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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