Liver steatosis, but not fibrosis, is associated with insulin resistance in nonalcoholic fatty liver disease

ORIGINAL ARTICLE

Liver steatosis, but not fibrosis, is associated with insulin resistance in nonalcoholic fatty liver disease

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Short title: Histology of fatty liver and insulin resistance

Abstract

Purpose: <u>To address the hypothesis that liver steatosis causes systemic insulin resistance, we sought to determine the liver histological feature that most strongly contributes to insulin resistance in patients with nonalcoholic fatty liver disease (NAFLD).</u>

Methods: Liver biopsy specimens were obtained from 131 patients with clinically suspected NAFLD. The stage, grade of nonalcoholic steatohepatitis (NASH), and levels of steatosis were scored and analyzed in relation to the homeostasis model assessment of insulin resistance (HOMA-IR), and the metabolic clearance rate (MCR) measured using the glucose clamp method.

Results: In the univariate analysis<u>, the degree of hepatic steatosis (r=0.458, p<0.001), stage (r=0.360, p<0.001), and grade (r=0.349, p<0.01) of NASH were significantly correlated with the HOMA-IR. Multiple regression analysis adjusting for age, sex, body mass index, and each other's histological score showed that steatosis was significantly and independently associated with HOMA-IR (coefficient=1.42, p<0.001), but not the stage (coefficient=0.33, p=0.307) or grade (coefficient=0.67, p=0.134) of NASH. Similar independent relationships were observed between steatosis and MCR, but the relationship was weaker (coefficient=-0.98, p=0.076).</u>

Conclusions: Steatosis of the liver, but not the stage or the grade of NASH, is associated with insulin resistance in patients with NAFLD.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is often associated with various

metabolic abnormalities, including obesity,[1,2] type 2 diabetes,[1-4] and dyslipidemia,[3-7] all

of which are closely associated with insulin resistance and are important risk factors for

cardiovascular diseases. Insulin resistance, which can be accurately measured using the

glucose clamp method,[8] is a condition in which the cells of the body become resistant to

the effects of insulin, resulting in a decreased response to a given amount of insulin. This

condition is associated with various underlying metabolic abnormalities, including

obesity, type 2 diabetes, dyslipidemia, and hypertension.[9,10] Insulin resistance is also

associated with NAFLD,[11-15] which can range from a simple fatty liver to nonalcoholic

steatohepatitis (NASH).[16-18] This latter condition can progress to cirrhosis of the liver

and hepatocellular carcinoma.[19-24]

The particular histological findings of NAFLD that are associated with the

metabolic abnormalities have not been elucidated fully.[11, 12, 25-31] Steatosis of the liver has

been reported to be associated with body mass index (BMI),[25] waist circumference,[11]

hypertension,[11,25] glucose intolerance,[11,25] dyslipidemia,[11,25] and insulin resistance.[11,12]

Furthermore, experiments have demonstrated that hepatic steatosis *per se* causes insulin

resistance *in vivo*.[32-35] Nevertheless, the association between the degree of hepatic

fibrosis and metabolic abnormalities is still controversial. Some reports suggest that, in

NAFLD patients, fibrosis is associated with insulin resistance and higher rates of diabetes,

dyslipidemia, and hypertension,[11,12,25-29] whereas other reports suggest that the stage of

fibrosis is not significantly associated with metabolic abnormalities.[30,31] These

discrepant findings may be due to differences in the severity of obesity and the

complicated metabolic abnormalities of the patients in these studies.

To test the hypothesis that liver steatosis causes systemic insulin resistance, we

sought to determine the liver histological feature that most strongly contributes to insulin

resistance in patients with NAFLD.

Methods

Patients

The study subjects were 131 patients with clinically suspected NAFLD, who were

recruited consecutively between 1997 and 2004 at Kanazawa University Hospital, Japan.

Twenty of the patients were referred to our hospital for evaluation of liver injury, while in

the remaining 111 patients, the liver injury was identified during the treatment of other

metabolic disorders, such as diabetes mellitus and obesity. Fatty liver was clinically

diagnosed based on ultrasound examination showing an increase in hepato-renal contrast,

defined as the ratio of the hepatic to the kidney echo levels of >1.0, also known as a

'bright liver.' In each patient, all other liver disorders were excluded, including viral

hepatitis B and C, primary biliary cirrhosis, autoimmune hepatitis, sclerosing cholangitis,

hemochromatosis, Wilson's disease, drug-induced liver injury, and biliary obstruction.

All patients reported drinking less than 20 g/day of ethanol.

The 131 patients had a mean age of 48 years and a mean BMI of 27.4 kg/m^2 (Table 1). Of the 131 patients, 108 (82%) had type 2 diabetes according to the American Diabetes Association criteria.[36] Among these patients, 54 were treated with diet alone; the remainder were treated with an α-glucosidase inhibitor (n=14), a rapid-acting insulin secretion agent (nateglinide, n=11), or a pre-meal rapid-acting insulin analogue (n=40). Hypertension and dyslipidemia were defined according to the definition of the National Cholesterol Education Program – Adult Treatment Panel-III metabolic syndrome.[37] Hypertension was observed in 65 patients (50%), 36 of whom had been treated with antihypertensive agents. High triglyceride levels were observed in 47 (36%) patients, and low HDL levels were observed in 33 (25.2%), 30 of whom had been treated with statins. None of the patients were on medication, such as sulfonylureas, thiazolidinediones, metformins, vitamin E, and ursodeoxycholic acid, that could influence the fasting insulin/glucose levels or induce histological changes in the liver. The patients gave their written informed consent for this study, which was approved by the hospital ethics board.

Laboratory studies

After an overnight fast, venous blood samples were withdrawn from each patient. Serum samples were assayed for total cholesterol, triglycerides, HDL cholesterol, and insulin. Plasma samples were assayed for glucose.

Evaluation of insulin sensitivity

Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula, HOMA-IR = [fasting insulin (µU/ml) × fasting plasma glucose (mmol/l)]/22.5.[38]

Insulin sensitivity was also evaluated using the glucose clamp method[8] in 46 patients (35 diabetic and 11 non-diabetic patients). These patients did not receive any medication on the morning of the examination. At approximately 9 a.m., after an overnight fast of at least 10 hours, an intravenous catheter was placed in an antecubital vein of each subject for infusion, while a second catheter was placed in the contralateral hand for blood sampling. The euglycemic hyperinsulinemic clamp technique was done using an artificial pancreas (model STG-22; Nikkiso, Tokyo, Japan), as described previously.[39] A solution of 0.8 U/ml insulin (Novolin R: Novo Nordisk, Copenhagen, Denmark) in normal saline was allowed to remain in the intravenous lines for at least 15 min, and the lines were then flushed before starting the insulin infusion. Insulin was infused at a rate of 3.0 mU/kg/min, resulting in a steady state insulin concentration of 290.3±61.7 µU/ml (mean±SD). Blood glucose levels were determined continuously during the clamp study and maintained by variable-rate infusion of 20% glucose at fasting levels or at 100 mg/dl, whichever was higher. The steady state period was maintained for 30 min or longer, during which the coefficients of variation for blood

glucose and the glucose infusion rate were each less than 5%. Glucose levels reached during the clamp study were 91.3±15.5 mg/dl. Insulin sensitivity was expressed as the glucose metabolic clearance rate (MCR) in mg/kg/min. The mean MCR in healthy subjects (n=9, age 26.6±2.9 years, BMI 22.3±2.1 kg/m^2) was 13.5±3.4 mg/kg/min.

Pathology

After obtaining informed consent, liver biopsy specimens were obtained from all 131 patients, 59 of whom had elevated aminotransferase levels on admission for the liver biopsy and 72 had normal aminotransferase levels at the time of biopsy, but a history of elevated aminotransferase levels before admission. Each specimen was stained with hematoxylin-eosin and silver reticulin stains and histologically examined by one pathologist who was blinded to the patient's clinical condition and biochemical data. Each biopsy was scored according to the standard criteria for grading and staging of NASH proposed by Brunt *et al*.[40,41] Steatosis of the liver was also scored from 0 to 3 (0, none; 1, <33%; 2, 33-66%; 3, >66%) according to the modified criteria of Brunt *et al*.[40,41]

Statistical analyses

The correlation coefficients between pairs of indices were calculated, with $p<0.05$ considered statistically significant. Multiple linear regression analysis was used to calculate age-, sex-, and BMI-adjusted coefficients between each histological score and

insulin resistance. The *t*-statistic was used to compare the strength of the relationship.

Results

The characteristics of the study subjects and the number of patients with each histological score are shown in Table 1. We found that the mean HOMA-IR was higher and the mean MCR was lower in NAFLD patients than in normal subjects. Scores of the stage and grade of NASH were strongly correlated (r=0.570, *p*<0.001), and each was weakly correlated with the hepatic steatosis score (stage, r=0.389, *p*<0.001; grade, r=0.397, *p*<0.001), suggesting that steatosis and inflammation or fibrosis are pathological attributes of patients with NAFLD.

We evaluated the association between markers for insulin resistance and histological scores of the liver. In univariate analysis (Table 2), the degree of hepatic steatosis, stage, and grade of NASH were significantly correlated with HOMA-IR. The degree of hepatic steatosis and grade of NASH were significantly correlated with MCR.

Multiple linear regression models were computed to assess the age-, sex-, and BMI-adjusted relative influence of each histological score on insulin resistance (Table 3). Steatosis, as well as the stage and grade of NASH, was associated with HOMA-IR, even after adjusting for age, sex, and BMI in the multiple regression analysis. When these three histological scores were adjusted for each other in a model that included all three simultaneously, only the steatosis score was associated with HOMA-IR, and the associations of the stage and grade of NASH with HOMA-IR were not significant. A similar independent relationship was observed between steatosis of the liver and MCR,

but the relationship was weaker.

Discussion

Patients with NAFLD have features of the insulin resistance-associated metabolic syndrome, which puts them at increased risk for developing cardiovascular diseases.[13-15] Patients with advanced NASH have also been reported to have a higher intra-abdominal fat mass, a higher prevalence of hypertriglyceridemia, a low HDL-cholesterol, and more severe insulin resistance than healthy subjects matched for age, sex, race, and BMI.[27] However, the association between the degree of the histological changes, especially liver fibrosis, and metabolic abnormalities is still controversial in NAFLD patients.[11,12,25-31] When we investigated the HOMA-IR and MCR calculated by using the glucose clamp method, which is the gold standard for evaluating insulin resistance,[8] we found that insulin resistance was increased in NAFLD patients. On examining the relationship between insulin resistance and NAFLD histological scores, we found that insulin resistance was associated with the severity of hepatic steatosis, but not with the severity of inflammation or fibrosis after adjusting for the hepatic steatosis score.

Our findings differ from those of a recent report showing that metabolic syndrome is associated with advanced NASH.[26] In that report, patients with advanced NASH had a higher BMI than patients who have a simple fatty liver. Since a higher BMI itself is strongly associated with insulin resistance and metabolic abnormalities, metabolic abnormalities associated with liver pathology should only be determined after adjusting for BMI. In fact, our study showed that BMI was independently associated with both

liver steatosis and the stage of NASH. When we evaluated the association between the

histological scores of the liver and insulin sensitivity using multiple regression models,

the hepatic steatosis score was associated with insulin sensitivity, even after adjusting for

age, sex, BMI, and each other's histological score, whereas the association between the

stage or grade of NASH and insulin resistance was no longer significant. These findings

clearly indicate that only the degree of hepatic steatosis is an independent predictor of

insulin resistance in patients with NAFLD.

We evaluated the insulin sensitivity using two methods: HOMA-IR and MCR. In

our results, steatosis of the liver was more strongly associated with HOMA-IR than MCR.

In this study, MCR was measured only in 46 of 131 NAFLD patients; therefore, the

statistical power would not be sufficient to detect a significant relationship. Furthermore,

organ-specific insulin resistance may also influence the results. MCR is a marker for

splanchnic insulin resistance, mainly muscle insulin resistance.[8] By contrast, HOMA-IR

is considered a marker for hepatic insulin resistance because HOMA-IR is calculated

using fasting glucose and insulin levels, which are determined by fasting hepatic glucose

production. Therefore, hepatic steatosis may be associated more strongly with the index

of 'hepatic' insulin resistance.

Recently, we used cDNA microarrays to characterize the changes that occur in the

moderate fatty liver of type 2 diabetic patients.[42,43] In the livers of patients with type 2

diabetes, the genes for mitochondrial oxidative phosphorylation and gluconeogenesis are

up-regulated, and are associated with fasting hyperglycemia.[43] These findings, together

with our results, suggest that hepatic steatosis *per se* causes insulin resistance in patients

with NAFLD.

The main limitation of this study is that most of the study subjects were diabetic

patients. Therefore, insulin resistance may be greater in these study subjects than in the

general population, and this may have influenced the results. A large-scale prospective

study is needed to confirm our conclusion and to clarify the prognosis of patients with

NAFLD.

In this paper, we showed that, steatosis of the liver *per se* is an independent

predictor for insulin resistance in patients with NAFLD. Simple fatty liver has been

considered as benign condition compared with advanced NASH, but we should regard the

patients with liver steatosis, irrespective with the degree of inflammation and fibrosis, as

high-risk group for cardiovascular disease because they have higher insulin resistance.

To prevent cardiovascular disease, patients with severe fatty liver should be evaluated for

insulin resistance-associated metabolic abnormalities and monitored intensively to

reduce the occurrence of risk factors for atherosclerosis.

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Table 1. Clinical characteristics of the study subjects

	(Male n=84; Female n=47; Total, n=131)	
	Mean ± SD	Normal range
Age (years)	48±14	
Body mass index (kg/m^2)	27.4±5.5	18-25
AST (IU/L)	39.4±38.5	10-48
ALT (IU/L)	62.1±74.6	3-50
Fasting plasma glucose (mg/dl)	127±40	70-110
Basal insulin (μ U/ml)	12.0±11.7	<20
HbA$_{1C}$ (%)	6.9±1.8	4.3-5.8
Total cholesterol (mg/dl)	205±44	132-220
Triglycerides (mg/dl)	139±77	32-150
HDL cholesterol (mg/dl)	48±13	40-97
HOMA-IR	3.4±3.2	<2.0
Metabolic clearance rate (mg/kg/min)[*]	6.1±2.7	9.9-16.9
Histological scores		
Stage (0/1/2/3/4)	22/45/38/23/3	
Grade (0/1/2/3)	48/61/19/3	
Steatosis (0/1/2/3)	7/82/27/15	

HOMA-IR, homeostasis model assessment of insulin resistance.

* Metabolic clearance rate was measured in a part of the subjects (n=46).

Table 2. Univariate correlation between histological scores and insulin resistance

	HOMA-IR (n=131)	Metabolic clearance rate (n=46)
Steatosis	0.46 ***	-0.42 **
Stage	0.36 ***	-0.17
Grade	0.35 ***	-0.30 *

HOMA-IR, homeostasis model assessment of insulin resistance.

*$p<0.05$, **$p<0.01$, *** $p<0.001$

Table 3. Age-, sex-, and BMI-adjusted association between insulin resistance and histological changes of the liver

	HOMA-IR (n=131)			Metabolic clearance rate (n=46)		
	Coefficient	*t*-statistic	*p*	Coefficient	*t*-statistic	*p*
Steatosis	1.81	4.59	<0.001	-1.23	-2.62	0.012
Stage	0.90	3.15	0.002	-0.23	-0.62	0.540
Grade	1.37	3.51	0.001	-1.07	-2.26	0.029
Steatosis*	1.42	3.40	<0.001	-0.98	-1.82	0.076
Stage	0.33	1.03	0.307	0.35	0.85	0.401
Grade	0.67	1.51	0.134	-0.86	-1.46	0.151

HOMA-IR, homeostasis model assessment of insulin resistance.

All models are adjusted for age, sex, and BMI by multiple linear regression.

*Three histological scores are included in the same model.