Table 3 Comparison between participants with simple steatosis, and mild and advanced NASH

Parameters	Simple steatosis (SS)	Mild NASH (mNASH)	Advanced NASH (aNASH)	*P*-value
n	43	73	17	
Sex (female)	16 (37%)	40 (55%)	14 (82%)	0.0020
Age (year)	48.7 (15.9)[a]	56.8 (14.9)	65.1 (7.9)	[a]0.0007 vs aNASH, 0.0169 vs. mNASH
BMI (kg/m^2)	27.0 (4.8)	28.5 (5.0)	27.2 (4.0)	NS
obesity (BMI > 25 kg/m^2)	30 (70%)	56 (77%)	12 (71%)	NS
Diabetes	10 (23%)	38 (52%)	9 (53%)	NS
Hypertension	8 (19%)	30 (41%)	5 (29%)	NS
Dyslipidemia	12 (43%)	26 (36%)	9 (53%)	NS
Hemoglobin (g/dL)	14.8 (1.5)	14.1 (1.4)[b]	13.9 (1.3)	[b]0.046 vs SS
Platelet (10^4/μL)	24.4 (5.5)	22.2 (6.3)	17.7 (5.6)[c]	[c]0.0007 vs SS, 0.0210 vs mNASH
AST (IU/L)	40.8 (24.0)[d]	64.1 (31.8)	75.6 (40.5)	[d]0.0001 vs mNASH, aNASH
ALT (IU/L)	72.2 (56.5)	93.8 (48.0)	84.4 (50.2)	NS
AST/ALT ratio	0.64 (0.20)	0.75 (0.31)	0.96 (0.28)[e]	[e]0.0003 vs SS, 0.0160 vs mNASH
γGT (IU/L)	75.6 (66.9)	84.4 (81.4)	93.4 (45.8)	NS
Cholinesterase (IU/L)	397.8 (69.2)	378.9 (67.6)[f]	324.9 (101.3)	[f]0.0039 vs SS, 0.0283 vs aNASH
Albumin(g/dL)	4.51 (0.32)	4.37 (0.30)	4.29 (0.38)	NS
Cholesterol (mg/dL)	214.5 (43.7)	208.3 (39.6)	189.9 (38.5)	NS
Triglyceride (mg/dL)	172.9 (83.6)	189.1 (105.0)	153.4 (86.7)	NS
HDL-C (mg/dL)	50.8 (14.7)	52.8 (31.5)	51.8 (10.7)	NS
Prothrombin time (%)	106.9 (16.3)	99.0 (17.8)	90.3 (17.7)[g]	[g]0.0061 vs SS
Ferritin (ng/mL)	179.0 (182.6)	241.0 (182.1)	278.1 (246.2)	NS
Hyaluronic acid (ng/mL)	26.1 (21.8)	69.8 (104.7)	169.1 (172.4)[h]	[h]<0.0001 vs SS, 0.0014 vs mNASH
Type IV collagen 7 s (ng/mL)	3.67 (0.61)	4.99 (1.51)[i]	6.86 (1.68)[j]	[i]<0.0001 vs SS, [j]<0.0001 vs SS, mNASH
FPG (mg/dL)	97.8 (17.8)	107.8 (49.2)	99.1 (24.7)	NS
IRI (μU/mL)	9.0 (5.6)[k]	16.6 (8.7)	21.0 (12.9)	[k]P < 0.0001 vs aNASH, 0.001 vs mNASH
HOMA-IR	2.15 (1.34)[l]	4.68 (4.51)	5.21 (3.45)	[l]0.0023 vs mNASH, 0.0160 vs aNASH
QUICKI	0.35 (0.03)[m]	0.32 (0.03)	0.31 (0.03)	[m]<0.0001 vs mNASH, 0.001 vs aNASH
Free fatty acid (mEq/L)	0.56 (0.18)	0.58 (0.20)	0.58 (0.16)	NS
DHEA-S (μg/dL)	170.4 (129.2)	121.2 (100.7)	55.7 (45.0)[n]	[n]0.0012 vs SS

Results are presented as numbers with percentages in parenthesis for qualitative data or as means with standard deviation in parenthesis for quantitative data.

P-values were calculated by Scheffe's method or χ^2-test analysis.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DHEA-S, dehydroepiandrosterone sulfate; FPG, fasting plasma glucose; HDL-C, high density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment for insulin resistance; IRI, immuno-reactive insulin; QUICKI, quantitative insulin sensitivity check index; γGT, γ-glutamyl transpeptidase.

levels of DHEA-S compared with mild NASH (Table 3). Though patients with SS had significantly higher levels of serum DHEA-S (170.4 ± 129.2 μg/dL) than the control group (113.6 ± 91.8 μg/dL, $P < 0.001$), the former was younger (48.7 ± 15.9 years) than the latter (55.6 ± 12.1 years, $P < 0.001$) (Tables 1,3). Thus, we selected 129 sex- and age-matched healthy people out of the control group to clarify whether the real difference exists. Serum DHEA-S levels of 129 sex- and age-matched healthy people (145.0 ± 114.8 μg/dL) were not different from patients with SS ($P = 0.225$).

Participants with advanced NAFLD (NASH with stage 3–4 fibrosis) had significantly lower levels of DHEA-S compared with participants with mild NAFLD (SS and NASH with stage 0–2 fibrosis) (55.7 ± 45.0 vs 139.4 ± 114.1 μg/dL, $P = 0.003$). None of the younger patients (<40 years, $n = 21$) had advanced NASH. We compared DHEA-S levels in patients with more advanced NASH, aged 40–65 years (mean 57.9 ± 4.2 years), with patients with mild NAFLD, aged 40–65 years (mean 53.8 ± 7.5 years, $P = 0.139$). DHEA-S levels tended to be lower in patients with advanced NASH than in patients with mild NAFLD (76.1 ± 55.9 vs 117.1 ± 66.2 μg/dL, $P = 0.098$) without reaching significant difference. Next, we compared DHEA-S levels in patients with more advanced NASH,

Table 4 Logistic regression models of the association of NAFLD (advanced vs mild) with DHEA-S levels and other clinical variables

Variables	OR	95% CI	*P*-value
Model 1			
DHEA-S ≤66 μg/dL	8.9113	2.7009–29.4014	0.0003
Model 2			
DHEA-S ≤66 μg/dL	7.1201	2.0811–24.3606	0.0018
Age ≥65 years	2.1324	0.6899–6.5910	0.1884
Model 3			
DHEA-S ≤66 μg/dL	5.4624	1.5555–19.1822	0.0081
Age ≥65 years	2.2978	0.7440–7.0964	0.1482
Sex (female)	2.7458	0.6797–11.0932	0.1562
Model 4			
DHEA-S ≤66 μg/dL	8.5274	2.2958–31.6740	0.0014
HOMA-IR ≥5	2.4319	0.6799–8.6982	0.1717
BMI ≥2 kg/m^2	0.9328	0.2546–3.4177	0.9164
Diabetes	1.5532	0.4324–5.5796	0.4998
Dyslipidemia	0.2547	0.0727–0.8926	0.0326
Hypertension	0.5488	0.1473–2.0446	0.3713
Model 5			
DHEA-S ≤66 μg/dL	4.9549	1.1691–20.9996	0.0229
Age ≥65 years	2.8962	0.7843–10.6948	0.1106
Sex (female)	1.9494	0.3765–10.0935	0.4264
HOMA-IR ≥5	2.3671	0.6276–8.9273	0.2033
BMI ≥28 kg/m^2	1.0446	0.2619–4.1658	0.9508
Diabetes	1.6007	0.3904–6.5023	0.5107
Dyslipidemia	0.2500	0.0682–0.9162	0.0364
Hypertension	0.4184	0.1022–1.7126	0.2256

BMI, body mass index; CI, confidence interval; DHEA-S, dehydroepiandrosterone sulfate; HOMA-IR, Homeostasis Model Assessment for Insulin Resistance; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio.

aged more than 65 years (mean 71.4 ± 3.4 years), with patients with mild NAFLD, aged more than 65 years (mean 72.0 ± 5.5 years, $P = 0.887$). DHEA-S levels were significantly lower in patients with advanced NAFLD than in patients with mild NAFLD (37.6 ± 22.8 vs 68.4 ± 37.5 μg/dL, $P = 0.026$). As levels of DHEA-S are different between men and women and lower in older individuals as mentioned above, DHEA-S levels were adjusted for age and sex. Several multivariate logistic regression models were run in order to determine the association of DHEA levels with the presence or absence of advanced NAFLD while adjusting for the effect of age and sex. As shown in Table 4, the unadjusted (model 1) association of DHEA levels with severity of NAFLD remained highly significant when adjusted by age (model 2) and age plus sex (model 3). The AUC for DHEA in separating patients with and without advanced fibrosis was 0.788 (Fig. 1b). The sensitivity of a DHEA-S-value of 66 μg/dL or less for the presence of more advanced NAFLD was 76.5% (13/17) and specificity was 73.3% (85/116). The positive predictive value (PPV) of the cut-off value was 29.5 % (13/44) and negative predictive value (NPV) was 95.5% (85/89). Almost all of the predictivity for histological severity of NAFLD could be attributed to DHEA-S levels independent of age and sex. DHEA levels remained highly significantly associated with advanced NAFLD after adjustment by metabolic disease or insulin resistance (Table 4, models 4 and 5). A "dose effect" of lower DHEA-S and advanced fibrosis was observed with a mean DHEA-S of 170.4 ± 129.2, 137.6 ± 110.5, 96.2 ± 79.3, 61.2 ± 46.3 and 30.0 ± 32.0 μg/dL for fibrosis stages 0, 1, 2, 3 and 4,

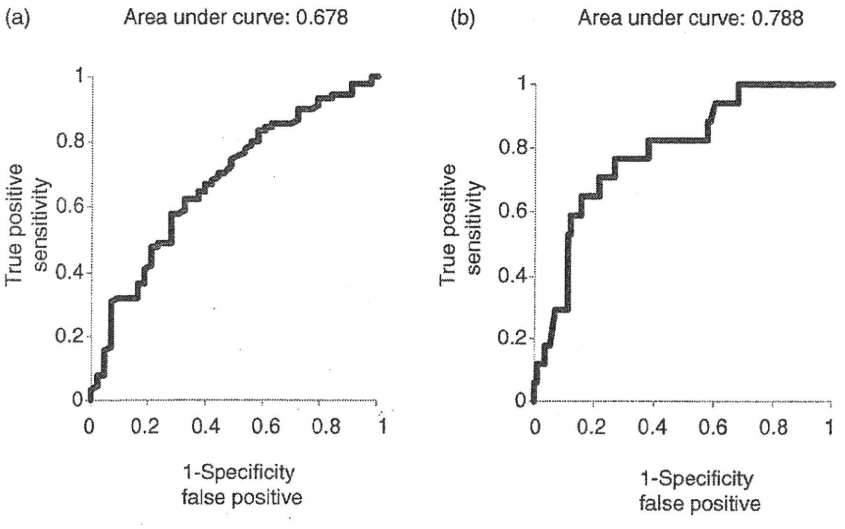

Figure 1 The area under the receiver-operator curve for dehydroepiandrosterone in separating patients with and without non-alcoholic steatohepatitis (a) or separating patients with and without advanced fibrosis (b).

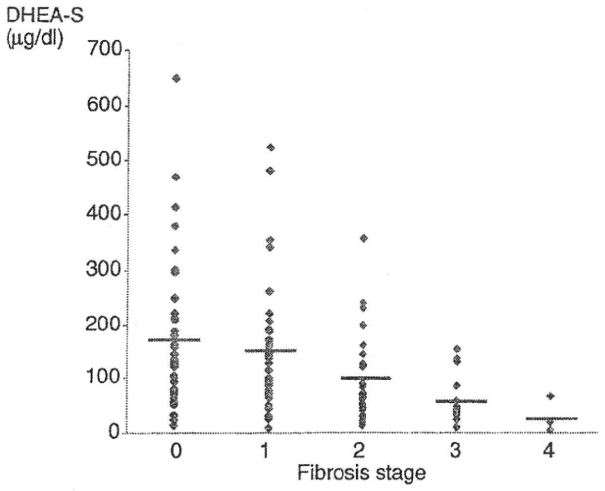

Figure 2 Variation in dehydroepiandrosterone sulfate (DHEA-S) levels with fibrosis stage for participants with non-alcoholic fatty liver disease. Mean DHEA-S levels are indicated by horizontal lines. A "dose effect" of lower DHEA-S and advanced fibrosis was observed, with a mean DHEA-S of 170.4 ± 129.2, 137.6 ± 110.5, 96.2 ± 79.3, 61.2 ± 46.3 and 30.0 ± 32.0 for fibrosis stages 0, 1, 2, 3 and 4, respectively. Significant inverse correlations were detected by Spearman's rank correlation analysis (correlation coefficient value −0.4141, $P < 0.0001$).

respectively (Fig. 2). DHEA-S levels were inversely correlated with the progression of fibrosis by Spearman's rank correlation analysis (correlation coefficient value −0.4141, $P < 0.0001$).

DISCUSSION

THE PRINCIPAL FINDING of this study is that circulating DHEA-S levels are strongly associated with the most important feature of histologically advanced NAFLD, though DHEA-S levels in a total of NAFLD patients were not different from those in sex- and age-matched healthy people. DHEA, and its interchangeable sulfated form, DHEA-S, are the most abundant circulating steroid hormones in healthy individuals. They are derived from the zona reticularis of the adrenal cortex. Both cross-sectional and longitudinal data[26] have clearly indicated that serum concentrations of DHES-S decrease with age. DHEA and DHEA-S levels peak at approximately age 25 years and decrease progressively thereafter, falling to 5% of peak levels by the ninth decade. Though it is important to consider whether the lower DHEA levels observed in patients with advanced NAFLD in our study were simply a surrogate of older age, age was less predictive of severity of NAFLD than DHEA-S by logistic regression analysis (Table 4). In an middle-aged population, there was no significant difference in serum DHEA-S levels between mild and advanced NAFLD, but in aged people, DHEA-S was significantly lower in advanced NAFLD than in mild NAFLD.

The role of DHEA-S deficiency in histological progression of NAFLD is likely to involve effects on insulin sensitivity, hepatic susceptibility to oxidative stress injury and/or stimulation of fibrosis. Hyperinsulinemia and increased insulin resistance may have important roles in the pathogenesis of NASH in both Western and Asian countries.[6,27–29] Hyperinsulinemia in NASH patients is attributable to increased insulin secretion compensatory to reduced insulin sensitivity, and is not the consequence of decreased hepatic extraction of insulin that occurs in all forms of chronic liver diseases at the stage of advanced fibrosis or cirrhosis.[27,28] The HOMA model[23] or the QUICKI model[24] have been validated and widely used for determining the degree of insulin resistance and strongly predicts the development of type 2 DM.[2] In agreement with our result, patients with NASH have higher HOMA index or lower QUICKI index compared with those with SS.[27,30] With regard to results obtained from the logistic regression model (Table 4), we intended to support the concept that the association between low levels of DHEA and worsening histology is independent of age, sex and insulin resistance. Though Charlton *et al.* observed that levels of DHEA are significantly lower in patients with histologically advanced NASH, independent of age or sex, they did not capture a specific index of insulin resistance, such as HOMA or QUICKI. We examined whether serum DHEA-S is correlated with these indices, but did not find any correlations. Therefore, it is highly likely that the association of DHEA levels and severity of NAFLD found in our patients was not confounded by the degree of insulin resistance. Several studies found an association between a decline in serum DHEA concentration and reduced insulin sensitivity.[31] In this way, the published work concerning the role of DHEA in mediating insulin sensitivity in humans is conflicting.

On the other hand, DHEA has been shown to exert a protective effect in hepatocytes against oxidative injury by decreasing malondialdehyde concentration and increasing superoxide dismutase activity and total glutathione concentrations in animal models of oxidative stress.[32,33] FFA, which lead to oxidative stress in NASH, are the major source of DHEA. In the presence of severe insulin resistance, increased circulating FFA are not converted into DHEA. It is suggested that the inability to

produce appropriate amounts of DHEA in response to FFA may translate into a more rapid and worsening progression toward NASH.[34] Although we found no correlations between serum DHEA-S and serum FFA (Table 2), it remains unknown whether serum DHEA-S is correlated with hepatic FFA.

Although several relatively non-invasive parameters have been identified as predictive of more advanced fibrosis stage in patients with NAFLD, none has sufficient sensitivity or specificity to be of clinical utility to negate the need for liver biopsy.[35,36] By ROC analysis, serum DHEA-S levels seem to be useful for differentiating advanced NAFLD rather than for detecting NASH. Our data suggest that patients with DHEA-S levels greater than 66 μg/dL are highly unlikely to have advanced NAFLD (4/89 patients, sensitivity 76% and specificity 73%). This cut-off value was lower than that proposed by Charlton *et al.* (<100 μg/dL).[19] First, racial/ethnic differences in DHEA-S levels could account for this difference.[37] DHEA-S seems to be significantly higher in white versus Chinese men, but not in the women.[38] However, the PPV is too low (29.5%) to pick up advanced NAFLD, because the prevalence of advanced NAFLD is extremely low in our population (12.7%, 17/133). Thus, serum DHEA-S levels can be applicable to exclude advanced NAFLD rather than to detect the stage.

It was also important to consider whether low levels of DHEA-S might occur as a result of chronic liver disease in general versus a specific phenomenon of histologically more advanced NAFLD. Serum DHEA-S levels depend on adrenal DHEA production and its hepatic metabolism mediated by DHEA sulfotransferase (DHEA-ST) which catalyzes sulfonation of DHEA to form DHEA-S. The relationship between adrenal function and liver function remains unclear. It is probable that adrenal DHEA production may decrease in accordance with fibrosis progression, because adrenal insufficiency is increasingly reported with end-stage liver disease.[39] In the present study, however, we excluded patients with decompensated LC. In cirrhotic patients, serum DHEA-S levels are lower than normal control subjects.[40] It is hypothesized that a low level of DHEA-S was due to a defect in sulfurylation in patients with hepatic cirrhosis. However, that study has a few limitations: the number of subjects was very small, and cirrhotic patients were older (mean 49 years, range 21–70) than normal men (mean 25 years, range 21–38). On the other hand, histochemical analysis revealed that the immunopositive area for DHEA-ST was significantly larger in chronic hepatitis than in normal liver, but was not different between LC and normal liver.[41] In another study,[42] DHEA-ST activity and concentration were significantly reduced in PBC, primary sclerosing cholangitis (PSC), chronic active hepatitis and alcoholic cirrhosis, but not in cryptogenic cirrhosis when compared to normal liver. Based on these controversial results, it is unknown whether reduced activity of DHEA-ST is responsible for low levels of DHEA-S in the advanced stage of NAFLD. According to Charlton *et al.*[19] DHEA-S levels were not significantly predictive of severity of disease in patients with cholestatic liver disease, PBC and PSC. In the future, we should examine the serum levels of DHEA-S in other chronic liver diseases and hepatic expression of DHEA-ST in NAFLD patients.

There are thus several potential mechanisms for DHEA deficiency to promote histological progression in NAFLD. DHEA deficiency (patients with advanced NAFLD had levels of DHEA-S associated with hypoadrenalism) presents an appealing new therapeutic target for the treatment and prevention of NASH. A protective effect of DHEA was reported in an orotic acid-induced animal model of fatty liver disease.[33] However, therapeutic benefits of hormone supplementation for the treatment of aging, insulin resistance and cardiovascular disease remain obscure and controversial.[43]

Our study has a few important limitations. Patient selection bias can also exist, because liver biopsy might be considered for NAFLD patients who are likely to have NASH. First, the proportion of subjects with advanced fibrosis was small as reported in other Asian series.[44,45] We acknowledge that pathological diagnosis was mainly determined using liver tissues received by percutaneous liver biopsy, which is prone to sampling error or inter-observer variability.[46,47] Due to these limitations, the present results need to be validated in independent populations by other investigators.

In conclusion, we have found that patients with more advanced NAFLD have low circulating levels of DHEA-S. These data provide novel evidence for relative DHEA deficiency in Japanese patients with histologically advanced NASH.

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The fatty acid composition of plasma cholesteryl esters and estimated desaturase activities in patients with nonalcoholic fatty liver disease and the effect of long-term ezetimibe therapy on these levels

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ABSTRACT

Background: The aim of this study was to investigate the relationship between fatty acid composition of plasma cholesteryl esters (CEs) and estimated desaturase activity and the development and progression of nonalcoholic fatty liver disease (NAFLD). The study also assessed the effect of ezetimibe on CE levels.
Methods: Plasma CEs fatty acid composition was analyzed in 3 groups: patients with a NAFLD activity score (NAS) ≤4 (n=31) or NAS ≥5 (n=32) and normal controls (n=25). The estimated desaturase activities were calculated using ratios of 16:1n−7/16:0 (D9-16D), 18:1n−9/18:0 (D9-18D), 18:3n−6/18:2n−6 (D6D) and 20:4n−6/20:3n−6 (D5D).
Results: Compared with controls, the levels of palmitate, palmitoleate, γ-linoleate, D9-16D and D6D were significantly increased, whereas levels of linoleate and D5D were significantly decreased. Patients with NAS ≥5 had significantly higher palmitate levels than patients with NAS ≤4. The levels of these fatty acids, especially palmitate and palmitoleate, correlated with NAFLD-related lipid, metabolic, and inflammatory parameters. Long-term therapy with ezetimibe caused significant improvements in the levels of these fatty acids, estimated desaturase activity index and NAFLD-related parameters.
Conclusions: Our results suggest that fatty acids and desaturase activity associate with the development and progression of NAFLD, and that ezetimibe may be a novel treatment for this disorder.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver injury in the world [1–3]. NAFLD is a metabolic condition which encompasses a wide spectrum of liver disease, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). Although the intricacies of the molecular and cellular mechanisms responsible for progression from simple steatosis to NASH have not been fully elucidated, hyperlipidemia, insulin resistance and oxidative stress are known to be major contributing factors in the initiation and progression of NAFLD [4–6]. It has been proposed that steatosis, the earliest and most prevalent stage of NAFLD, often referred to as the "first hit", increases the vulnerability of the liver to a "second hit" that in turn lead to the inflammation, fibrosis and cellular death characteristic of NASH.

Excessive accumulation of lipid substrates in the liver has serious adverse effects on cell functions and is termed lipotoxicity [7]. Studies of lipid accumulation in tissue have usually involved measuring triglycerides (TG) content, although recent studies have shown clearly that the deleterious effects are due not only to TG accumulation but also to other lipid metabolites such as palmitate, diacylglycerols (DAG) and ceramide [7–11]. Recent studies have shown NAFLD is also characterized by increased DAG, free cholesterol, decreased phosphatidylcholine (PC), and altered n−3 and n−6

Abbreviations: CEs, Cholesteryl esters; DAG, Diacylglycerols; HOMA-R, Homeostasis model assessment of insulin resistance; IRI, Immunoreactive insulin; NAS, NAFLD activity score; emlDL, Electronegative charge modified-LDL; NAFLD, Nonalcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; oxLDL, Oxidized LDL; PC, Phosphatidylcholine; PUFA, Polyunsaturated fatty acid.

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polyunsaturated fatty acid (PUFA) metabolism [12,13]. However, it is not yet established whether these changes are reflected by circulating lipidome levels and also whether or not NASH is associated with a distinct lipidomic profile. A change in the proportions of fatty acids in the diet is reflected mainly by serum triglyceride levels within the first hours. On the other hand, the fatty acids composition of serum cholesterol esters (CEs) is related to the average dietary fatty acid composition during the last 3 to 6 weeks and also reflects endogenous fatty acid metabolism [14]. Fatty acid composition is used as an indicator of disease risk, because its alteration has been related to metabolic disease and cardiovascular disease [15,16]. Low concentrations of linoleic acid (18:2n−6) and high concentrations of palmitic (16:0), palmitoleic (16:1n−7) and dihomo-g-linolenic (20:3n−6) acids in plasma lipid esters have been reported to be associated with metabolic syndrome [17,18]. However, to our knowledge, only two studies have assessed the relationship between plasma fatty acid composition between histopathologically-proven NAFLD/NASH [19,20]. Desaturases are involved in the endogenous synthesis of PUFAs. The delta 9, 6, and 5 desaturases (D9D, D6D and D5D) introduce a double bond at specific position on long-chain fatty acids. D9D synthesizes monounsaturated fatty acids (MUFA), palmitoleic (16:1n−7) and oleic acids from palmitic (16:0) and stearic (18:0) acids, respectively. D5D and D6D catalyze the synthesis of long-chain n−6 and n−3 PUFAs. In human studies, the estimated desaturase activities are generally used, since it is not possible to directly measure desaturase activities in human. Therefore, the estimated desaturase activities of D9D, D6D and D5D calculated by the plasma ratio of 16:1n−7/16:0, 18:3n−6/18:2n−6 and 20:4n−6/20:3n−6, respectively, can be used as surrogates of the measure of the true desaturase activity [14].

The aim of this study was to assess whether the levels of fatty acid components of plasma CEs and their estimated desaturase activities were associated with the development and progression of NAFLD. We also investigated the effect of long-term ezetimibe, a cholesterol absorption inhibitor, on CE levels, as it has been reported that this drug causes significant reduction in the absorption of several saturated fatty acids in diet-induced obese and diabetic mice [21].

2. Patients and methods

2.1. Patients

The study protocol was approved by the ethics committee of Saiseikai Suita Hospital and the Kyoto Prefectural University of Medicine. Informed consent was obtained from all subjects prior to enrollment in the study. A total of 63 patients at Saiseikai Suita Hospital and Kyoto Prefectural University Hospital who had been diagnosed histologically with NAFLD between 2007 and 2009 were evaluated in the study.

All liver biopsy specimens were examined by 2 experienced pathologists blinded to the patients' clinical and laboratory data and liver biopsy sequence. In this study, the NAFLD activity score (NAS) system was used to classify NAFLD into 2 groups; NAS ≤ 4 (n = 31, "simple steatosis" and "borderline NASH") and NAS ≥ 5 (n = 32, "definite NASH"). The NAS system was reported as a reliable scoring system for diagnosing NASH by Kleiner et al. [22]. Prior to evaluation of liver histology we excluded patients with an alcohol intake exceeding 20 g/day and those who reported signs, symptoms and/or history of known liver disease including viral, genetic, autoimmune, and drug-induced liver disease, and previous use of anti-diabetic medication including insulin-sensitizing agents such as metformin and pioglitazone.

After enrollment, all the patients were asked to adhere to a dietary plan tailored to their energy requirements and metabolic control. The dietary plans were formulated by a registered dietitian and/or medical doctor using the current Japan Diabetes Society recommendations (JDSR) and were maintained throughout the study. Blood samples were obtained in the morning after an overnight fasting. Blood samples were also obtained from 25 age- and sex-matched normal control subjects who were on the clinical staff at our hospitals. To investigate the efficacy of long-term ezetimibe therapy on CE levels, all patients received ezetimibe (10 mg/day) for 24 months. Fatty acid composition in plasma CEs and liver histological examination was determined before and after the 24-month ezetimibe therapy.

2.2. Analysis of fatty acid composition in plasma CEs

Blood was drawn into tubes containing ethylenediaminetetraacetic acid disodium salt. Plasma was separated by centrifugation of the samples at 1600×g for 15 min at 4 °C and then stored at −80 °C until assayed. Total lipid was extracted from plasma by using the method of Bligh and Dyer [23]. CEs were separated by thin-layer chromatography on silica gel plates (Silica Gel 60, Merck, Darmstadt, Germany) using a solvent system of petroleum ether:ethyl ether:acetic acid (80:20:1, v/v/v). The spot corresponding to the CEs was scraped from the plate and transmethylated with 2 ml of acetyl chloride:methanol (5:50, v/v) at 90 °C for 2 h. Heptadecanoic acid (17:0) was used as an internal standard. Fatty acid methyl esters were quantified using a model GC14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 25-m×0.5-mm capillary column (HR-SS-10, Shinwa Chemical Industries, Ltd., Kyoto, Japan).

2.3. Estimation of desaturase activity

Desaturase and elongase activities were estimated as the ratio product of individual precursor fatty acids in plasma CEs according to the following criteria: D9-16D = 16:1n−7/16:1, D9-18D = 18:1n−9/18:1, D6D = 18:3n−6/18:2n−6 and D5D = 20:4n−6/20:3n−6 [14].

2.4. Other laboratory investigations

Plasma glucose (PG) was measured by the glucose oxidase method and HbA1c determined by high performance liquid chromatography (HPLC: Arkray Inc., Kyoto, Japan). Plasma insulin (immunoreactive insulin: IRI) concentrations were measured by an immunoradiometric assay (Insulin-RIAbead II, Abbott, Japan). The homeostasis model assessment of insulin resistance (HOMA-R) was calculated from fasting insulin and plasma glucose levels by the following equation: HOMA-R = fasting IRI (μU/ml) × fasting PG (mg/dl)/405. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-Ch), high-density lipoprotein cholesterol (HDL-Ch), low-density lipoprotein cholesterol (LDL-Ch) and triglyceride (TG) were measured by enzymatic methods using a chemical autoanalyzer (Hitachi Co., Tokyo, Japan). Serum type IV collagen 7S was measured by a radioimmunoassay kit (Mitsubishi Chemical Group, Tokyo, Japan), serum high-sensitivity C-reactive protein (hs-CRP) was measured by latex particle-enhanced nephelometry (Dade Behring, Tokyo, Japan) and serum oxidized LDL (oxLDL) was measured by an enzyme-linked immunoassay (ELISA) kit (Kyowa Medex Co., Ltd., Tokyo, Japan).

Net electronegative charge modified-LDL (emlDL) was analyzed using an agarose gel electrophoresis lipoprotein fraction system, according to the manufacturer's instructions (Chol/Trig Combo System™; Helena Labs, Saitama, Japan). The percentage frequency of emlDL was calculated on a computer using the migration distance (b) of the LDL fraction in the test samples and the migration distance (a) of normal control sera according to the following formula: emlDL density = [b − a/a] × 100% [24]. Production of LDL with an increased net electronegative charge, caused by modification of lysine residues by either acetylation, carbamylation, glycation, glycoxidation or oxidation all result in increased uptake of the lipids by macrophages via the scavenger receptor system [25–27]. This uptake is thought to

be a key process in the formation of foam cells, the hallmark of early atherosclerotic lesions.

2.5. Statistical analysis

All statistical analyses were performed using Statview version 5.0 (Abacus Concepts, Berkeley, CA), with data expressed as the means ± SD. When the data were not normally distributed, logarithmical transformation was performed. Differences between the groups were determined by one-way ANOVA with Scheffé's multiple comparison test. The nonparametric Wilcoxon signed rank test was used to compare data of pre- and post-ezetimibe treatment. The risk of progression of NAFLD was estimated by logistic regression analysis with the standardized (SD = 1.0) odds ratio (OR) and 95% confidence interval (CI) being calculated. Spearman's correlation coefficients were used to evaluate the relationship between NAFD-related metabolic risk factors and plasma CEs fatty acids and index of estimated desaturase activity. A P-value <0.05 was considered statistically significant.

3. Results

3.1. Subject characteristics

The baseline clinical and laboratory characteristics of the normal controls and two patient groups classified according to the NAS scores are shown in Table 1. Compared with normal controls, both patient groups (NAS ≤4 and NAS ≥5) had significantly higher values of body mass index (BMI), waist circumference (WC), visceral fat area (VFA), systolic blood pressure (sBP), diastolic blood pressure (dBP), HbA1c, PG, IRI, HOMA-R, AST, ALT, TG, T-Ch, LDL-Ch, oxLDL, emlDL, type IV collagen 7S, leptin and hsCRP, and significantly lower HDL-Ch and adiponectin levels. As expected, a significant proportion of NAFLD subjects had features of the metabolic syndrome. The patients with a NAS ≥5 had significantly higher serum emlDL and type IV collagen 7S levels than patients with NAS ≤4 (both P<0.05). There was no significant difference in any other clinical or laboratory parameter between the two patient groups.

3.2. Fatty acid composition in plasma CEs

The fatty acid composition of the plasma CEs and their estimated desaturase activity was shown in Table 2. In both patient groups, the levels of palmitic acid (C16:0), palmitoleic acid (16:1n−7) and γ-linoleic (C18:3n−6) acid were significantly higher, and linoleic acid (C18:2n−6) was significantly lower, compared to controls. Oleic (18:1n−9) and dihomo-γ-linoleic acid (C20:3n−6) levels in patients with a NAS ≤4 were significantly higher than in control subjects. In contrast, the levels of arachidoric (C20:4n−6), eicosapentaenoic (C20:5n−3) and docosahexaenoic (C22:6n−3) acids showed no significant differences between the groups. The levels of oleic and dihomo-γ-linoleic acid in the patients with NAS ≥5 were also higher than in control subjects, although this difference was not statistically significant. The levels of palmitic acid in patients with a NAS ≥5 were significantly higher in patients with a NAS ≤4 (P<0.05). No other significant difference in fatty acid composition of plasma CEs was measured between the two groups. Logistic regression analysis showed that high palmitic acid levels were a risk factor for the development (OR = 1.980, 95% CI = 1.109–3.536, P = 0.0209) and progression (OR = 2.040, 95% CI = 1.180–3.526, P = 0.0107) of NAFLD. Both patient groups had significantly higher levels of D9-16D and D6D activities, and significantly lower levels of D5D activity compared to controls. There was no significant difference in D9-16D, D9-18D, D6D and D5D activity between patients with a NAS ≤4 or NAS ≥5 (Table 3).

3.3. Relationship between NAFLD-related metabolic factors and CE fatty acid composition and estimated desaturase activity

As shown in Table 4, there were significant and positive associations between palmitic acid and ALT, TG, T-Ch, emlDL and hsCRP, while palmitoleic acid showed positive associations with BMI, VFA, IRI, ALT,

Table 1
Baseline clinical and laboratory characteristics of the healthy control subjects and two patient groups classified according to NAS scores.

	Controls (n = 25)	NAS ≤4 (n = 31)	NAS ≥5 (n = 32)
Male/female	14/11	16/15	18/14
Age (y)	52.2 ± 9.2	53.3 ± 10.4	54.5 ± 11.1
Body mass index (kg/m^2)	22.6 ± 2.8	27.5 ± 3.1*	27.3 ± 3.4*
Waist circumference (cm)	80.1 ± 3.7	93.6 ± 5.8*	92.1 ± 5.9*
Visceral fat area (cm^2)	96.7 ± 34.6	158.5 ± 41.5*	152.9 ± 40.2*
Systolic blood pressure (mm Hg)	118 ± 14	137 ± 8*	140 ± 18*
Diastolic blood pressure (mm Hg)	77 ± 9	88 ± 11*	89 ± 12*
HbA1c (%)	4.9 ± 0.3	6.4 ± 0.7*	6.3 ± 0.8*
Fasting glucose (mg/dl)	93 ± 32	125 ± 31*	121 ± 34*
Fasting insulin (μU/ml)	4.8 ± 1.6	11.9 ± 4.7*	13.3 ± 7.6*
HOMA-R	1.1 ± 08	3.7 ± 2.1*	3.9 ± 2.7*
Aspartate aminotransferase (U/l)	24 ± 9	38 ± 16*	40 ± 22*
Alanine aminotransferase (U/l)	25 ± 11	49 ± 23*	58 ± 25*
Triglyceride (mg/dl)	96 ± 56	166 ± 85*	160 ± 94*
Total cholesterol (mg/dl)	188 ± 33	218 ± 36*	216 ± 34*
HDL cholesterol (mg/dl)	61 ± 22	51 ± 7*	48 ± 12*
LDL cholesterol (mg/dl)	110 ± 25	134 ± 31*	131 ± 32*
Oxidized LDL (U/ml)	8.7 ± 3.0	13.8 ± 2.5*	14.1 ± 2.9*
Electronegative charge modified-LDL (ecd)	1.2 ± 3.0	3.4 ± 3.2*	6.4 ± 3.5*, #
Type IV collagen 7S (ng/dl)	3.8 ± 0.7	4.1 ± 1.6	5.2 ± 1.9*, #
Adiponectin (mg/ml)	8.5 ± 3.6	5.4 ± 2.9*	5.7 ± 3.1*
Leptin (ng/ml)	2.4 ± 1.1	4.1 ± 3.0*	3.6 ± 2.7*
High-sensitivity CRP (ng/ml)	388 ± 244	585 ± 377*	788 ± 457*
75 g OGTT (NGT/IGT/DM)	25/0/0	7/17/7*	7/15/10*

Data are expressed as the mean ± standard deviation.
ecd, electronegative-charge density; OGTT, oral glucose tolerance test; NGT, normal glucose tolerance; GT, impaired glucose tolerance; DM, diabetes mellitus.
* P<0.05 vs. controls.
# P<0.05 vs. NAS ≤4.

Table 2
Plasma cholesterol esters fatty acid composition and estimated desaturase activities in the control subjects and two patient groups classified according to NAS scores.

Fatty acids (% of total fatty acids)	Controls (n = 25)	NAS ≤4 (n = 31)	NAS ≥5 (n = 32)
C14:0 (miristic acid)	0.55 ± 0.14	0.57 ± 0.11	0.57 ± 0.23
C16:0 (palmitic acid)	11.14 ± 1.05	11.83 ± 0.99*	12.55 ± 1.03*, #
C16:1n−7 (palmitoleic acid)	2.38 ± 0.77	3.24 ± 1.00**	3.13 ± 1.02*
C18:0 (stearic acid)	1.04 ± 0.25	1.09 ± 0.29	1.09 ± 0.30
C18:1n−9 (oleic acid)	17.82 ± 2.60	19.61 ± 2.53*	19.20 ± 2.41
C18:2n−6 (linoleic acid)	53.20 ± 4.64	48.08 ± 4.41*	48.67 ± 4.32*
C18:3n−6 (γ-linoleic acid)	0.59 ± 0.18	0.85 ± 0.25**	0.82 ± 0.24**
C18:3n−3 (α-linoleic acid)	0.77 ± 0.38	0.76 ± 0.32	0.77 ± 0.36
C20:3n−6 (dihomo-γ-linoleic acid)	0.63 ± 0.18	0.79 ± 0.22*	0.75 ± 0.23
C20:4n−6 (arachidonic acid)	5.54 ± 1.24	5.71 ± 0.95	5.65 ± 1.18
C20:5n−3 (eicosapentaenoic acid)	2.19 ± 0.69	2.15 ± 0.57	2.13 ± 0.72
C22:6n−3 (docosahexaenoic acid)	0.90 ± 0.27	0.86 ± 0.31	0.84 ± 0.30
Estimated desaturase index			
D9-16D (16:1n−7/16:0)	0.21 ± 0.07	0.28 ± 0.09*	0.26 ± 0.08*
D9-18D (18:1n−9/18:0)	17.13 ± 4.13	17.99 ± 4.06	17.77 ± 4.01
D6D (18:3n−6/18:2n−6)	0.011 ± 0.007	0.019 ± 0.011**	0.017 ± 0.009**
D5D (20:4n−6/20:3n−6)	9.05 ± 2.60	7.22 ± 2.45*	7.48 ± 2.38*

Data are expressed as the mean ± standard deviation.
* P<0.05 vs. controls.
** P<0.01 vs. controls.
# P<0.05 vs. NAS ≤4.

Table 3
Spearmen's correlation between 17 clinical variables and fatty acid composition of plasma cholesterol esters nonalcoholic fatty liver disease (n = 63).

	C16:0	C16:1 n−7	C18:1 n−9	C18:2 n−6	C18:3 n−6	C20:3 n−6	D9-16D[a]	D6D[b]	D5D[c]
Body mass index (kg/m^2)	NS	0.257*	NS	−0.252*	NS	0.275*	0.252*	0.252*	−0.318**
Waist circumference (cm)	NS	NS	NS	NS	NS	NS	0.260*	NS	−0.266*
Visceral fat area (cm^2)	NS	0.255*	NS	NS	NS	NS	0.264*	0.260*	−0.263*
Fasting glucose (mg/dl)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Fasting insulin (μU/ml)	NS	0.261*	NS	NS	NS	NS	NS	0.263*	−0.277**
Aspartate aminotransferase (U/l)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Alanine aminotransferase (U/l)	0.259*	0.258*	NS	NS	NS	NS	NS	NS	NS
Triglyceride (mg/dl)	0.253*	0.305**	NS	−0.251*	0.288**	0.265*	0.313**	0.281**	−0.260*
Total cholesterol (mg/dl)	0.252*	0.260*	NS	NS	NS	NS	0.259*	0.251*	−0.255*
HDL cholesterol (mg/dl)	NS	−0.254*	NS	NS	NS	NS	NS	NS	0.271*
LDL cholesterol (mg/dl)	NS	NS	NS	NS	NS	NS	NS	NS	−0.260*
Oxidized LDL (U/ml)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Electronegative charge modified-LDL (ecd)	0.266*	NS	NS	−0.254*	NS	NS	NS	NS	−0.270*
Type IV collagen 7S (ng/dl)	NS	NS	NS	NS	NS	NS	NS	NS	NS
Adiponectin (mg/ml)	NS	NS	NS	NS	NS	NS	NS	NS	0.262*
Leptin (ng/l)	NS	NS	NS	NS	NS	NS	NS	NS	NS
High-sensitivity CRP (ng/ml)	0.255*	NS	NS	NS	NS	NS	NS	NS	NS

* $P<0.05$.
** $P<0.01$.
[a] D9-16D = 16:1n−7/16:1.
[b] D6 = 18:3n−6/18:2n−6.
[c] D5D = 20:4n−6/20:3n−6.

TG, and inverse associations with HDL-Ch. Linoleic acid was significantly and inversely associated with BMI, TG and emlDL. Significant and positive associations were also observed between γ-linoleic acid and TG, dihomo-γ-linoleic acid and BMI and TG, between D9-16D and BMI, WC, VFA, TG and T-Ch, and between D6D and BMI, VFA, TG and T-Ch. D5D showed significant inverse associations with BMI, WC, VFA, IRI, TG, T-Ch, LDL-Ch and emlDL, and positive associations with HDL-Ch and adiponectin.

3.4. Effect of long-term ezetimibe therapy on CE fatty acid composition, estimated desaturase activity and histological changes

We next investigate the effect of long-term ezetimibe therapy on CE fatty acid composition and estimated desaturase activity. As shown in Fig. 1, a significant decrease was observed in miristic acid (−3.9%, $P<0.05$), palmitic acid (−7.0%, $P<0.01$), palmitoleic acid (−10.3%, $P<0.01$), oleic acid (−4.1%, $P<0.05$), dihomo-γ-linoleic acid (−6.1%, $P<0.01$) and D9-16D activity (−9.8%, $P<0.01$), associated with a significant increase in linoleic acid (9.2%, $P<0.01$) and D5D activity (+10.0%, $P<0.01$). Long-term ezetimibe therapy also caused significant decreases in the levels of VFA (−3.5%, $P<0.05$), ALT (−4.8%, $P<0.05$), TG (−4.1%, $P<0.05$), T-Ch (−10.5%, $P<0.01$), LDL-Ch (−11.5%, $P<0.01$), emlDL (−20.5%, $P<0.01$), and hsCRP (−4.5%, $P<0.05$). Follow-up liver biopsies were performed on 33 patients at the end of the 24 months of EZ treatment. Table 4 shows the histologic changes before and after treatment. The mean level of steatosis grade (from 2.3 ± 0.7 to 1.9 ± 0.8, $P=0.0003$), necroinflammatory grade (from 1.9 ± 0.7 to 1.8 ± 0.7, $P=0.0456$), ballooning score (from 1.4 ± 0.5 to 1.3 ± 0.5, $P=0.0253$) and NAS score (from 5.6 ± 1.6 to 5.1 ± 1.8, $P=0.0007$) improved significantly during the study. Of 33 patients, 24 had a one or more point improvement in the NAS score, 8 had no change and one had a one-point increase. In contrast, the mean level of fibrosis stage level did not change significantly (from 2.0 ± 0.8 to 2.1 ± 0.9, P=NS).

Table 4
Histological changes in 33 patients with NAFLD.

	Baseline	After treatment	*P*-value
Steatosis grade	2.3 ± 0.7	1.9 ± 0.8	$P=0.0003$
0	0 (0)	1 (3)	
1	5 (16)	9 (31)	
2	14 (44)	16 (44)	
3	14 (41)	7 (22)	
Necroinflammatory grade	1.9 ± 0.7	1.8 ± 0.7	$P=0.0456$
1	10 (30)	12 (36)	
2	16 (48)	16 (45)	
3	7 (21)	5 (18)	
Fibrosis stage	2.0 ± 0.8	2.1 ± 0.9	$P=0.6547$
0	1 (3)	1 (33)	
1	6 (18)	8 (24)	
2	17 (52)	12 (42)	
3	9 (27)	12 (30)	
4	0 (0)	0 (0)	
Ballooning score	1.4 ± 0.5	1.3 ± 0.5	$P=0.0253$
0	0 (0)	1 (3)	
1	19 (64)	22 (70)	
2	14 (36)	10 (27)	
NAS score	5.5 ± 1.6	5.0 ± 1.8	$P=0.0007$

4. Discussion

In this study, we demonstrated patients with NAFLD had significantly higher levels of palmitic acid, palmitoleic acid, oleic acid, γ-linoleic acid, D9-16D and D6D, and significantly lower levels of D5D compared with normal controls. We did not observe significant differences in total, n−3 and n−6-PUFA levels or n−6/n−3 ratio between our three study groups, despite an increase in the ratio

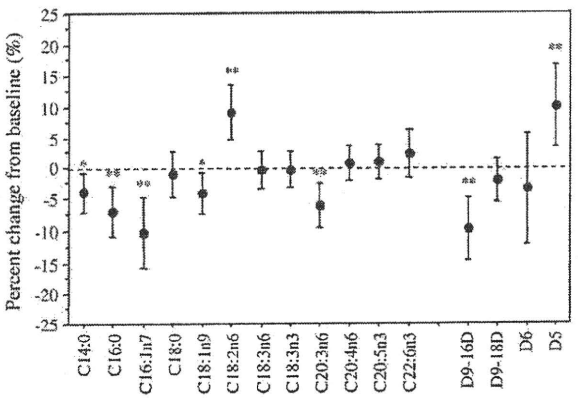

Fig. 1. Effects of ezetimibe on plasma cholesterol fatty acid component and estimated desaturase index in patients with nonalcoholic fatty liver disease. Percentage changes from baseline are least-square means adjusted for the baseline level. Error bars indicate the 95% confidence interval. *$P<0.05$, **$P<0.01$ vs. baseline.

having been reported previously in liver lipids of patients with NASH [12,13]. Our findings essentially agree with the report of Puri et al. [19] that the levels of palmitic acid, palmitoleic acid, oleic acid, and D9-16D were increased significantly in NAFLD across multiple plasma lipid classes, including CEs. Similar findings have also been reported by de Almeida et al. [20].

A noteworthy finding in our study was that patients with a NAS ≥ 5 had significantly higher palmitic acid levels than patients with a NAS ≤ 4, and that these levels were significantly and positively correlated with, not only TG, T-Ch and emIDL, but also ALT and hsCRP. Furthermore, logistic regression analysis showed that a high level of palmitic acid was predictive of NAFLD progression. Joshi-Barve et al. [9] demonstrated that palmitic acid induced production of interleukin-8 by hepatocytes, leading ultimately to liver injury. Several recent studies have also demonstrated palmitic acid induces apoptosis in liver cells [28–30]. Taken together, these earlier data and the findings of the present study suggest strongly that increased levels of palmitic acid are involved in the development and progression of NAFLD.

Stearoyl-CoA desaturase 1 (SCD1; alternatively known as D9D) is the final step in de novo lipogenesis and converts saturated fatty acids to monounsaturated fatty acids, whereas Δ5- and Δ6-desaturases participate in the metabolism of PUFAs [31]. In this study, higher levels of D9-16D and D6D activity and lower levels of D5D activity were observed in patients with NAFLD. Miyazaki et al. [32] have shown that the biosynthesis of hepatic CEs and triglycerides is highly dependent on the expression of the SCD1 gene, while Attie et al. [33] reported that D9D activity was correlated with serum TG levels in human subjects. In this study, we observed significant and positive correlations between D9-16D activity and TG, T-Ch and metabolic syndrome-related anthropometric parameters. With regard to the activities of D5D and D6D, it has been reported that they are both related to insulin sensitivity and insulin levels [34–37]. We also found that insulin resistance parameters correlated positively with D6D levels and negatively with D5D levels. However, no significant difference in the levels of these estimated desaturase activities was observed between NAS ≤ 4 and NAS ≥ 5. Taken together, it is possible to speculate that increasing levels of D9-16D and D6D and decreasing levels of D5D may be major factors in the development and progression of liver steatosis.

Ezetimibe, a lipid-lowering drug, selectively inhibits intestinal cholesterol absorption by binding to Niemann–Pick C1 Like 1 (NPC1L1) protein [38]. Recently, Labonté et al. [21] reported that ezetimibe caused significant reductions in absorption of several saturated fatty acids in diet-induced obese and diabetic mice. In the current study, we demonstrated for the first time that long-term therapy with ezetimibe was also associated with significant decreases in the levels of miristic acid, palmitic acid, palmitoleic acid, oleic acid, dihomo-γ-linoleic acid and D9-16D activity, and significant increases in linoleic acid levels and D5D activity. Several recent studies in an experimental NAFLD model have shown that ezetimibe monotherapy not only protects against diet-induced hyperlipidemia, but also attenuates liver steatosis in an experimental NAFLD model [39,40]. In this study, liver histologic findings were also improved in steatosis grade, necroinflammatory grade, ballooning score and NAS score, although the mean level of fibrosis stage level did not change significantly. In addition, we also found long-term ezetimibe therapy not only decreased T-Ch, LDL-Ch and TG but also emIDL, the most atherogenic lipoprotein that has a strong relationship to insulin resistance. Our findings therefore suggest that ezetimibe may be a useful therapy for NAFLD-related dyslipidemia and hepatic insulin resistance.

In conclusion, the results of the present study suggest that fatty acids composition and desaturase activity associate with the development and progression of NAFLD, and that ezetimibe may be a novel treatment for NAFLD. An appropriately designed, large-scale, prospective clinical study, including histopathological evaluation, is necessary to confirm our findings.

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Cell Metabolism

A Liver-Derived Secretory Protein, Selenoprotein P, Causes Insulin Resistance

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SUMMARY

The liver may regulate glucose homeostasis by modulating the sensitivity/resistance of peripheral tissues to insulin, by way of the production of secretory proteins, termed hepatokines. Here, we demonstrate that selenoprotein P (SeP), a liver-derived secretory protein, causes insulin resistance. Using serial analysis of gene expression (SAGE) and DNA chip methods, we found that hepatic SeP mRNA levels correlated with insulin resistance in humans. Administration of purified SeP impaired insulin signaling and dysregulated glucose metabolism in both hepatocytes and myocytes. Conversely, both genetic deletion and RNA interference-mediated knockdown of SeP improved systemic insulin sensitivity and glucose tolerance in mice. The metabolic actions of SeP were mediated, at least partly, by inactivation of adenosine monophosphate-activated protein kinase (AMPK). In summary, these results demonstrate a role of SeP in the regulation of glucose metabolism and insulin sensitivity and suggest that SeP may be a therapeutic target for type 2 diabetes.

INTRODUCTION

Insulin resistance is an underlying feature of people with type 2 diabetes and metabolic syndrome (Saltiel and Kahn, 2001), but is also associated with risk for cardiovascular diseases (Després et al., 1996) and contributes to the clinical manifestations of nonalcoholic steatohepatitis (Ota et al., 2007). In an insulin-resistant state, impaired insulin action promotes hepatic glucose production and reduces glucose uptake by peripheral tissues, resulting in hyperglycemia. The molecular mechanisms underlying insulin resistance are not fully understood, but are now known to be influenced by the secretion of tissue-derived factors, traditionally considered separate from the endocrine system. Recent work in obesity research, for example, has demonstrated that adipose tissues secrete a variety of proteins, known as adipocytokines (Friedman and Halaas, 1998; Maeda et al., 1996; Scherer et al., 1995; Steppan et al., 2001; Yang et al., 2005), which can either enhance or impair insulin sensitivity, thereby contributing to the development of insulin resistance.

SeP (in humans encoded by the *SEPP1* gene) is a secretory protein primarily produced by the liver (Burk and Hill, 2005; Carlson et al., 2004). It contains ten selenocysteine residues and functions as a selenium supply protein (Saito and Takahashi, 2002). However, the role of SeP in the regulation of glucose metabolism and insulin sensitivity has not yet been established. Furthermore, the clinical significance of SeP in human diseases has not been well defined, although studies of SeP knockout mice showed SeP deficiency to be associated with neurological injury and low fertility (Hill et al., 2003; Schomburg et al., 2003).

The liver plays a central role in glucose homeostasis and is also the site for the production of various secretory proteins. For example, recent work in our laboratory has revealed that genes encoding secretory proteins are abundantly expressed in the livers of people with type 2 diabetes (Misu et al., 2007). Moreover, genes encoding angiogenic factors, fibrogenic factors, and redox-associated factors were differentially expressed in the livers of people with type 2 diabetes (Takamura et al., 2004; Takeshita et al., 2006), possibly contributing to the pathophysiology of

type 2 diabetes and its clinical manifestations. On the basis of these findings, we hypothesize that, analogous to adipose tissues, the liver may also contribute to the development of type 2 diabetes and insulin resistance, through the production of secretory proteins, termed hepatokines.

RESULTS

Identification of a Hepatic Secretory Protein Involved in Insulin Resistance

To identify hepatic secretory proteins involved in insulin resistance, we performed liver biopsies in humans and conducted a comprehensive analysis of gene expression profiles, using two distinct methods. First, we obtained human liver samples from five patients with type 2 diabetes and five nondiabetic subjects who underwent surgical procedures for malignant tumors, and we subjected them to serial analysis of gene expression (SAGE) (Velculescu et al., 1995). Consequently, we identified 117 genes encoding putative secretory proteins with expression levels in people with type 2 diabetes, 1.5-fold or greater higher than those in normal subjects. Next, we obtained ultrasonography-guided percutaneous needle liver biopsies from ten people with type 2 diabetes and seven normal subjects (Table S1 available online), and we subjected them to DNA chip analysis to identify genes whose hepatic expression was significantly correlated with insulin resistance (Table S2). We performed glucose clamp experiments on these human subjects and measured the metabolic clearance rate (MCR) of glucose (glucose infusion rate divided by the steady-state plasma glucose concentration) as a measure of systemic insulin sensitivity. As a result, we found that *SEPP1* expression levels were upregulated 8-fold in people with type 2 diabetes compared with normal subjects, as determined by SAGE (Table S2). Additionally, there was a negative correlation between hepatic *SEPP1* messenger RNA (mRNA) levels and the MCR of glucose, indicating that elevated hepatic *SEPP1* mRNA levels were associated with insulin resistance (Figure 1A). As a corollary, we found a positive correlation between the levels of hepatic *SEPP1* mRNA and postloaded or fasting plasma glucose (Figures 1B and 1C).

Elevation of SeP in Type 2 Diabetes

To characterize the role of SeP in the development of insulin resistance, we measured serum SeP levels in human samples (Table S3), using enzyme-linked immunosorbent assays (ELISA), as described previously (Saito et al., 2001). Consistent with elevated hepatic *SEPP1* mRNA levels, we found a significant positive correlation between serum SeP levels and both fasting plasma glucose and hemoglobin A_{1c} (HbA_{1c}) levels (Figures 1D and 1E). HbA_{1c} is a clinical marker of protein glycation due to hyperglycemia, and elevated HbA_{1c} levels generally reflect poor glucose control over a 2–3 month period. Additionally, serum levels of SeP were significantly elevated in people with type 2 diabetes compared with normal subjects (Figure 1F and Table S4). Similar to data derived from clinical specimens, in rodent models of type 2 diabetes, including OLETF rats and KKAy mice, hepatic *Sepp1* mRNA and serum SeP levels were elevated (Figures 1G–1J and Table S5).

SeP Expression in Hepatocytes Is Regulated by Glucose, Palmitate, and Insulin

To clarify the pathophysiology contributing to the hepatic expression of SeP in type 2 diabetes, we investigated the effects of nutrient supply on *Sepp1* mRNA expression in cultured hepatocytes. We found that the addition of glucose or palmitate upregulated *Sepp1* expression, whereas insulin downregulated it in a dose- and time-dependent manner (Figures 2A, 2C, 2E, and 2F). Similar effects on SeP protein levels were observed in primary mouse hepatocytes (Figures 2B, 2D, and 2G). Consistent with the negative regulation of *Sepp1* by insulin in hepatocytes, *Sepp1* mRNA levels were elevated in the livers of fasting C57BL6J mice, compared with those that had been fed (Figure 2H). Thus, multiple lines of evidence suggest that elevated SeP is associated with the development of insulin resistance.

SeP Impairs Insulin Signaling and Dysregulates Glucose Metabolism In Vitro

Because there is no existing cell culture or animal model in which SeP is overexpressed, we purified SeP from human plasma using chromatographic methods (Saito et al., 1999; Saito and Takahashi, 2002) to examine the effects of SeP on insulin-mediated signal transduction. Treatment of primary hepatocytes with purified SeP induced a reduction in insulin-stimulated phosphorylation of insulin receptor (IR), and Akt (Figures 3A and 3B). SeP exerts its actions through an increase in cellular glutathione peroxidase (Saito and Takahashi, 2002). Coadministration of BSO, a glutathione synthesis inhibitor, rescued cells from the inhibitory effects of SeP (Figure 3C). Moreover, SeP increased phosphorylation of IRS1 at Ser307, the downregulator of tyrosine phosphorylation of IRS (Figure S1A). Similar effects of SeP were also observed in C2C12 myocytes (Figure S1B). Next, we assessed whether SeP dysregulated cellular glucose metabolism. In H4IIHEC hepatocytes, treatment with SeP upregulated mRNA expression of *Pck1* and *G6pc*, key gluconeogenic enzymes, resulting in a 30% increase in glucose release in the presence of insulin (Figures 3D–3F). Treatment with SeP alone had no effects on the levels of mRNAs encoding gluconeogenic enzymes or on glucose production in the absence of insulin, suggesting that SeP modulates insulin signaling. Additionally, treatment with SeP induced a reduction in insulin-stimulated glucose uptake in C2C12 myocytes (Figure 3G). These in vitro experiments indicate that, at physiological concentrations, SeP impairs insulin signal transduction and dysregulated cellular glucose metabolism.

SeP Impairs Insulin Signaling and Disrupts Glucose Homeostasis In Vivo

To examine the physiological effects of SeP in vivo, we treated female C57BL/6J mice with two intraperitoneal injections of purified human SeP (1 mg/kg body weight), 12 and 2 hr before the experiments. Injection of purified human SeP protein resulted in serum levels of 0.5–1.5 μg/mL (data not shown). These levels correspond to the incremental change of SeP serum levels in people with normal glucose tolerance to those with type 2 diabetes (Saito et al., 2001). Glucose and insulin tolerance tests revealed that treatment of mice with purified SeP induced glucose intolerance and insulin resistance (Figures 3H and 3I). Blood insulin levels were significantly elevated in

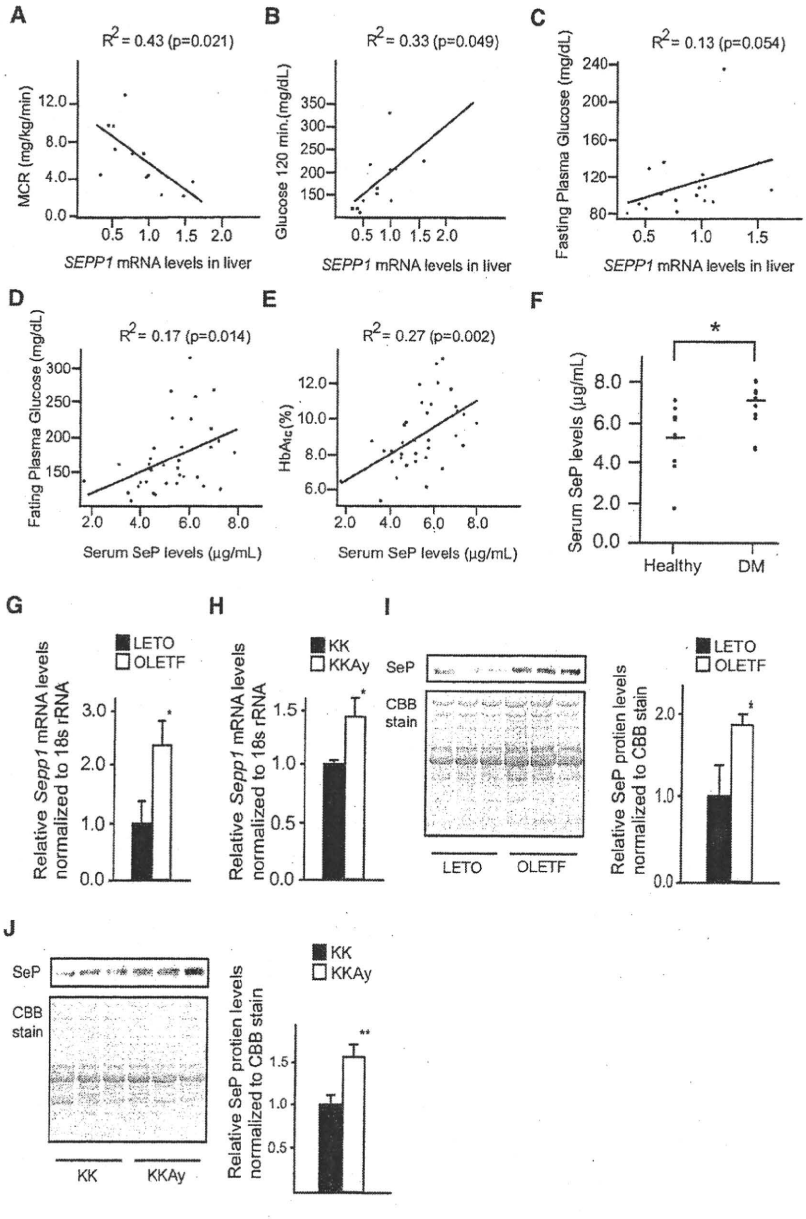

Figure 1. Elevation of Serum SeP Levels and Hepatic *Sepp1* Expression in Type 2 Diabetes

(A–C) Individual correlations between hepatic *SEPP1* mRNA levels and metabolic clearance rate (MCR) of glucose (A), postloaded plasma glucose levels (B), and fasting plasma glucose levels (C) in humans (n = 12–17). MCR equals the glucose infusion rate divided by the steady-state plasma glucose concentration, and is a measure of systemic insulin sensitivity. MCR values were determined by glucose clamp. *SEPP1* mRNA levels were quantified with DNA chips.

(D and E) Correlations between serum levels of SeP and fasting plasma glucose levels (D) and HbA_{1c} (E) in people with type 2 diabetes (n = 35).

(F) Serum levels of SeP in people with type 2 diabetes and healthy subjects (n = 9–12). Age and body weight were not significantly different between the two groups. Data represents the means ± SEM from two groups. *p < 0.05.

(G and H) Hepatic *Sepp1* mRNA levels in an animal model of type 2 diabetes (n = 5–6).

(I and J) Serum SeP levels in an animal model of type 2 diabetes. SeP was detected by western blotting. Coomassie brilliant blue (CBB)-stained gel is used as a control for protein loading. Graphs display the results of densitometric quantification, normalized to CBB-stained proteins (n = 5).

Data represent the mean ± SEM from five to six mice per group. *p < 0.05, **p < 0.01. See also Tables S1–S5.

SeP-injected mice, although those of glucagon and GLP-1 were unaffected during a glucose tolerance test (Figure S1C). Western blot analysis showed a reduction in insulin-induced serine phosphorylation of Akt in both liver and skeletal muscle of SeP-injected mice (Figures 3J and 3K). Hyperinsulinemic-euglycemic clamp studies showed that treatment with SeP significantly increased endogenous glucose production and decreased peripheral glucose disposal (Figure S1D and Figures 3L and 3M). Additionally, serum levels of injected human SeP protein negatively correlated with rates of peripheral glucose disposal (Figure S1E). These data indicate that SeP impairs insulin signaling in the liver and skeletal muscle and induces glucose intolerance in vivo.

Knockdown of *Sepp1* in Liver Improves Glucose Intolerance and Insulin Resistance in Mice with Type 2 Diabetes

To determine whether knockdown of endogenous *Sepp1* enhances insulin signaling, we transfected H4IIEC hepatocytes with *Sepp1*-specific small interfering RNA (siRNA), and we observed a reduction in endogenous *Sepp1* mRNA and SeP protein levels (Figures 4A and 4B). Insulin-stimulated serine phosphorylation of Akt was enhanced in these treated cells (Figure 4C). Similarly, delivery of *Sepp1*-specific siRNAs into KKAy mice via a hydrodynamic transfection method (McCaffrey et al., 2002; Zender et al., 2003) resulted in a 30% reduction in SeP protein levels in the liver and blood (Figures 4D–4G and Figure S2). Knockdown of *Sepp1* improved both glucose intolerance (Figures 4H and 4I) and insulin resistance (Figures 4J and 4K) in KKAy mice.

SeP-Deficient Mice Show Improved Glucose Tolerance and Enhanced Insulin Signaling in Liver and Muscle

We further confirmed the long-term effects of lowered SeP using *Sepp1* knockout mice (Hill et al., 2003). SeP knockout mice were viable and displayed normal body weights when maintained on a selenium-sufficient diet. Body weight, food intake, and O_2 consumption were unaffected by SeP knockout (Figures S3A

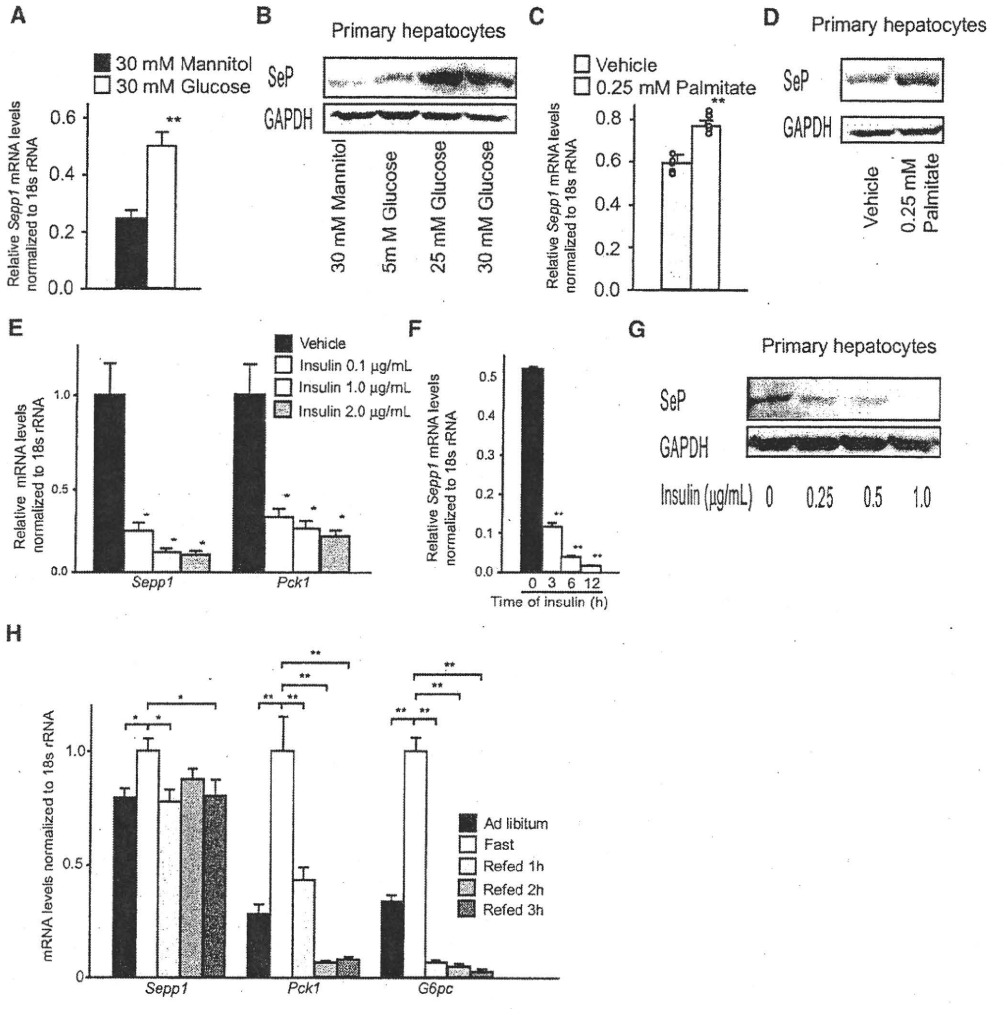

Figure 2. SeP Expression Is Regulated by Glucose, Palmitate, and Insulin

(A) *Sepp1* mRNA levels in H4IIEC hepatocytes treated with glucose or mannitol (30 mM) for 6 hr (n = 4).

(B) SeP protein levels in primary hepatocytes treated with glucose or mannitol for 6 hr.

(C) *Sepp1* mRNA levels in H4IIEC hepatocytes treated with palmitate (0.25 mM) for 16 hr (n = 5).

(D) SeP protein levels in primary hepatocytes treated with palmitate (0.25 mM) for 16 hr.

(E) *Sepp1* and *Pck1* mRNA levels in H4IIEC hepatocytes treated with various concentrations of insulin for 6 hr (n = 4).

(F) *Sepp1* mRNA levels in H4IIEC hepatocytes treated with insulin (0.1 μg/ml) for the indicated periods of time (n = 4).

(G) SeP protein levels in primary hepatocytes treated with various concentrations of insulin for 6 hr.

(H) Liver *Sepp1*, *Pck1*, and *G6pc* mRNA levels in C57BL/6J mice following fasting for 12 hr and subsequent refeeding (n = 4).

Data in (A), (C), (E), and (F) represent the means ± SEM from four to five cells per group, and data in (H) represent the means ± SEM from four mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$.

and S3B). Lipid accumulation in the liver and adipose tissues was also unaffected (Figure 5A). However, postprandial plasma levels of insulin were reduced in *Sepp1*$^{-/-}$ mice, although blood glucose levels remained unchanged (Figures 5B and 5C). Glucose loading test revealed that *Sepp1*$^{-/-}$ mice showed improved glucose tolerance (Figure 5D). Insulin loading test revealed that *Sepp1*$^{-/-}$ mice showed lower blood glucose levels 60 min after insulin injection (Figure 5E). Insulin signaling, including phosphorylation of Akt and insulin receptor, was enhanced in the liver and skeletal muscle of *Sepp1*$^{-/-}$ mice (Figures 5F–5K). Additionally, *Sepp1*$^{+/-}$ tended to show enhanced insulin sensitivity. Plasma levels of glucagon, active GLP-1, and total GIP were unaffected by the loss of SeP in both fasted and fed mice (Figure S3C), suggesting that SeP dysregulated glucose metabolism in vivo primarily by modulating the insulin pathway, but not by affecting other hormones, including glucagon, GLP-1, and GIP.

SeP Deficiency Attenuates Adipocyte Hypertrophy and Insulin Resistance in Dietary Obese Mice

To determine whether SeP deficiency reduces insulin resistance caused by diet-induced obesity, we fed SeP knockout mice

a high-fat, high-sucrose diet (HFHSD) that is known to induce obesity, insulin resistance, and steatosis (Maeda et al., 2002). HFHSD tended to induce body weight gains in wild-type and *Sepp1*-deficient mice, although there was no significance between the three groups of animals (Figure 6A). Daily food intake was significantly increased in $Sepp1^{-/-}$ mice compared with wild-type animals (Figure 6B). Basal energy expenditure, as measured by O_2 consumption through indirect calorimetry, was also increased in $Sepp1^{-/-}$ mice (Figure 6C). Liver triglyceride content and epididymal fat mass were unaffected by *Sepp1* gene deletion (Figures S4A and 6D). However, diet-induced hypertrophy of adipocytes was attenuated in $Sepp1^{-/-}$ mice (Figures 6E and 6F and Figure S4B). Additionally, serum levels of free fatty acid and insulin were significantly reduced in these animals (Figures 6G–6I). Glucose and insulin loading tests revealed that $Sepp1^{-/-}$ mice were protected against glucose intolerance and insulin resistance even when on an obesity-inducing diet (Figures 6J and 6K).

SeP Reduces Phosphorylation of AMPKα Both In Vitro and In Vivo

Adenosine monophosphate-activated protein kinase (AMPK) is a serine/threonine kinase that phosphorylates a variety of energy-associated enzymes and functions as a metabolic regulator that promotes insulin sensitivity (Kahn et al., 2005). In this study, we found that SeP treatment reduced phosphorylation of AMPKα and ACC in both H4IIEC hepatocytes and mouse liver (Figures S5A and 7A). Fatty acid β oxidation and β oxidation-related gene expression were also suppressed by SeP (Figures S5B–S5D). The levels of AMP and ATP were unchanged in hepatocytes treated with SeP (Figure S5E). In contrast, *Sepp1*-deficient mice exhibited increased phosphorylation of AMPKα and ACC in the liver (Figure 7B). To determine whether AMPK pathways were involved in the action of SeP, we infected H4IIEC hepatocytes with an adenovirus encoding dominant-negative (DN) or constitutively active (CA) AMPK. Transduction with DN-AMPK reduced insulin-stimulated Akt phosphorylation such that it could not be further decreased by SeP (Figures 7C–7E). In contrast, when CA-AMPK was overexpressed, SeP was unable to impair insulin-stimulated Akt phosphorylation (Figures 7F–7H). Additionally, coadministration of 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAR), a known activator of AMPK, rescued cells from the inhibitory effects of SeP on insulin signaling (Figure 7I). These results suggest that reduced phosphorylation of AMPK mediates, at least in part, the inhibitory effects of SeP on insulin signal transduction. Next, we examined the effects of SeP on some of the proteins that regulate the phosphorylation of AMPK. SeP dose-dependently increased the levels of protein phosphatase 2C (PP2C), a negative regulator of AMPK phosphorylation, in H4IIEC hepatocytes (Figure 7J). Expression of LKB1 and CaMKKβ, two positive regulators of AMPK, was unaffected by SeP treatment.

DISCUSSION

A Liver-Derived Secretory Protein, SeP, Causes Insulin Resistance

Our research reveals that hepatic overproduction of SeP contributes to the development of insulin resistance in the liver and skeletal muscle (Figure S5F). The liver plays a central role in glucose homeostasis, mainly via glycogen storage and glucose release into the blood stream. In addition, the liver is a major site for the production of secretory proteins. Therefore, we hypothesized that the liver would maintain glucose homeostasis by producing liver-derived secretory protein(s) termed hepatokines. In fact, several studies have shown that hepatic secretory factors, including the angiopoietin-like protein family (Oike et al., 2005; Xu et al., 2005) and fetuin-A (Auberger et al., 1989; Srinivas et al., 1993), are involved in insulin sensitivity. However, we speculated that the identification of the liver-derived proteins that directly contribute to the pathogenesis of insulin resistance or type 2 diabetes may not be adequate. Specifically, our comprehensive approach using global gene expression analyses revealed that numerous genes encoding secretory proteins are expressed and altered in the human type2 diabetic liver (Misu et al., 2007). Thus, by comparing the expression levels and clinical parameters for glycemic control and insulin resistance, we selected candidate genes for liver-produced secretory proteins that cause insulin resistance. The current study sheds light on a previously underexplored function of the liver that is similar to adipose tissue; the liver may participate in the pathogenesis of insulin resistance through hormone secretion.

Suppression of SeP Expression by Insulin in Hepatocytes

Our results indicate that insulin negatively regulates SeP expression in hepatocytes. These findings are consistent with recent reports that the SeP promoter is a target of FoxO (forkhead box, class O) and PGC-1α (peroxisome proliferator-activated receptor-γ coactivator 1α), both of which are negatively regulated by insulin in hepatocytes (Speckmann et al., 2008; Walter et al., 2008). Consistent with these findings in vitro, we showed that hepatic SeP expression was upregulated in mice in the fasting state. Under hypoinsulinemic conditions, such as a fasting state, upregulation of SeP might prevent hypoglycemia by decreasing glucose uptake in peripheral tissues and by increasing hepatic glucose production. Our results raise the possibility that the liver regulates systemic insulin sensitivity by sensing blood insulin levels and altering the production of SeP.

SeP Decreases Phosphorylation of AMPK and ACC in Hepatocytes

Identification of SeP receptor(s) in insulin-target organs is necessary to clarify the action mechanisms of SeP. Several lines of evidence have shown that apolipoprotein E receptor 2 (ApoER2) functions as an SeP receptor in the testis (Olson et al., 2007) and brain (Burk et al., 2007), both by acting as a cellular uptake receptor and by inducing intracellular signaling (Masiulis et al., 2009). It remains unknown whether ApoER2 acts as the SeP receptor in the liver or skeletal muscle. However, in this study, technical difficulties in the identification of a SeP receptor(s) led us to screen for well-established pathways associated with metabolic derangement to clarify the specific mechanisms of SeP action. As a result, our experiments reveal that SeP reduces phosphorylation of AMPK and its target ACC in H4IIEC hepatocytes and the livers of C57BL6J mice, possibly in an AMP/ATP ratio-independent manner. AMPK functions as a regulator of

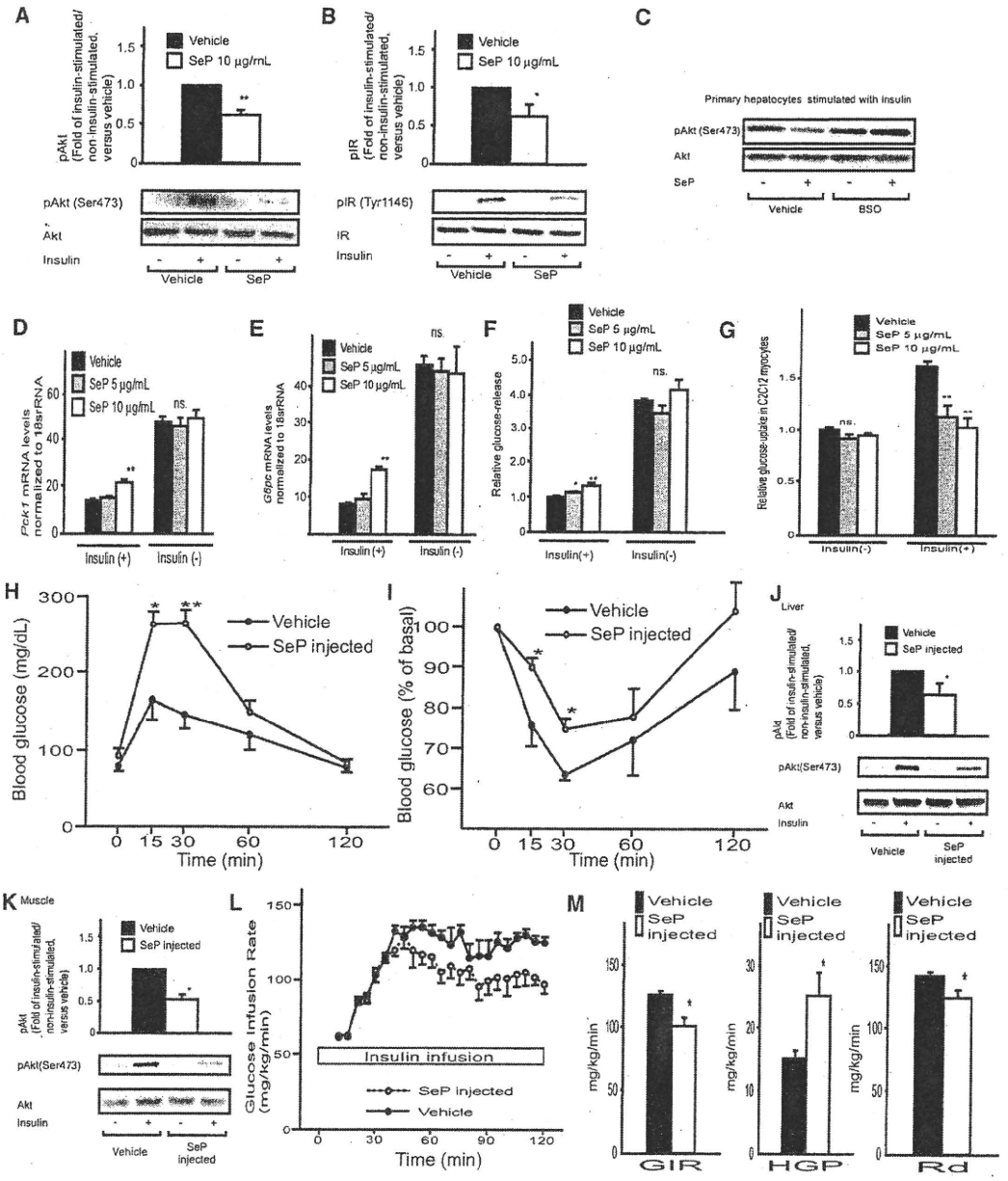

Figure 3. SeP Impairs Insulin Signaling In Vitro and In Vivo

(A and B) Effects of SeP on serine phosphorylation of Akt (A) and tyrosine phosphorylation of insulin receptor (B) in insulin-stimulated primary hepatocytes. Data represent the means ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$ (versus vehicle-treated cells). Primary hepatocytes were treated with SeP or vehicle for 24 hr, and then the cells were stimulated with 1 ng/ml insulin for 15 min.

(C) Effects of BSO on SeP-induced changes in insulin-stimulated Akt phosphorylation in primary hepatocytes.

(D and E) Effects of SeP on the expression of mRNAs encoding gluconeogenic enzymes in H4IIEC hepatocytes (n = 5).

(F) Release of glucose from H4IIEC hepatocytes treated with SeP for 24 hr (n = 6).

(G) Effects of SeP on glucose uptake in C2C12 myocytes (n = 6).

(H and I) Glucose (H) and insulin (I) tolerance tests in mice injected with SeP or vehicle (n = 5). Glucose (1.5 g/kg body weight) and insulin (0.5 unit/kg body weight) were administered intraperitoneally.

(J and K) Effects of SeP on serine phosphorylation of Akt in liver (J) and skeletal muscle (K) in mice injected with purified human SeP or vehicle. Mice (n = 3 or 4) were stimulated with insulin (administered intraperitoneally). At 20 min after insulin stimulation, mice were anesthetized, and liver and hind-limb muscle samples removed for analysis.

(L) Time course of glucose infusion rate (GIR) during hyperinsulinemic-euglycemic clamp in mice injected with SeP or vehicle (n = 6).

(M) GIR, endogenous glucose production (EGP), and rate of glucose disposal (Rd) during hyperinsulinemic-euglycemic clamp (n = 6).

cellular energy homeostasis (Kahn et al., 2005) and mediates some effects of peripheral hormones such as leptin (Minokoshi et al., 2002) and adiponectin (Yamauchi et al., 2002); however, the mechanisms by which these adipokines alter AMPK phosphorylation are not fully understood. Our present findings demonstrate that SeP increases the levels of PP2C in H4IIEC hepatocytes. PP2C is a phosphatase that inactivates AMPK by dephosphorylating a threonine residue (Thr172) that lies in its α-catalytic subunit (Davies et al., 1995). Tumor necrosis factor α (TNF-α), a representative inflammatory cytokine linked to insulin resistance, is known to reduce AMPK phosphorylation by upregulating PP2C (Steinberg et al., 2006). Similar to TNF-α, SeP may reduce AMPK phosphorylation, at least partly, by upregulating PP2C. Further characterization of SeP and SeP-receptor-mediated interactions will provide insights into the involvement of SeP in PP2C upregulation and AMPK dephosphorylation.

Mechanism Underlying SeP-Mediated Insulin Resistance Varies between Liver and Skeletal Muscle

Given that plasma SeP is derived mainly from the liver (Carlson et al., 2004), our results suggest that AMPK mediates, at least in part, the autocrine/paracrine action of SeP. One limitation of our study is that the mechanism by which SeP acts on skeletal muscle remains unknown. Unlike in the liver, SeP-induced inhibitory effects on AMPK were not observed in either the skeletal muscle of C57BL6J mice or C2C12 myocytes (data not shown). Additionally, we showed that SeP reduces tyrosine phosphorylation of insulin receptors in primary hepatocytes. In contrast, SeP acts on serine phosphorylation of IRS1, but not tyrosine phosphorylation of insulin receptors, in C2C12 myocytes (data not shown). These results suggest that SeP disrupts the insulin signal cascade at different levels between hepatocytes and myocytes. SeP might induce insulin resistance in skeletal muscle, possibly through AMPK-independent pathways. The mechanisms that connect SeP to insulin resistance likely exhibit tissue specificity.

We showed that SeP heterozygous mice have no phenotype in glucose- and insulin-loading tests, whereas a 30% decrease in SeP levels caused by the injection of siRNA improves glucose tolerance and insulin resistance in KKAy mice. In general, multiple compensatory changes are observed in knockout mice, because the target gene has been absent since conception. In contrast, compensation may be inadequate in adult animals in which the target gene has been knocked down with siRNA. In fact, real-time PCR analysis showed that expression of the gene encoding IL-6, a representative inflammatory cytokine linked to insulin resistance, shows compensatory upregulation in the liver of *Sepp1*$^{-/+}$ mice, but not in *Sepp1* siRNA-treated KKAy mice (data not shown). Induction of IL-6 might compensate for the 50% reduction in SeP levels in *Sepp1*$^{-/+}$ mice.

Actions of SeP on the central nervous system may contribute to the in vivo phenotype. We did find that SeP-deficient mice fed a high-fat, high-sucrose diet display increases in food intake and O2 consumption (Figures 6B and 6C), suggesting that SeP acts on the central nervous system. Additionally, an earlier report described the colocalization of SeP and amyloid-β protein in the brains of people with Alzheimer's disease, suggesting the potential involvement of SeP in this condition's pathology (Bellinger et al., 2008). More recently, Takeda et al. reported that amyloid pathology in Alzheimer's disease may adversely affect diabetic phenotypes in mice (Takeda et al., 2010). Further experiments are necessary to determine whether the actions of SeP on the central nervous system involve the in vivo phenotype seen in this study.

We cannot exclude the possibility that the current phenotype in *Sepp1*-deficient mice is affected by the abnormal distribution of selenium. In fact, selenium levels in plasma and several tissues have been reported to be reduced in *Sepp1*-deficient mice fed a selenium-restricted diet (Schomburg et al., 2003). However, Burk et al. reported that the selenium levels in all tissues except the testis were unchanged in these mice fed a diet containing adequate amounts of selenium (Hill et al., 2003). In this study, we performed experiments using *Sepp1*-deficient mice fed a diet containing adequate amounts of selenium. Thus, we speculate that the effects of abnormal selenium distribution on our results in *Sepp1*-deficient mice may be insignificant.

A limitation of this study is that we could not match age, gender, or body weight completely between people with type 2 diabetes and normal subjects when comparing the serum SeP levels, as a result of the limited sample numbers. However, a previous large-scale clinical report showed that the age-, gender-, race-, and BMI-adjusted mean serum selenium levels were significantly elevated in participants with diabetes compared with those without diabetes in the US population (Bleys et al., 2007). Additionally, several lines of evidence showed that serum selenium levels are positively correlated with those of SeP in humans (Andoh et al., 2005; Persson-Moschos et al., 1998). In combination with our result, these reports lead us to speculate that serum SeP levels are also elevated in people with type 2 diabetes compared with normal subjects. However, additional large-scale clinical trials are needed to address this.

In summary, our experiments have identified SeP as a liver-derived secretory protein that induces insulin resistance and hyperglycemia. Our findings suggest that the secretory protein SeP may be a target for the development of therapies to treat insulin resistance-associated diseases, including type 2 diabetes.

EXPERIMENTAL PROCEDURES

Animals

Eight-week-old c57BL/6J mice were obtained from Sankyo Lab Service (Tokyo, Japan). Male Otsuka Long-Evans Tokushima Fatty (OLETF) rats and Long-Evans Tokushima Otsuka (LETO) rats were obtained from the Otsuka Pharmaceutical Tokushima Research Institute (Tokushima, Japan). OLETF

C57BL/6J mice were twice injected intraperitoneally with purified human SeP (1 mg/kg body weight) or vehicle in (H)–(M). Injections were administered 12 and 2 hr before the each experiment. Data in (D)–(G) represent the means ± SEM from five to six cells per group, and data in (H)–(M) represent the means ± SEM from three to six mice per group. *$p < 0.05$, **$p < 0.01$ versus cells treated with vehicle in (D)–(G). *$p < 0.05$, **$p < 0.01$ versus mice treated with vehicle in (H)–(M). See also Figure S1.

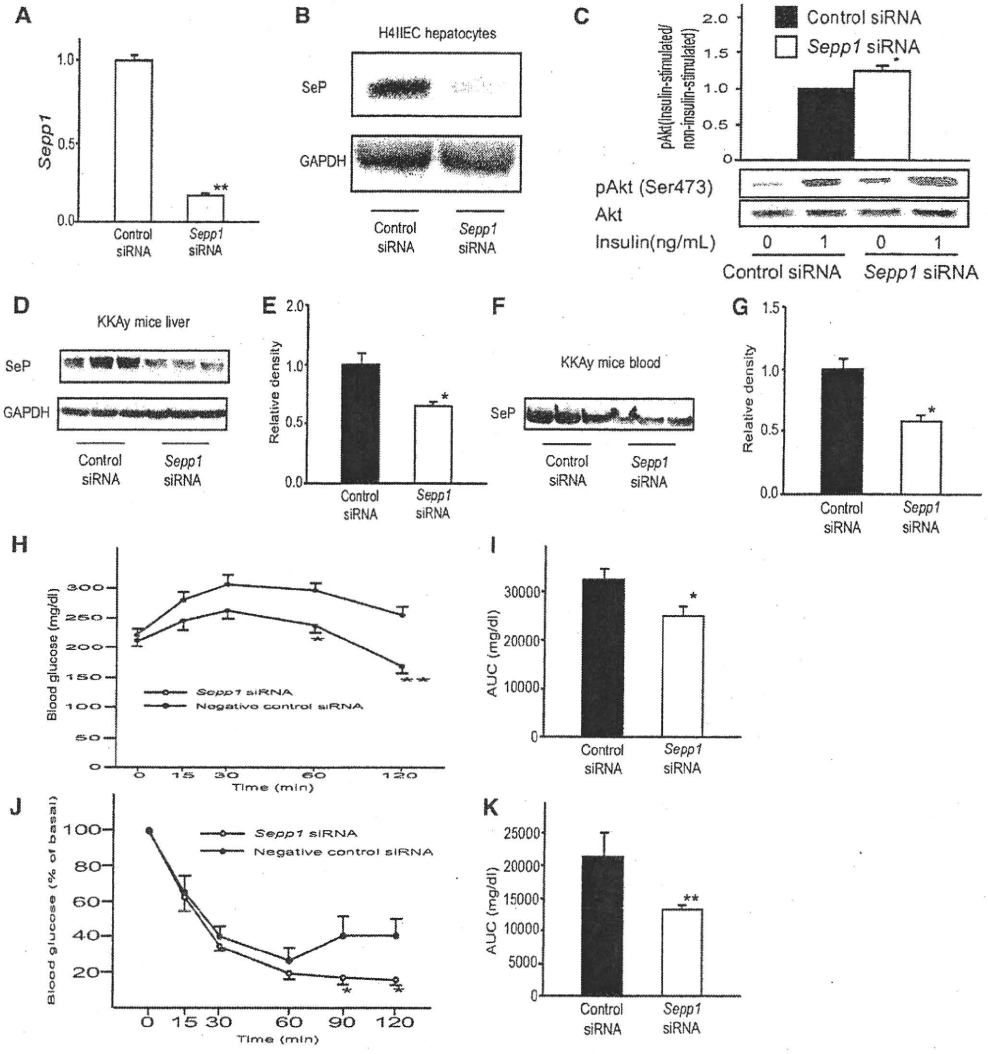

Figure 4. *Sepp1* Knockdown in the Liver Improves Insulin Sensitivity

(A) *Sepp1* mRNA levels in H4IIEC hepatocytes transfected with control or *Sepp1*-specific siRNA (n = 4).

(B) SeP protein production in H4IIEC hepatocytes transfected with *Sepp1*-specific siRNA. SeP production was detected in whole cell lysates by western blotting.

(C) Effects of SeP knockdown on insulin-stimulated serine phosphorylation of Akt in H4IIEC hepatocytes. Data represent the mean ± SEM of three independent experiments.

(D and E) Liver SeP production in KKAy mice injected with control or *Sepp1*-specific siRNA (n = 6). SeP protein levels were measured by western blotting 4 days after injection of siRNA.

(F and G) Blood SeP levels in KKAy mice injected with siRNA. Blood samples were obtained 4 days after siRNA injection (n = 6).

(H–K) Intraperitoneal glucose (H and I) and insulin (J and K) tolerance tests in KKAy mice (n = 6–8) injected with control or *Sepp1*-specific siRNA. Glucose and insulin was administered at doses of 0.3 g/kg body weight and 4 units/kg body weight, respectively.

Area under the curve (AUC) for blood glucose levels is shown in (I) and (K). Data in (A) represent the means ± SEM from four cells per group, and data in (E) and (G)–(K) represent the means ± SEM from six to eight mice per group. *$p < 0.05$ versus cells transfected with control siRNA in (A) and (C). *$p < 0.05$, **$p < 0.01$ versus mice injected with control siRNA in (E) and (G)–(K). See also Figure S2.

rats have been established as an animal model of obesity-related type 2 diabetes (Kawano et al., 1992). Female KKAy mice were obtained from CLEA Japan (Tokyo, Japan). All animals were housed in a 12 hr light/dark cycle and allowed free access to food and water. High-fat and high-sucrose diet (D03062301) was purchased from Research Diets (New Brunswick, NJ). The experiments with OLETF and LETO rats were performed with frozen blood and liver samples obtained in our previous study (Ota et al., 2007).

Purification of SeP

SeP was purified from human plasma via conventional chromatographic methods, as previously described (Saito et al., 1999; Saito and Takahashi, 2002). Homogeneity of purified human SeP was confirmed by analysis of both amino acid composition and sequence (Saito et al., 1999). Concentrations of purified SeP were measured by the Bradford method, using bovine immunoglobulin G as a standard.