Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Possible involvement and the mechanisms of excess *trans*-fatty acid consumption in severe NAFLD in mice

Noriyuki Obara[1], Koji Fukushima[1], Yoshiyuki Ueno[1,*], Yuta Wakui[1], Osamu Kimura[1], Keiichi Tamai[1], Eiji Kakazu[1], Jun Inoue[1], Yasuteru Kondo[1], Norihiko Ogawa[2], Kenta Sato[3], Tsuyoshi Tsuduki[3], Kazuyuki Ishida[4], Tooru Shimosegawa[1]

[1]*Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryo, Aobaku, Sendai 980-8574, Japan;* [2]*Division of Advanced Surgical Science and Technology, Graduate School of Medicine, Tohoku University, Sendai, Japan;* [3]*Laboratory of Food and Biomolecular Science, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan;* [4]*Department of Pathology, Tohoku University Hospital, Sendai, Japan*

Background & Aims: Excessive *trans*-fatty acids (TFA) consumption has been thought to be a risk factor mainly for coronary artery diseases while less attention has been paid to liver disease. We aimed to clarify the impact of TFA-rich oil consumption on the hepatic pathophysiology compared to natural oil.

Methods: Mice were fed either a low-fat (LF) or high-fat (HF) diet made of either natural oil as control (LF-C or HF-C) or partially hydrogenated oil, TFA-rich oil (LF-T or HF-T) for 24 weeks. We evaluated the liver and body weight, serological features, liver lipid content and composition, liver histology and hepatic lipid metabolism-related gene expression profile. In addition, primary cultures of mice Kupffer cells (KCs) were evaluated for cytokine secretion and phagocytotic ability after incubation in *cis*- or *trans*-fatty acid-containing medium.

Results: The HF-T-fed mice showed significant increases of the liver and body weights, plasma alanine-aminotransferase, free fatty acid and hepatic triglyceride content compared to the HF-C group, whereas the LF-T group did not differ from the LF-C group. HF-T-fed mice developed severe steatosis, along with increased lipogenic gene expression and hepatic TFA accumulation. KCs showed increased tumor necrosis factor secretion and attenuated phagocytotic ability in the TFA-containing medium compared to its *cis*-isomer.

Conclusions: Excessive consumption of the TFA-rich oil up-regulated the lipogenic gene expression along with marked hepatic lipid accumulation. TFA might be pathogenic through causing severe steatosis and modulating the function of KCs. The quantity and composition of dietary lipids could be responsible for the pathogenesis of non-alcoholic steatohepatitis.

Keywords: *trans*-Fatty acid; NASH; NAFLD; Metabolic syndrome; Kupffer cell.
Received 16 September 2009; received in revised form 18 January 2010; accepted 26 February 2010; available online 22 April 2010
*Corresponding author. Tel.: +81 22 717 7171; fax: +81 22 717 7177.
E-mail address: yueno@mail.tains.tohoku.ac.jp (Y. Ueno).

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; FFA, free fatty acid; LPS, lipopolysaccharide; TFA, *trans*-fatty acid; ALT, alanine-aminotransferase; LF(-C or -T), low-fat (control or TFA-rich) diet; HF(-C or -T), high-fat (control or TFA-rich) diet; KCs, Kupffer cells (KCs); AST, aspartate-aminotransferase; TG, triglyceride; ELISA, Enzyme-Linked ImmunoSorbent Assay; HDL, high density lipoprotein; (V)LDL, (very) low density lipoprotein; NAS, NAFLD activity score; TBARS, thiobarbituric acid reactive substances; TNFα, tumor necrosis factor α; IL-6, interleukin-6; SD, standard deviation; iNOS, inducible nitric oxide synthase; TGF-β, transforming growth factor-β; SREBP-1, sterol regulatory element-binding protein-1; FAS, fatty acid synthase; ACC, acetyl CoA carboxylase; PPAR, peroxisome proliferator activated receptor; PGC-1β, PPARγ coactivator-1β; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid.

Introduction

In concordance with the prevalence of obesity, the incidence of non-alcoholic fatty liver disease (NAFLD) has increased and is nowadays recognized as the most common liver disease [2]. It is known that a part of NAFLD can progress to non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis and hepatocellular carcinoma [9]. Nevertheless, the mechanisms of NAFLD-to-NASH transition remain to be clarified; NAFLD appears to originate from the dysregulation of hepatic lipid metabolism as a part of the metabolic syndrome accompanied by visceral obesity, dyslipidemia, atherosclerosis, and insulin resistance [25]. According to the hypothetical theory named the 2-hit theory [5], the secondary hit to NAFLD that can be due to free fatty acid (FFA)s, oxidative stress, lipopolysaccharide (LPS) and inflammatory cytokines, causes NASH as a consequence.

In terms of the "first hit", the lipid accumulation in the liver is induced by high-fat diets [6,23] that include various lipid species. Such dietary lipid species uniquely affect the obesity phenotype, liver histology and gene expression pattern in the rat liver [3]. In this context, lipid species could play a potential role in the pathogenesis of NAFLD and/or NASH.

trans-Fatty acid (TFA) is produced through the industrial hardening of the vegetable oils to make the products more stable and robust, and thus easier to handle or store. Excess consumption of TFA is known as a risk factor for coronary artery diseases, insulin resistance and obesity accompanied by systemic inflammation, the features of metabolic syndrome [20,29]. Nevertheless, little is known about the effects on the liver induced by lipids.

Fast-foods, containing large amount of TFA in the form of margarine, spreads or frying oils, cause body-weight gain and abnormal serum alanine-aminotransferase (ALT) elevations in healthy subjects [15]. In addition, TFA-rich chow leads to hepatic steatosis [30], ALT elevations and insulin resistance in mice [17]; although the mechanisms have not been completely clarified. Therefore, we aimed to investigate the impact of the dietary lipid species and their quantities on the pathogenicity of hepatic inflammation and steatosis in mice comparing in particular natural oil and industrially produced partially hydrogenated TFA-rich oil of the same origin.

Materials and methods

Animal treatment

All the animal experiments were conducted under the approval of the Institutional Animal Care and Use Committees of Tohoku University. Female C57BL/6Njcl mice (8–10 weeks) were randomly assigned to four groups ($n = 6$ per group) and fed the designated chows (ORIENTAL YEAST Co. Ltd., Tokyo, Japan) *ad libitum* for 24 weeks, respectively. Low-fat diet (LF) and high-fat diet (HF) were made of either natural canola oil as control oil (LF-C and HF-C) or industry produced partially hydrogenated canola oil as TFA-rich oil (28.5% TFA/total fat, LF-T and HF-T), respectively (Table 1). After 12 h of fasting, the mice were sacrificed under diethylether anesthesia and the livers were removed and weighed. The divided livers were either stored at −80 °C for lipid, protein and gene expression analysis, or fixed in 4% paraformaldehyde and embedded in paraffin for histological evaluation. Standard chow-fed female C57BL/6Njcl mice (6–10 weeks) were used as a source of primary Kupffer cells (KCs).

Chemistry

Plasma aspartate-aminotransferase (AST), ALT, triglyceride (TG) and total cholesterol were measured with FUJI DRI-CHEM 7000 (FUJIFILM, Tokyo, Japan) at Biomedical Research Core of Tohoku University Graduate School of Medicine. Plasma adiponectin (AdipoGen, Seoul, Korea) and leptin (RayBio, GA, USA) were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA). Plasma FFA, high density lipoprotein (HDL)-cholesterol and (very) low density lipoprotein ((V)LDL)-cholesterol were measured by enzymatic assay kits (BioVision, CA, USA).

Histology and immunohistochemistry

The thin-sliced specimens were stained with hematoxylin and eosin to evaluate steatosis and inflammation or Sirius red to evaluate fibrosis of the liver. The histology was scored by the NAFLD activity score (NAS) [16]. KCs were stained with anti-F4/80 monoclonal antibody (Abcam, Cambridge, UK) and neutrophils were detected by myeroperoxidase immunostaining (Abcam). Apoptosis was evaluated by TUNEL method using an ApopTag kit (Chemicon, CA, USA).

Table 1. Diet compositions.

	Low-fat diet		High-fat diet	
	Control oil (LF-C) kcal%	TEA-rich oil (LF-T) kcal%	Control (HF-C) kcal%	TEA-rich (HF-T) kcal%
Diet compositions				
Protein	13.8	13.8	18.8	18.8
Carbohydrate	74.4	74.4	17.6	17.6
Over all fat	11.8	11.8	63.6	63.6
Fat composition (g/100 g)				
Saturated	7.8	21.7	7.8	21.7
(*cis*-)Monounsaturated	62.5	45.3	62.5	45.3
Polyunsaturated	29.7	4.5	29.7	4.5
trans- (%)		28.5		28.5

Immunoblot analysis and real-time RT-PCR

Liver protein extracts were evaluated by immunoblot analysis with the following primary antibodies: phosphor-AKT (Thr308 and Ser473), total AKT (Cell Signaling Technology, Danvers, MA) and β-actin (Sigma, MO, USA). RNA extracted from the livers was subjected to real-time RT-PCR analysis using the specifically designed primer sets purchased form TAKARA BIO Perfect Real Time Support System (TAKARA BIO INC., Tokyo, Japan) and One Step SYBR Prime Script RT-PCR Kit II (TAKARA BIO INC.), and only PGC-1β was analyzed using the specifically designed TaqMan primer set and 1-step kit (Applied Biosystems, CA, USA). All results were normalized by GAPDH as the internal control.

Lipidomic analysis of the liver

Hepatic TG and FFA content were measured by enzymatic assay kit (BioVision) and were normalized by the liver weight. Hepatic lipid peroxide was evaluated by measuring TBARS (thiobarbituric acid reactive substances, Cayman Chemical Company, USA) in the liver and was normalized by the protein level [18]. Total lipids from the liver were extracted by Folch's procedure [10]. The lipids were methylated and evaluated by gas chromatography as previously reported [31].

Isolation and culture of primary Kupffer cells

KCs were isolated as reported previously [28]. Briefly, the mice livers were digested by two-step collagenase perfusion. The minced livers were subjected to the gradient centrifugation of Percoll (Sigma) and succeeding counterflow centrifugal elutriation. The viabilities of the obtained cells evaluated by trypan blue staining were more than 85%, and the purity was more than 90% determined by the population of CD11b positive cells counted by FACS Calibur (Becton Dickinson, Tokyo, Japan). KCs were suspended in RPMI1640 medium with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin sulfate) and incubated overnight at 37 °C in 5% CO_2 incubator for the succeeding examinations.

Fatty acid treatment

Fatty acids (Larodan Fine Chemicals, Malmo, Sweden) were dissolved in RPMI1640 medium with 1% fatty acid-free bovine serum albumin (Calbiochem, Darmstadt, Germany) and adjusted to a final concentration of 200 μM with 1% bovine serum albumin, 1% ITS-A supplement (GIBCO, CA, USA) and antibiotics same as above. After overnight incubation, KCs were washed and the medium was changed to fatty acid-containing medium or fatty acid-free medium as the control, and incubated for another 24 h.

Cytokine production by KCs stimulated with lipopolysaccharide

After 24 h incubation, KCs were stimulated by LPS (100 ng/ml, SIGMA) combined with LPS-binding protein (200 pg/ml, ALEXIS BIOCHEMICALS, Lausanne, Switzerland) for 6 h, and the cell viability was determined by MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt and phenazine ethosulfate, Promega, Tokyo, Japan). The supernatants were subjected to ELISA (Thermo Fisher Scientific Inc., IL, USA) for the evaluation of the tumor necrosis factor-alfa (TNFα) and interleukin-6 (IL-6) production.

Phagocytotic ability of KCs

After 24 h incubation, KCs were incubated at 37 °C for 1 h with 1 μm latex beads (75 ng/ml, SIGMA) or at 4 °C in the fatty acid-free medium as control. After incubation, the cells were washed 3 times, detached with trypsin/EDTA and analyzed by FACS calibur [1].

Statistical analysis

The results are shown as the mean ± standard deviation (SD), and were analyzed by SPSS software (SPSS INC., Tokyo, Japan).

The differences between the groups were tested by ANOVA, followed by Tukey post hoc test. A p values less than 0.05 were considered statistically significant.