,19). In the present pilot study, plasma PAI-1 and endotoxin concentrations were positively associated as were plasma PAI-1 concentration and hepatic expression of TLR4. However, hepatic PAI-1 expression and plasma endotoxin concentration or hepatic TLR4 expression were not associated. The lack of association of the latter could be due to a biphasic induction of PAI-1 previously shown in animal experiments (9,19) or a posttranscriptional regulation of PAI-1. Indeed, results of in vitro studies suggest that PAI-1 can be regulated by intracellular iron and hypoxia, respectively, through post-transcriptional mechanisms (20,21). However, if similar mechanisms play a role in the present study remains to be determined. Taken together, the results of the present study suggest that plasma PAI-1 and endotoxin concentrations as well as hepatic TLR4 expression are related in humans. However, if PAI-1 in humans is induced directly through TLR4-/endotoxin-dependent signaling pathways or indirectly through proinflammatory cytokines such as TNFα or interleuken-1 will have to be addressed in future studies.

Carbohydrate intake and PAI-1 concentration are related. Results of studies investigating the relation of diet and PAI-1 in animals as well as the results of dietary interventions (e.g. low-carbohydrate diet and high-fat diet) suggest that PAI-1 can be modulated by diet (22–25). Results of in vitro studies further suggest that PAI-1 expression can be induced by high glucose concentrations (26). Indeed, in the present study, total intakes of carbohydrates, fructose, glucose, and sucrose were related to hepatic PAI-1 mRNA expression. However, plasma endotoxin and PAI-1 concentrations or hepatic TLR4 expression and intake of carbohydrates of NAFLD patients and controls were not related. The results of the present study suggest that carbohydrates and particularly monosaccharides might influence PAI-1 expression in the liver through mechanisms not yet clarified. These data do not preclude that other factors such as the content of trans- and (n-3) fatty acids in the diet might also be a potential contributor to the development of NAFLD in humans. As discussed above, PAI-1 may be regulated through post-transcriptional mechanisms (20,21); therefore, the lack of association of PAI-1 plasma concentration with carbohydrate intake might have resulted from other intracellular factors yet to be determined.

The results of the present study suggest that endotoxin and its receptor TLR4 and plasma PAI-1 concentration, dietary fructose intake, and PAI-1 are associated with NAFLD in humans. These results also suggest that hepatic TLR4 expression, plasma PAI-1, and endotoxin concentrations are related. Furthermore, our data indicate that hepatic PAI-1 expression might be related to total carbohydrate and sugar intake. Although further studies will be needed to explore the molecular mechanisms responsible, the results of the present study are compatible with the concept that intestinal permeability and flora as well as dietary pattern

and PAI-1 are important in the pathogenesis of NAFLD in humans.

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