Liver steatosis is associated with insulin resistance in skeletal muscle rather than in the liver in Japanese patients with non-alcoholic fatty liver disease

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Liver steatosis is associated with insulin resistance in skeletal muscle rather than in the liver in Japanese patients with non-alcoholic fatty liver disease

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ABSTRACT

Aims/Introduction: To examine the association between liver histological features and organ-specific insulin resistance indices calculated from 75-g oral glucose tolerance test data in patients with non-alcoholic fatty liver disease.

Materials and Methods: Liver biopsy specimens were obtained from 72 patients with non-alcoholic fatty liver disease, and were scored for steatosis, grade and stage. Hepatic and skeletal muscle insulin resistance indices (hepatic insulin resistance index and Matsuda index, respectively) were calculated from 75-g oral glucose tolerance test data, and metabolic clearance rate was measured using the euglycemic hyperinsulinemic clamp method.

Results: The degree of hepatic steatosis, and grade and stage of non-alcoholic steatohepatitis were significantly correlated with Matsuda index (steatosis $r = -0.45$, $P < 0.001$; grade $r = -0.54$, $P < 0.001$; stage $r = -0.37$, $P < 0.01$), but not with hepatic insulin resistance index. Multiple regression analyses adjusted for age, sex, body mass index and each histological score showed that the degree of hepatic steatosis (coefficient = –0.22, $P < 0.05$) and grade (coefficient = –0.40, $P < 0.01$) were associated with Matsuda index, whereas the association between stage and Matsuda index (coefficient = –0.07, $P = 0.593$) was no longer significant. A similar trend was observed for the association between steatosis and metabolic clearance rate (coefficient = –0.62, $P = 0.059$).

Conclusions: Liver steatosis is associated with insulin resistance in skeletal muscle rather than in the liver in patients with non-alcoholic fatty liver disease, suggesting a central role of fatty liver in the development of peripheral insulin resistance and the existence of a network between the liver and skeletal muscle.

INTRODUCTION

Insulin resistance is a central pathology, and is associated with various metabolic abnormalities, including obesity, type 2 diabetes, dyslipidemia and non-alcoholic fatty liver disease (NAFLD), all of which are important risk factors for cardiovascular diseases[1,2]. Whole-body insulin resistance is a composite of hepatic and peripheral insulin resistance, and is best measured by the euglycemic hyperinsulinemic clamp technique[3]. We previously showed that liver steatosis, but not fibrosis, is associated with whole-body insulin resistance, independent of body mass index (BMI), in patients with NAFLD[4]. However, it remains unclear how liver histological features are associated with organ-specific insulin resistance. When combined with radiolabeled glucose, the euglycemic hyperinsulinemic clamp technique allows one to quantify the individual contribution of organ-specific (hepatic and skeletal muscle) insulin resistance to the defect in whole-body insulin-mediated glucose disposal[5]. However, because this technique is time-consuming and technically difficult to carry out, it cannot be applied in daily clinical practice or in

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large-scale epidemiological studies. In recent years, surrogate measures of insulin resistance from measurements of glucose and insulin concentrations during the 75-g oral glucose tolerance test (OGTT) have been developed[6–10]. Furthermore, some of these indices can selectively quantify organ-specific (hepatic and muscle) insulin resistance[7,10].

The aim of the present study was to examine the association between liver histological features (steatosis, inflammation, fibrosis) and organ-specific (hepatic and skeletal muscle) insulin resistance indices calculated from OGTT data in patients with NAFLD.

MATERIALS AND METHODS

Participants and Study Design

We studied 72 patients clinically diagnosed with NAFLD, who were recruited consecutively between 1999 and 2009 at Kanazawa University Hospital, Kanazawa, Japan. They were in good general health without evidence of any acute or chronic diseases (other than NAFLD, type 2 diabetes, hypertension or dyslipidemia) as determined by history, physical examination, routine blood chemistry, urinalysis and electrocardiography. In almost all patients, the liver injury was identified during the treatment of other metabolic disorders, such as diabetes mellitus and obesity. In each patient, all other liver diseases 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 <20 g/day of ethanol. Of the 72 patients, 48 (66%) had type 2 diabetes according to the American Diabetes Association criteria[11]. Among these patients, 25 were treated with diet alone; the remainder were treated with an α-glucosidase inhibitor (n = 3), a rapid-acting insulin secretion agent (nateglinide, n = 3), or a pre-meal rapid-acting insulin analog (n = 17). None of the patients were on medication (e.g. long-acting insulin, sulfonylureas, thiazolidinediones, metformin, vitamin E or ursodeoxycholic acid) that could influence 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 medical ethics committee of Kanazawa University.

Evaluation of Insulin Sensitivity

After an overnight fast (10–12 h), an OGTT was carried out at 08.30 hours. Blood samples were obtained 0, 30, 60, 90 and 120 min after the glucose load for the measurement of plasma glucose and insulin concentrations. The patients did not receive any medication on the morning of the examination. Insulin resistance indices were calculated from OGTT data as proposed by Matsuda and DeFronzo[7,10]. The Matsuda index, an index of whole-body (mainly skeletal muscle) insulin sensitivity, was calculated using the following formula: Matsuda index = 10,000 / √ (fasting plasma glucose × fasting insulin [FPI]) × (mean glucose × mean insulin during OGTT)]. The hepatic insulin resistance index was defined as the product of the total areas under the curve (AUC) for glucose and insulin during the first 30 min of the OGTT, and was calculated using the following formula: hepatic insulin resistance = (AUC[glucose]0–30) × (AUC[insulin]0–30).

Insulin sensitivity was also evaluated using the euglycemic hyperinsulinemic clamp method in 16 patients (seven with diabetes and nine without diabetes). The patients did not receive any medication on the morning of the examination. At approximately 09.00 hours, after an overnight fast of at least 10 h, an intravenous catheter was placed in an antecubital vein of each patient for infusion, and a second catheter was placed in the contralateral hand for blood sampling. The euglycemic hyperinsulinemic clamp technique was carried out using an artificial pancreas (model STG-22; Nikkiso, Tokyo, Japan), as described previously. A solution of 0.8 U/mL insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA) 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 317.7 ± 13.3 μU/mL (mean ± standard error of the mean), a level that might be sufficient to suppress hepatic glucose production (HGP). Blood glucose levels were continuously determined during the clamp study, and maintained with variable-rate infusion of 20% glucose at a concentration of 100 mg/dL (or 90 mg/dL for baseline values under 90 mg/dL). 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 both <5%. The glucose level reached during the clamp study was 95 ± 4 mg/dL. Because steady-state plasma glucose level affects the glucose infusion rate (GIR) level, insulin sensitivity was expressed as the glucose metabolic clearance rate (MCR), which was calculated by adjusting GIR by steady-state plasma glucose. 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

Ultrasound-guided liver biopsy specimens were obtained from all 72 patients. Each specimen was stained with hematoxylin–eosin and silver reticulin stains, and was examined histologically by a pathologist who was blinded to the patient's clinical condition and biochemical data. The biopsied tissues were scored for steatosis (0, none; 1, <33%; 2, 33–66%; 3, >66%), grade and stage, according to the standard criteria for grading and staging of non-alcoholic steatohepatitis (NASH) proposed by Brunt *et al.*[12,13] in their studies.

Statistical Analyses

All analyses were carried out using the SPSS software version 11.0 (SPSS Inc., Chicago, IL, USA). All values are expressed as the means ± standard error of the mean, unless stated otherwise. The relationship between individual variables was assessed by Pearson's correlation for parametric variables and by Spearman's correlation for non- parametric variables. Multiple linear

regression analysis was used to calculate age-, sex-, and BMI-adjusted coefficients for histological score and insulin resistance. The *t*-statistic was used to compare the strength of the relationship. Statistical significance was defined as $P < 0.05$.

RESULTS

Liver Histological Features and Clinical Characteristics in Patients with NAFLD

The characteristics of the study participants and the number of patients with each histological score are shown in Table 1. Hepatic steatosis score was correlated with grade score ($r = 0.372$, $P = 0.001$), but not with stage score ($r = 0.224$, $P = 0.059$). Grade score and stage score were strongly correlated with each other ($r = 0.607$, $P < 0.001$), suggesting that inflammation causes fibrosis in NAFLD.

Liver Histological Features and Organ-Specific (Hepatic and Muscle) Insulin Resistance

We evaluated the associations between indices of insulin resistance and histological scores of the liver. In a univariate analysis, the degree of hepatic steatosis, and grade and stage of NASH were significantly correlated with Matsuda index (mainly skeletal muscle insulin resistance; steatosis $r = -0.45$, $P < 0.001$; grade $r = -0.54$, $P < 0.001$; stage $r = -0.37$, $P < 0.01$), but were not correlated with hepatic insulin resistance index (Table 2). The degree of hepatic steatosis was significantly correlated with MCR ($r = -0.728$, $P = 0.001$).

Multiple linear regression models were computed to assess the relative age-, sex- and BMI-adjusted influence of each histological score on insulin resistance (Table 3). Steatosis (coefficient = –0.36, $P < 0.01$), grade (coefficient = –0.51, $P < 0.001$) and stage of NASH (coefficient = –0.37, $P < 0.01$) were associated with Matsuda index, but not with hepatic insulin resistance index. When these three histological scores were adjusted for each other in a model that included all three simultaneously, the degree of hepatic steatosis (coefficient = –0.22, $P < 0.05$) and grade of NASH (coefficient = –0.40, $P < 0.01$) were associated with the Matsuda index. A similar independent trend was observed between steatosis of the liver and MCR (coefficient = –0.62, $P = 0.059$).

Table 1 | Clinical characteristics of the study participants

n	72
Age (years)	46.2 ± 1.8
Sex (male/female)	39/33
BMI (kg/m^2)	29.9 ± 1.2
Fasting plasma glucose (mg/dL)	111 ± 2
2 h glucose (mg/dL)	208 ± 9
Basal insulin (μU/mL)	14.0 ± 1.2
Insulinogenic index [(μU/mL)/(mg/dL)]	0.77 ± 0.10
HOMA-IR	3.8 ± 0.3
Matsuda index	2.8 ± 0.2
Hepatic insulin resistance index × 10^6	5.9 ± 0.5
HbA1c (%)	6.8 ± 0.2
Total cholesterol (mg/dL)	200 ± 4
Triglycerides (mg/mL)	140 ± 9
HDL cholesterol (mg/mL)	46 ± 1
Aspartate aminotransferase (IU/L)	44 ± 3
Alanine aminotransferase (IU/L)	72 ± 7
Historogical scores	
Stage (0/1/2/3/4)	2/17/20/7/6
Grade (0/1/2/3)	37/16/14/5
Steatosis (0/1/2/3)	0/31/23/18
Statin (+/–)	17/55
Angiotensin receptor blocker (+/–)	13/59
Insulin (+/–)	17/55

BMI, body mass index; HbA1c, hemoglobin A1c; HDL cholesterol, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance. Data are presented as mean ± standard error of the mean or absolute numbers.

DISCUSSION

In the present study, we investigated the associations between liver histological features and indices of hepatic and skeletal muscle insulin resistance in patients with NAFLD. We found that liver steatosis was more strongly associated with skeletal muscle insulin resistance than with hepatic insulin resistance. The present results are consistent with a previous report in which organ-specific (hepatic and skeletal muscle) insulin resistance was assessed using the glucose clamp technique[14]. A novel aspect of the present study was our evaluation of insulin resistance using 75-g OGTT-derived indices, which are more simple and practical in daily clinical practice than the glucose clamp. Our findings suggest that hepatic steatosis per se is a central surrogate pathology indicative of skeletal muscle insulin resistance in patients with NAFLD. In addition, there could be a network between the liver and skeletal muscle to maintain whole-body energy homeostasis.

We evaluated insulin sensitivity using three indices: Matsuda index, hepatic insulin resistance index and MCR. Various indices derived from 75-g OGTT data have been proposed as indices of skeletal muscle insulin sensitivity and hepatic insulin resistance[6–10]. Above all, Matsuda index is strongly correlated

Table 2 | Univariate correlation between histological scores and insulin resistance

	Matsuda index		Hepatic insulin resistance index		MCR†	
	r	*P*	*r*	*P*	*r*	*P*
Steatosis	–0.452	<0.001*	0.195	0.101	–0.728	0.001*
Grade	–0.544	<0.001*	0.215	0.069	–0.149	0.581
Stage	–0.369	0.001*	0.078	0.515	0.017	0.949

MCR, metabolic clearance rate. *A *P*-value <0.05 is considered statistically significant. †A euglycemic hyperinsulinemic clamp was carried out in 16 patients.

Table 3 | Age-, sex- and body mass index-adjusted association between insulin resistance and histological scores of the liver

	Matsuda index			Hepatic insulin resistance index			MCR†		
	Coefficient	*t*-statistic	*P*	Coefficient	*t*-statistic	*P*	Coefficient	*t*-statistic	*P*
Steatosis	−0.36	−3.08	0.003*	−0.60	−0.50	0.617	−0.62	−2.39	0.036*
Grade	−0.51	−5.18	<0.001*	0.20	1.91	0.061	−0.08	−0.31	0.761
Stage	−0.37	−3.47	0.001*	0.45	0.41	0.685	−0.08	−0.27	0.790
Steatosis‡	−0.22	−2.02	0.047*	−1.44	−1.19	0.237	−0.62	−2.16	0.059
Grade†	−0.40	−3.00	0.004*	0.36	2.43	0.018*	−0.02	−0.04	0.969
Stage‡	−0.07	−0.54	0.593	−0.17	−1.21	0.229	−0.05	−0.12	0.909

MCR, metabolic clearance rate. All models were adjusted for age, sex and body mass index (BMI) by multiple linear regression. *A *P*-value <0.05 is considered statistically significant. †A euglycemic hyperinsulinemic clamp was carried out in 16 patients. ‡Three histological scores are included in the model.

with insulin-stimulated total glucose disposal during the euglycemic clamp, the gold standard of skeletal muscle insulin sensitivity, and the correlation coefficient is greater than for other OGTT-derived indices of insulin sensitivity[7,10]. In contrast, there has been no established gold standard marker of hepatic insulin sensitivity. Considering evaluation of skeletal muscle and hepatic insulin sensitivity in equal condition, the OGTT-derived hepatic insulin sensitivity index should be based on HGP suppression in hyperinsulinemic state. However, the Defronzo group defines the product of fasting plasma insulin × basal endogenous glucose production as a hepatic insulin resistance index, and also observed that the hepatic insulin resistance index (glucose0–30[AUC] insulin0–30[AUC]) strongly correlated with it[10]. Unfortunately, the conventional index that correlates with HGP suppression under hyperinsulinemic state still remains to be defined. Therefore, we used the Matsuda index and the hepatic insulin resistance index as indices of skeletal muscle insulin sensitivity and hepatic insulin resistance, respectively. MCR represents the difference between insulin-stimulated glucose disposal and HGP, and therefore reflects insulin resistance in both skeletal muscle and the liver to varying degrees depending on the insulin infusion rate in the clamp study[15]. In the present clamp study, insulin was infused at a rate of 3.0 mU/kg/min, resulting in a steady-state insulin concentration of 317.7 ± 13.3 μU/mL. Such supraphysiological hyperinsulinemic condition might occupy the insulin receptors and therefore could reflect postreceptor defects in insulin signaling. In addition, that level might be sufficient to suppress HGP, and MCR mainly reflects skeletal muscle insulin resistance under this condition (insulin infusion at rate: 3.0 mU/kg/min). However, because suppression of HGP is impaired in type 2 diabetes, we cannot rule out the possibility that MCR might reflect insulin resistance not only in skeletal muscle, but also to some extent in the liver[15]. This might explain the relatively weak association between steatosis score and MCR in the present study.

We recently reported the similar association between liver fat and organ-specific insulin resistance assessed by a euglycemic hyperinsulinemic clamp with tracer infusion ([6,6-$^2H_2$]glucose)[16]. Unlike the present study, we observed the significant correlation between hepatic steatosis and HGP × FPI[16]. However, also in that study, hepatic steatosis was more strongly correlated with the skeletal muscle insulin resistance. These accumulating data, together with the present findings, will clarify the significance of hepatic steatosis in energy homeostasis. Also, it should be determined in future which index reflects best for authentic hepatic insulin resistance among the proposed indices for hepatic insulin resistance (the OGTT-derived hepatic insulin resistance index, HGP × FPI, and %HGP).

In the present study, we were unable to find any association between liver histological score and insulin resistance in the liver, although that score was associated with insulin resistance in the distant skeletal muscle. Although the detailed mechanisms still remain unclear, possible interorgan network, as described later, might regulate the change in insulin resistance resulting from pathogenesis of NAFLD greater in skeletal muscle than in the liver. Whether hepatic steatosis is a consequence or cause of skeletal muscle insulin resistance remains unclear. One hypothesis is that skeletal muscle insulin resistance causes obesity and subsequent hepatic steatosis, as shown experimentally in mice with muscle-selective insulin resistance[17]. Indeed, Flannery *et al.*[18] reported that skeletal muscle insulin resistance promotes increased hepatic de novo lipogenesis and hepatic steatosis in the elderly. The second hypothesis is that a skeletal muscle-derived hormone (myokine) could be overproduced in patients with skeletal muscle insulin resistance, and might induce liver steatosis[19]. Conversely, the third hypothesis is that a liver-derived hormone (hepatokine)[20] is overproduced in patients with NAFLD, and mgiht affect the insulin sensitivity of distant organs. We previously isolated the hepatokine, selenoprotein P, which is overproduced in overnutrition, and causes insulin resistance in both the liver and skeletal muscle[21]. Assaying the levels of myokines and hepatokines will help us to understand their contribution to the pathology of NAFLD. In addition, complex mechanisms might underlie the causal role of hepatic steatosis in the development of hepatic insulin resistance. Recent studies suggest that triglyceride itself is not a toxic lipid[22]. Rather, the accumulation of triglycerides might be a

protective mechanism to prevent toxic effects of free fatty acids. Hepatic steatosis can occur independently of insulin resistance[20]. In this regard, the quality of, rather than quantity of, the accumulating lipid could determine hepatic insulin signaling, and searching for toxic lipids that cause hepatic insulin resistance should be required for understanding the pathophysiology of fat-induced insulin resistance. We previously identified cholesterol[23] and palmitate[24] as toxic lipids that cause mitochondria-derived oxidative stress and hepatic insulin resistance. These might be the reasons why hepatic steatosis correlates with insulin resistance in the skeletal muscle rather than in the liver in the present study.

The present study had several limitations. First, most of the participants were diabetes patients. Therefore, insulin resistance might be greater in these study subjects than in the general population, which could have influenced the results. In future studies, direct evaluation of organ-specific insulin resistance by using the euglycemic hyperinsulinemic clamp with tracer will better validate the present conclusion in NAFLD patients with and without diabetes. Second, the number of study participants with severe fibrosis (stage 3 or 4) was relatively small, and the study participants might therefore not have fully shown pathophysiological conditions of fibrosis. Third, we could not evaluate insulin sensitivity in equal condition (hepatic insulin resistance index and Matsuda index are based on index in a fasting and hyperinsulinemic state, respectively). Fourth, the present study was an observational design, and so we could not evaluate causal associations. A large-scale longitudinal study is required to clarify whether hepatic steatosis is a consequence or cause of skeletal muscle insulin resistance.

In summary, the present study showed that liver steatosis is associated with insulin resistance in skeletal muscle rather than in the liver, suggesting a central role of fatty liver in the development of insulin resistance, and that a network between the liver and skeletal muscle maintains whole-body energy homeostasis.

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