Hepatology Research

Hepatology Research 2016 doi: 10.1111/hepr.12720

Original Article

L-carnitine prevents metabolic steatohepatitis in obese diabetic KK-A^y mice

Kazuyoshi Kon, Kenichi Ikejima, Maki Morinaga, Hiromi Kusama, Kumiko Arai, Tomonori Aoyama, Akira Uchiyama, Shunhei Yamashina and Sumio Watanabe

Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan

Aim: Pharmacological treatment for metabolic syndrome-related non-alcoholic steatohepatitis has not been established. We investigated the effect of L-carnitine, an essential substance for β-oxidation, on metabolic steatohepatitis in mice.

Methods: Male KK-A^y mice were fed a high-fat diet (HFD) for 8 weeks, with supplementation of L-carnitine (1.25 mg/mL) in drinking water for the latter 4 weeks.

Results: Serum total carnitine levels were decreased following HFD feeding, whereas the levels were reversed almost completely by L-carnitine supplementation. In mice given L-carnitine, exacerbation of hepatic steatosis and hepatocyte apoptosis was markedly prevented even though HFD feeding was continued. Body weight gain, as well as hyperlipidemia, hyperglycemia, and hyperinsulinemia, following HFD feeding were also significantly prevented in mice given L-carnitine. High-fat diet feeding elevated hepatic expression levels of carnitine palmitoyltransferase 1A mRNA; however, production of β-hydroxybutyrate in the liver was not affected by HFD alone. In contrast, L-carnitine treatment significantly increased hepatic β-hydroxybutyrate contents in HFD-fed mice. L-carnitine also blunted HFD induction in sterol regulatory element binding protein-1c mRNA in the liver. Furthermore, L-carnitine inhibited HFD-induced serine phosphorylation of insulin receptor substrate-1 in the liver. L-carnitine decreased hepatic free fatty acid content in 1 week, with morphological improvement of swollen mitochondria in hepatocytes, and increases in hepatic adenosine 5'-triphosphate content.

Conclusions: L-carnitine ameliorates steatohepatitis in KK-A^y mice fed an HFD, most likely through facilitating mitochondrial β-oxidation, normalizing insulin signals, and inhibiting de novo lipogenesis in the liver. It is therefore postulated that supplementation of L-carnitine is a promising approach for prevention and treatment of metabolic syndrome-related non-alcoholic steatohepatitis.

Key words: free fatty acid, insulin signal, metabolic syndrome, non-alcoholic steatohepatitis (NASH), β-oxidation

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is a broad spectrum of hepatic disorders characterized by evidence of hepatic steatosis either by imaging or histology, and appropriate exclusion of other etiologies such as heavy alcohol consumption, hepatitis viruses, and autoimmunity.[1] Non-alcoholic steatohepatitis (NASH) is a progressive form of NAFLD, eventually leading to liver cirrhosis and cancer. The incidence of NASH is increasing worldwide,[2] and is currently the third most common indication for liver transplantation.[3] Non-alcoholic steatohepatitis is recognized as the hepatic manifestation of metabolic syndrome, as obesity, insulin resistance, and dyslipidemia contribute profoundly to the development of NASH.[4–6] The pathogenesis of NASH still remains to be fully elucidated, and the pharmacotherapy of metabolic syndrome-related NASH has not been well established.

L-carnitine, a vitamin-like constituent of protein, is indispensable for mitochondrial β-oxidation, by which free fatty acids (FFA) are catabolized.[7] Systemic primary carnitine deficiency is typically characterized by episodes of hypoketotic hypoglycemia and hepatomegaly due to hepatic steatosis with elevated serum aminotransferases and ammonia levels in infants, which are markedly resolved by L-carnitine intake.[8] Acquired deficiency of L-carnitine is often seen in patients undergoing hemodialysis, in which supplementation of L-carnitine has been proved to enhance the efficacy of erythropoietin on renal anemia, and ameliorate intradialytic symptoms.[9,10] Depletion of L-carnitine also occurs following long-term treatment with

Correspondence: Dr Kenichi Ikejima, Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo, 113-8421 Japan. Email: ikejima@juntendo.ac.jp

Conflict of interest: The authors' affiliation received grant support (scholarship contribution) from Otsuka Pharmaceutical Co., Ltd (Tokyo, Japan).

Received 4 March 2016; revision 3 April 2016; accepted 5 April 2016.

certain drugs such as pivalate-generating prodrugs and valproic acid, and L-carnitine supplementation has been shown to improve hyperammonemia in psychiatric patients receiving valproic acid.[9,1111] Some clinical trials have recently suggested the potential efficacy of L-carnitine in patients with NAFLD.[12,13] It has been reported that L-carnitine prevents streptozotocin-induced steatohepatitis and subsequent hepatocarcinogenesis in a murine model (STAM mice);[14] however, the effect of L-carnitine on metabolic syndrome-related NASH is still unclear.

KK-A^y mice are a strain generated from diabetic KK mice by introducing lethal yellow (A^y) mutation, which causes aberrant expression of the agouti gene on chromosome 2. KK-A^y mice show a phenotype similar to human metabolic syndrome including obesity, dyslipidemia, and insulin resistance, in combination with spontaneous development of steatohepatitis; thus this animal is potentially useful as a model of NAFLD/NASH.[15] We have reported that KK-A^y mice show increased susceptibility to methionine- and choline-deficiency diet-induced steatohepatitis,[16] and acetaminophen-induced liver injury.[17] We also reported that KK-A^y mice show proportional and functional alterations in hepatic natural killer T cells,[18] and impaired in liver regeneration after partial hepatectomy.[19] KK-A^y mice fed a high-fat diet (HFD) develop more severe steatohepatitis,[20,21] resembling metabolic syndrome-related NASH.

Therefore, our aim in this study was to investigate the effect of L-carnitine on metabolic syndrome-related steatohepatitis in KK-A^y mice fed HFD.

METHODS

Animal experiments

THE EXPERIMENTAL PROTOCOLS were approved by the Committee of Laboratory Animals according to institutional guidelines. Male, 7-week-old KK-A^y mice were purchased from CLEA Japan (Tokyo, Japan). Mice were housed in air-conditioned, specific pathogen-free animal quarters with lighting from 0800 to 2000 throughout this study, and were given unrestricted access to a standard laboratory chow and water during the 1-week acclimation period. After acclimation, 8-week-old KK-A^y mice were fed an HFD (HFD32; CLEA Japan) (Table 1) for 8 weeks. Following an initial 4-week feeding period, some animals were treated with L-carnitine hydrochloride solution (1.25 mg/mL in drinking water; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for 4 weeks. KK-A^y mice fed normal chow for 4 weeks were used as controls. After dietary treatment with/without L-carnitine supplementation, overnight-fasted mice were killed by exsanguination from the inferior vena cava under ether anesthesia, and liver and serum samples were obtained. Serum and tissue samples for RNA preparation were kept frozen at −80°C until assayed.

Table 1 Ingredients of high-fat diet fed to KK-A^y mice

	%		%
Milk casein	24.500	Lactose	6.928
Dried albumen	5.000	Sucrose	6.750
L-cystine	0.430	Vitamin mix	1.400
Beef fat	15.880	Mineral mix	5.000
Safflower oil	20.000	Choline bitartrate	0.360
Cellulose	5.500	Butylhydroquinone	0.002
Maltodextrin	8.250		

Histological analysis and immunohistochemistry

For histological evaluation, liver tissues were fixed in 10% buffered formalin and embedded in paraffin, and hematoxylin–eosin staining was carried out. Caspase cleavage product of cytokeratin (ccCK) 18 was detected by immunohistochemistry using M30 CytoDEATH monoclonal antibody (Roche, Basel, Switzerland) as described previously.[22] Briefly, deparaffinized tissue sections were incubated with a monoclonal anti-M30 antibody and secondary biotinylated anti-mouse immunoglobulin G, and the specific binding was visualized with avidin–biotin complex solution followed by incubation with a 3, 3-diaminobenzidine tetrahydrochloride solution using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Specimens were observed under an optical microscope (DM7000; Leica, Wetzlar, Germany) equipped with a digital microscope camera (MC120HD; Leica), and digital images were captured.

Electron microscope analysis

A portion of liver specimen was immersed in fixative containing 3% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 4°C for 24 h. Ultrathin sections of liver were prepared, and observed by transmission electron microscopy as described previously.[23]

Blood biochemistry

Serum aspartate aminotransferase, alanine aminotransferase, triglyceride, and glucose levels were determined using a Fuji DRI-CHEM system (Fuji Film Medical Co. Ltd., Tokyo, Japan).

Measurement of carnitine

Serum total and free carnitine were measured by the enzymatic cycling method using commercial kits (KAINOS Laboratories Inc., Tokyo, Japan) following the manufacturer's instructions. Acyl-carnitine was calculated as the difference between total and free carnitine.

Measurement of FFA

Serum and hepatic FFA contents were measured by an enzyme-based method using the Free Fatty Acid Quantification Kit (BioVision, Milpitas, CA, USA) following the manufacturer's instructions. The amount of hepatic FFA content was normalized by weight of tissue samples.

Measurement of ketone bodies

β-Hydroxybutyrate in liver tissue was measured using a β-hydroxybutyrate (ketone body) Fluorometric Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, 50 mg liver tissue was homogenized in 300 mL buffer using a Teflon homogenizer. Homogenate was mixed with the equivalent volume of 1 mol/L metaphosphoric acid, and centrifuged at 10 000 g for 5 min. The pellet was dissolved in 60 μL of 5 mol/L potassium carbonate solution. After centrifugation at 10 000 g for 5 min, supernatant was collected, and 25 μL supernatant was then mixed with the equal volume of β-hydroxybutyrate dehydrogenase solution. After 30 min incubation at 37°C, fluorescence was measured using a fluorescence spectrophotometer with excitation and emission wavelength of 530–540 nm and 585–595 nm, respectively. The amount of hepatic β-hydroxybutyrate content was normalized by weight of tissue samples.

Measurement of adenosine 5'-triphosphate

The adenosine 5'-triphosphate (ATP) content in liver tissue was measured by luciferase assay using an ATP assay kit for animal tissues (TOYO B-Net Co., Ltd., Tokyo, Japan) following the manufacturer's instructions.

Enzyme-linked immunosorbent assay

Serum insulin levels were measured using the Mouse Insulin enzyme-linked immunosorbent assay (ELISA) Kit (Morinaga Institute of Biological Sciences, Inc., Kanagawa, Japan). Serine phosphorylation of insulin receptor substrate (IRS)-1 in liver tissue was determined using the PathScan Phospho-IRS-1 (Ser307) Sandwich ELISA Kit (Cell Signaling Technology, Danvers, MA, USA).

RNA preparation and real-time reverse transcription–polymerase chain reaction

Total RNA was prepared from frozen tissue samples using the illustra RNAspin Mini RNA Isolation kit (GE Healthcare, Waukesha, WI, USA) as described previously.[24] The concentration and purity of isolated RNA were determined by measuring optical density at 260 and 280 nm. For real-time reverse transcription–polymerase chain reaction, total RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus transcriptase (SuperScript II; Invitrogen, Carlsbad, CA, USA) and an oligo (dT) 12–18 primer at 42°C for 1 h. Obtained cDNA (1 μg) was amplified using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and specific primers for carnitine palmitoyltransferase 1A (CPT1A; forward, 5'-GCC TCA GCG TGG AAC ACT CA-3'; reverse, 5'-CTG TGG CAT AAC CCA ACT GGA AG-3'), sterol regulatory element binding protein-1c (SREBP1c; forward, 5'-ACA GTC CAG CCT TTG AGG ATA G-3'; reverse, 5'-GAC ACA GAA AGG CCA GTA CAC A-3'), and glyceraldehyde-3-phosphate dehydrogenase (forward, 5'-TGT GTC CGT CGT GGA TCT GA-3'; reverse, 5'-TTG CTG TTG AAG TCG CAG GAG-3'). After a 10-s activation period at 95°C, 40 cycles of 95°C for 5 s and 60°C for 31 s, followed by a final cycle of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s, were carried out using the ABI PRISM 7700 sequence detection system (Applied Biosystems), and the threshold cycle values were obtained.

Statistical analysis

Morphometric and densitometric analyses were carried out using Scion Image (version Beta 4.0.2; Scion Corp., Fredrick, MD, USA). Data were expressed as mean ± stanndard error of the mean. Statistical differences between means were determined using one-way ANOVA or Kruskal–Wallis ANOVA on ranks followed by an all pairwise multiple comparison procedure (Student–Newman–Keuls method) as appropriate. $P < 0.05$ was selected before the study to reflect significance.

RESULTS

L-carnitine blunts body weight gain and increases serum carnitine levels in KK-A^y mice

HIGH-FAT DIET-FED KK-A^Y mice gained body weight rapidly, the values reaching 54.5 ± 0.9 g and 61.4 ± 0.9 g after 4 and 8 weeks, respectively. Treatment with L-carnitine, started after 4 weeks of prefeeding, significantly prevented body weight gain almost completely, with values 8 weeks after HFD feeding only reaching 54.2 ± 2.0 g ($P < 0.05$) (Fig. 1a).

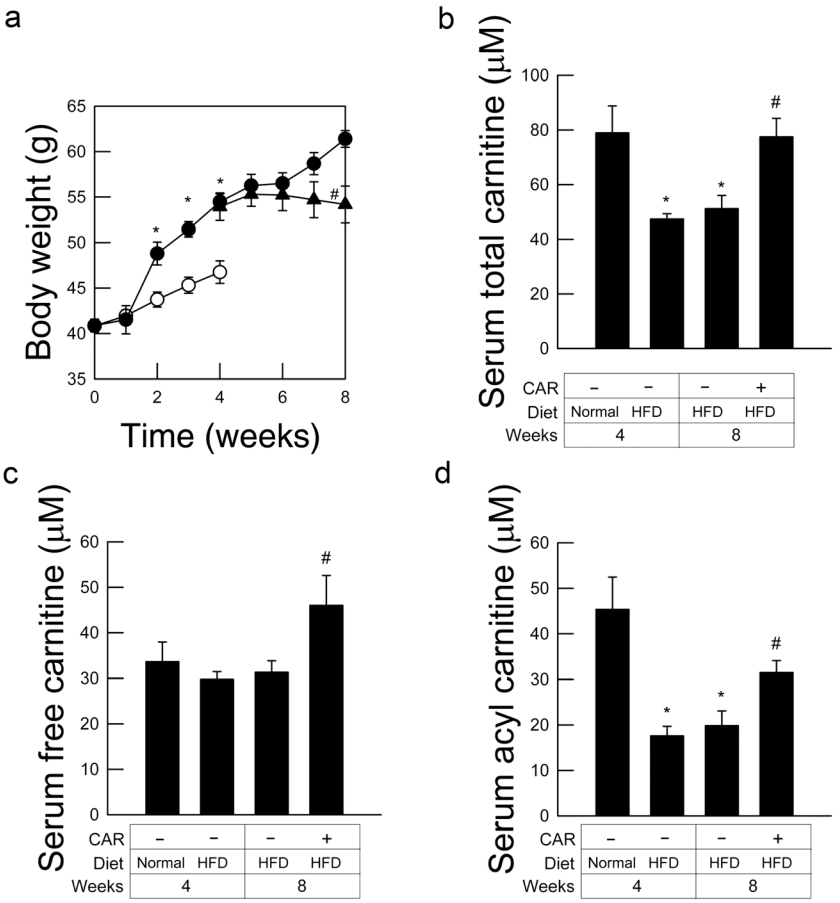

Figure 1 Treatment with L-carnitine (CAR) prevents body weight gain and reduction of serum carnitine contents following high-fat diet (HFD) feeding. KK-A^y mice were fed HFD for 8 weeks. Following the initial 4-week feeding period, some animals were treated with L-carnitine (1.25 mg/mL in drinking water) for 4 consecutive weeks. Changes in body weight are plotted (a) ($n = 5$; $*P < 0.05$ vs control; $\#P < 0.05$ vs HFD by one-way ANOVA and Student–Newman–Keuls post-hoc test). Serum levels of total carnitine (b) and free carnitine (c) were measured with the enzymatic cycling method. Acyl-carnitine was calculated as the difference between total and free carnitine (d). The average values are plotted (mean ± standard error of the mean) ($n = 5$; $*P < 0.05$ vs controls; $\#P < 0.05$ vs 8-week HFD alone, by ANOVA on ranks and Student–Newman–Keuls post-hoc test).

To evaluate the status of carnitine insufficiency, we measured serum total and free carnitine levels. Total carnitine levels were significantly lower in KK-A^y mice fed HFD for 4 weeks compared with mice fed a control diet. However, serum free carnitine levels, which reflect the amount of dietary intake and synthesis in liver/kidney,[9] were not decreased following HFD feeding. In turn, serum levels of acyl-carnitine were significantly lower in HFD-fed mice, indicating that systemic consumption of carnitine was increased by HFD feeding. As expected, total carnitine levels were elevated to near basal levels when mice were given L-carnitine for 4 consecutive weeks. Treatment with L-carnitine also significantly increased both acyl and free carnitine (Fig. 1b–d).

L-carnitine ameliorates hepatic steatosis in KK-A^y mice

As expected, massive fat accumulation in hepatocytes with ballooning degeneration was observed 8 weeks after HFD feeding, which was dramatically prevented by administration of L-carnitine (Fig. 2a–d). Liver / body ratio was increased 8 weeks after HFD feeding, whereas the levels were significantly decreased in mice given L-carnitine ($P < 0.05$) (Fig. 2e). There was no significant difference in food consumption between mice treated with/without L-carnitine. Serum aspartate aminotransferase and alanine aminotransferase levels were significantly elevated in mice fed HFD, the values reaching 112.2 ± 26.5 IU/L and 113.0 ± 31.5 IU/L after 8 weeks, respectively. These elevations were significantly blunted in mice treated with L-carnitine to 77.2 ± 3.4 IU/L and 28.8 ± 4.4 IU/L, respectively ($P < 0.05$) (Fig. 2f,g).

L-carnitine prevents HFD-induced apoptosis of hepatocytes in KK-A^y mice

To evaluate apoptosis of hepatocytes, immunohistological staining for ccCK18 was carried out using an M30 CytoDEATH antibody. High-fat diet feeding increased the number of ccCK18-positive cells to 20.9 ± 1.8 per field at 4 weeks, followed by further increase to 41.6 ± 0.9% after

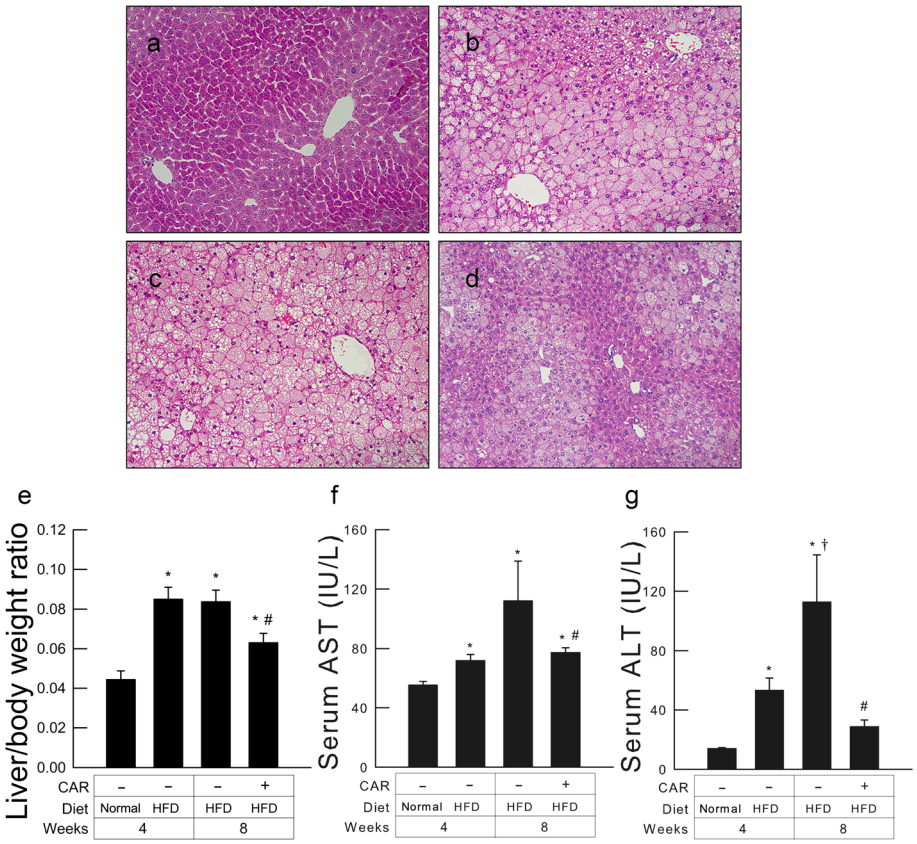

Figure 2 Treatment with L-carnitine (CAR) prevents hepatic steatosis following high-fat diet (HFD) feeding in KK-A^y mice. Representative photomicrographs of liver histology in mice after 4 weeks of feeding with control chow (a), 4-week HFD (b), and 8-week HFD (c). (d) Eight-week HFD with L-carnitine for the latter 4 weeks (hematoxylin–eosin staining, original magnification ×100). Liver/body weight ratio (e), and the average levels of serum aspartate aminotransferase (AST) (f) and alanine aminotransferase (ALT) (g) are plotted (means ± standard error of the mean) ($n = 5$; *$P < 0.05$ vs controls; †$P < 0.05$ vs. 4-week HFD; #$P < 0.05$ vs. 8-week HFD alone, by ANOVA on ranks and Student–Newman–Keuls post-hoc test).

8 weeks ($P < 0.05$). Surprisingly, the increase in apoptotic cell death was markedly prevented in mice treated with L-carnitine to 10.5 ± 2.4% ($P < 0.05$) (Fig. 3).

L-carnitine prevents hyperlipidemia following inhibition of de novo lipogenesis and activation of β-oxidation

In KK-A^y mice, HFD feeding for 8 weeks significantly increased serum FFA and triglyceride levels to 170.1 ± 14.9 μmol/L and 347.0 ± 66.8 mg/dL, which were significantly decreased by treatment with L-carnitine to 65.1 ± 7.1 μmol/L and 71.0 ± 4.3 mg/dL, respectively ($P < 0.05$) (Fig. 4a,b). Expression of SREBP1c mRNA in liver was measured to evaluate the effect of L-carnitine on de novo lipogenesis in HFD-fed mice. The treatment with L-carnitine completely prevented overexpression of SREBP1c mRNA induced by HFD to basal levels ($P < 0.05$) (Fig. 4c). To analyze the activity of lipolysis, expression of CPT1A mRNA, the liver isoform of CPT1,[25] and production of β-hydroxybutyrate in liver were evaluated. Exposure to HFD significantly increased expression of CPT1A mRNA (Fig. 4d) but not increased β-hydroxybutyrate in liver after 8 weeks. Treatment with L-carnitine markedly increased β-hydroxybutyrate to values more than twice that in mice fed HFD for 8 weeks ($P < 0.05$) (Fig. 4e).

L-carnitine improves hyperglycemia and hyperinsulinemia in HFD-fed KK-A^y mice

High-fat diet feeding significantly increased fasting serum glucose and insulin levels to 468.8 ± 64.5 mg/dL and 37.3 ± 5.5 ng/mL at 8 weeks, respectively, which were significantly decreased by treatment with L-carnitine to 217.2 ± 30.0 mg/dL and 4.4 ± 1.3 ng/mL, respectively ($P < 0.05$)

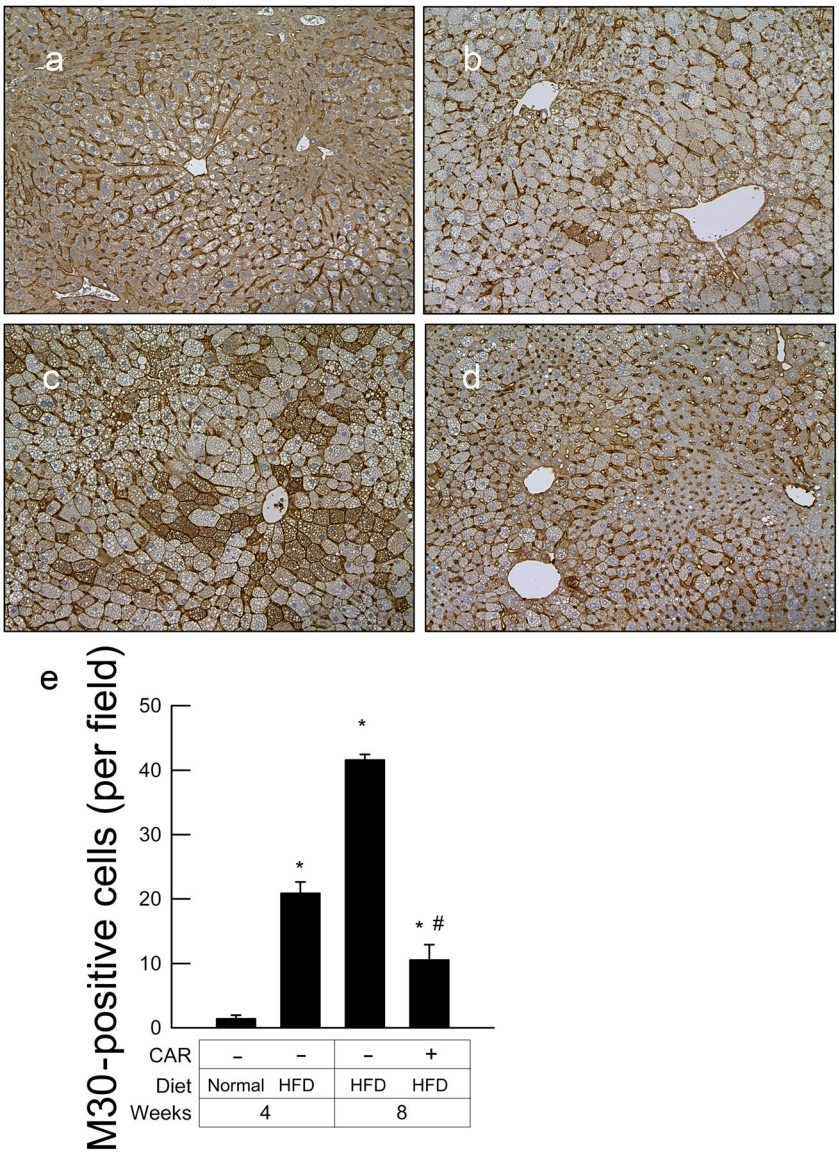

Figure 3 Treatment with L-carnitine prevents hepatocyte apoptosis in KK-A^y mice fed a high-fat diet (HFD). Representative photomicrographs of caspase cleavage product of cytokeratin (ccCK)18 immunohistochemistry in the liver after 4-week feeding with control chow (a) or HFD (b), and 8-week feeding with HFD (c), or HFD with L-carnitine for the latter 4 weeks (d) (original magnification ×100). Number of ccCK18-positive hepatocytes in 100× fields were counted in triplicate, and the average number of ccCK18-positive cells per field from five different animals are plotted (e). Data represent mean ± standard error of the mean. *$P < 0.05$ vs. controls; #$P < 0.05$ vs HFD alone, by ANOVA on ranks and Student–Newman–Keuls post-hoc test.

(Fig. 5a,b). Serine 307 phosphorylation of IRS-1, detected by sandwich ELISA, was significantly increased at 4 weeks of HFD feeding, which was completely reversed to basal levels by treatment with L-carnitine ($P < 0.05$) (Fig. 5c).

L-carnitine reduces FFA content and improves mitochondrial morphology and ATP production in liver

After 1 week of treatment, L-carnitine significantly reduced hepatic FFA content increased by HFD feeding ($P < 0.05$) (Fig. 6a). In morphological analysis using an electron microscope, hepatocyte mitochondria in mice fed HFD showed overt swelling in shape with few cristae. Treatment with L-carnitine dramatically improved the mitochondrial morphology to a near-normal, oval shape with more cristae (Fig. 6b,c). The HFD enhanced production of ATP in liver tissue, whereas addition of L-carnitine further increased the values, reaching more than twofold baseline levels ($P < 0.05$) (Fig. 6d).

DISCUSSION

IN THE PRESENT study, we showed that treatment with L-carnitine dramatically attenuates massive hepatic steatosis in KK-A^y mice, even under conditions of continuous HFD feeding (Fig. 2). L-carnitine primarily acts as a transport carrier of long-chain fatty acids across the inner

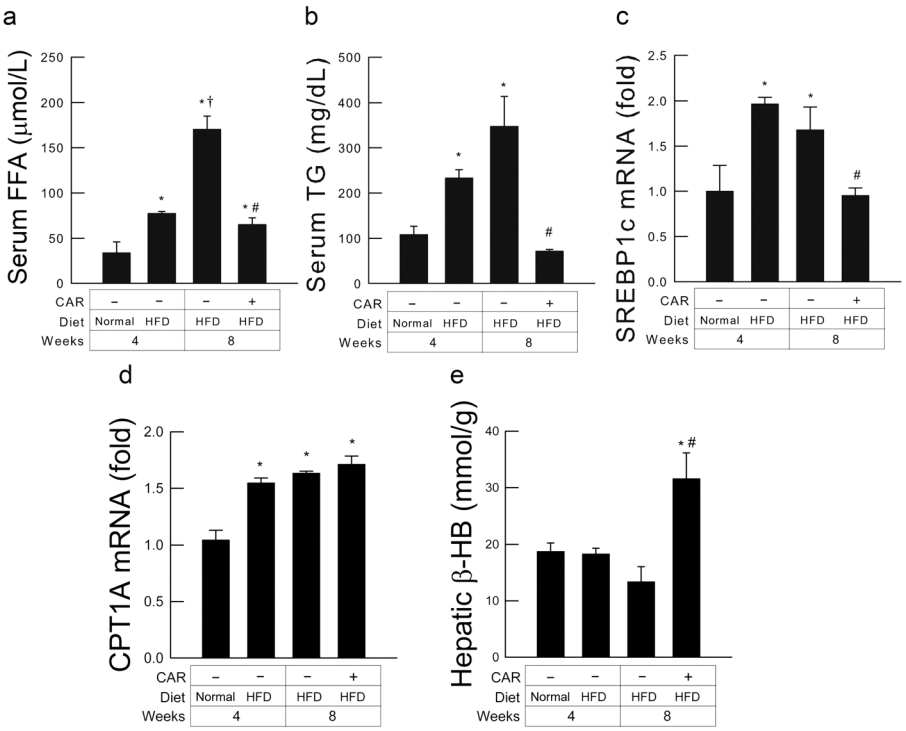

Figure 4 L-carnitine improves serum lipid profile and hepatic lipid metabolism in KK-A^y mice fed a high-fat diet (HFD). The average values of serum free fatty acids (FFA) (a) and triglyceride (TG) (b) are plotted. Hepatic mRNA levels for sterol regulatory element binding protein-1c (SREBP1c) and carnitine palmitoyltransferase 1A (CPT1A) were detected by real-time reverse transcription–polymerase chain reaction, and average values of fold-increase over controls for SREBP1c (c) and CPT1A (d) are plotted. Hepatic content of β-hydroxybutrate (β-HB) was measured by fluorometric assay. Obtained values were normalized by tissue weight, and average values are plotted (e). Data represent mean ± standard error of the mean. ($n = 5$; $*P < 0.05$ vs. controls; $\dagger P < 0.05$ vs. 4-week HFD; $\#P < 0.05$ vs. 8-week HFD alone, by ANOVA on ranks and Student–Newman–Keuls post-hoc test.

mitochondrial membrane from cytosol into the mitochondrial matrix, where fatty acids are catalyzed through β-oxidation.[26,275] Acyl–coenzyme A (CoA) units, formed by fatty acids and a CoA group, are converted into carnitine esters in a reaction catalyzed by CPT1, and subsequently cross the mitochondrial inner membrane.[27] Acetyl CoA produced by β-oxidation feeds into the citric acid cycle, and is converted to ketone bodies such as β-hydroxybutyrate. Here, the hepatic expression of CPT1A mRNA was increased following HFD intake (Fig. 4d); however, production of β-hydroxybutyrate in the liver was not increased by HFD alone. In contrast, HFD feeding supplemented with L-carnitine showed marked enhancement of β-hydroxybutyrate production in the liver (Fig. 4e). Furthermore, hepatic ATP content was significantly increased by L-carnitine treatment (Fig. 6d), suggesting the upregulation of ATP generation by the citric acid cycle and subsequent electron transport chain. Taken together, these findings clearly indicate that supplementation with L-carnitine, in concert with upregulation of CPT1A, is essential for the acceleration of mitochondrial β-oxidation in the liver under lipid overload.

L-carnitine markedly prevented overexpression of SREBP-1c mRNA induced by HFD feeding (Fig. 4c), which is the main transcription factor regulating de novo lipogenesis.[28] As the insulin signaling pathway involving IRS-2/phosphoinositide 3-kinase positively regulates the expression of SREBP1c,[29] hyperinsulinemia most likely contributes to upregulation of SREBP1c in KK-A^y mouse liver. It is therefore hypothesized that L-carnitine decreases expression of SREBP1c, at least in part, through normalization of hyperinsulinemia (Fig. 5b).

Our data clearly indicated that L-carnitine not only attenuates hepatic steatosis but also prevents the progression of steatohepatitis, as L-carnitine prevented hepatocyte apoptosis, the key event of the development of metabolic syndrome-related steatohepatitis.[30] The effect of L-carnitine on hepatic fibrogenesis, however, remains to be elucidated, because the model used in this study only represents the early phase of steatohepatitis.

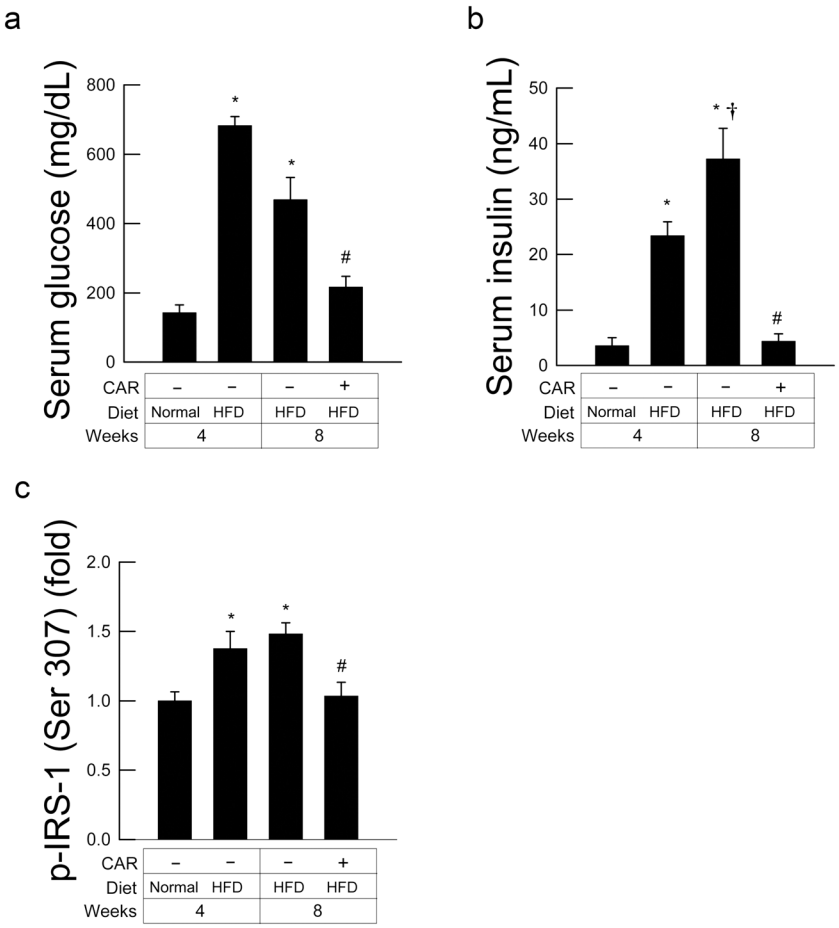

Figure 5 L-carnitine improves hyperglycemia/hyperinsulinemia and blunts serine phosphorylation of insulin receptor substrate (IRS)-1 in KK-A^y mice fed a high-fat diet (HFD). The average values of serum glucose levels are plotted (a). Serum insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (b). Serine phosphorylation of IRS-1 (Ser 307) in liver tissue was measured by sandwich ELISA, and average values of fold-increase over controls are plotted (c). Data represent mean ± standard error of the mean. ($n = 5$; $*P < 0.05$ vs. controls; $\dagger P < 0.05$ vs. 4-week HFD; $\#P < 0.05$ vs. 8-week HFD alone, by ANOVA on ranks and Student–Newman–Keuls post-hoc test.

Importantly, L-carnitine markedly reduced FFA levels in both serum and hepatic tissue (Figs. 4a,6a), most likely as a result of enhanced β-oxidation in liver and peripheral tissues including skeletal muscle. As FFA promotes lipotoxicity in the pathogenesis of steatohepatitis,[31] it is likely that L-carnitine prevented HFD-induced steatohepatitis in KK-A^y mice through amelioration of lipotoxicity by reduction of FFA content in hepatocytes. Furthermore, electron microscope analysis revealed that treatment with L-carnitine improved mitochondrial morphology in mice fed HFD (Fig. 6b,c). These findings are consistent with a previous study showing that L-carnitine reverses mitochondrial dysfunction caused by FFA *in vitro*.[32] Excess FFA induces mitochondrial injury, which results in the failure of ATP production, thus leading to cell death in hepatocytes.[33] Therefore, increases in hepatic ATP content following L-carnitine treatment (Fig. 6d) also supported the hypothesis that mitochondrial dysfunction caused by HFD feeding was rescued by supplementation with L-carnitine.

Another interesting observation in this study is that L-carnitine significantly prevented body weight gain in KK-A^y mice during HFD feeding (Fig. 1a), which is consistent with previous reports using mouse models fed HFD[34] and irregular diets.[35] The reduction of body weight by L-carnitine has also been reported in obese patients with metabolic syndrome[36] and polycystic ovary syndrome.[37] Moreover, L-carnitine improved hyperlipidemia, hyperglycemia, and hyperinsulinemia (Figs. 4a,b,5a,b), which are the hallmarks of metabolic syndrome and the established risk factors for NASH.[38,39] In terms of glucose metabolism, some reports have shown that L-carnitine improves insulin resistance in humans;[10,36,37,40] however, the mechanism underlying the effect of L-carnitine on insulin resistance is not fully understood. Here, we showed that L-carnitine prevents increases in serine phosphorylation of IRS-1 (Ser307; Ser312 in human IRS-1), which negatively regulates insulin signaling,[41] in the liver following HFD feeding (Fig. 5c). Lines of evidence have shown that serine phosphorylation of IRS-1 is induced by excess FFA.[42–44]

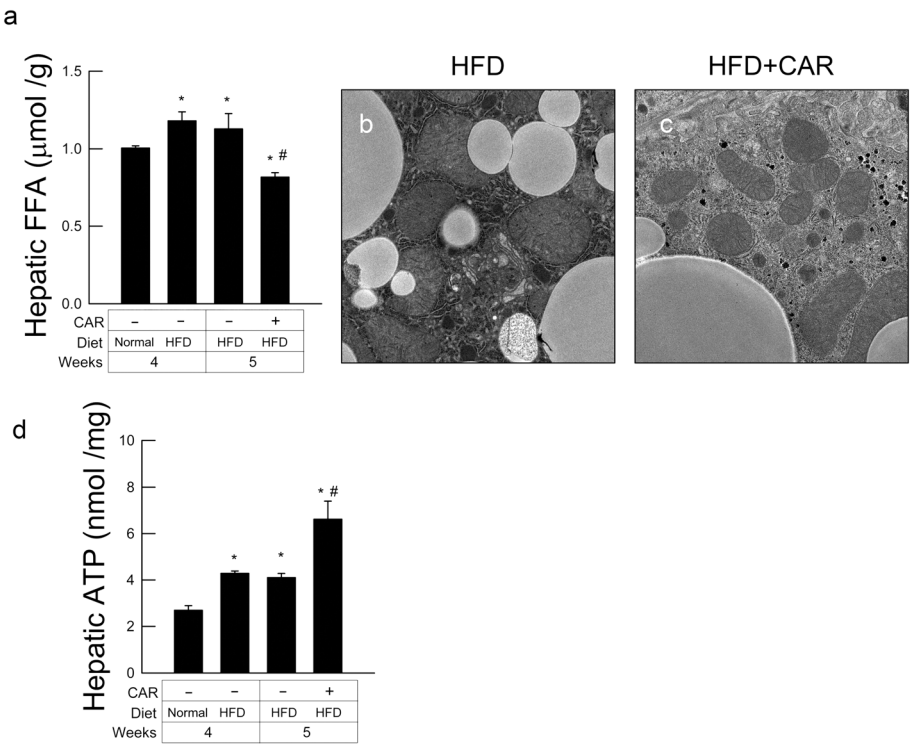

Figure 6 Treatment with L-carnitine decreases hepatic free fatty acid (FFA) content, improves mitochondrial morphology in hepatocytes, and increases hepatic adenosine 5′-triphosphate (ATP) content in KK-A^y mice fed a high-fat diet (HFD). FFA in liver tissue samples were measured by the enzyme-based method. Obtained values were normalized by tissue weight, and average values were plotted (a). Mitochondria in hepatocytes were observed by transmission electron microscopy. Representative photomicrographs of hepatocytes in mice 5 weeks after feeding with HFD (b) and those in combination with L-carnitine treatment for the latter 1 week (c) are shown (original magnification ×4000). Hepatic ATP content was measured by luciferase assay. Obtained values were normalized by protein concentration of homogenates, and average values are plotted (d). Data represent mean ± standard error of the mean. ($n = 5$; $*P < 0.05$ vs. controls; $\#P < 0.05$ vs. 5-week high-fat diet HFD alone, by ANOVA on ranks and Student–Newman–Keuls post-hoc test.

Therefore, it is likely that L-carnitine prevents HFD-induced serine phosphorylation of IRS-1, in part through decreasing hepatic FFA content (Fig. 6a), thereby improving insulin signaling. Taken together, our findings indicate that L-carnitine is promising to prevent development of NASH, in combination with systemic features of metabolic syndrome based on insulin resistance.

In conclusion, L-carnitine significantly ameliorates dietary steatohepatitis in KK-A^y mice, in concert with improvement of metabolic abnormalities. The underlying mechanisms most likely involve L-carnitine augmentation of mitochondrial β-oxidation, which reduces excess hepatic FFA content, thereby attenuating lipotoxicity that causes metabolic abnormalities and cellular damage in hepatocytes. It is therefore postulated that supplementation of L-carnitine is a promising approach for prevention and treatment of metabolic syndrome-related NASH.

ACKNOWLEDGMENTS

THE AUTHORS THANK Takako Ikegami and Tomomi Ikeda (Laboratory of Molecular and Biochemical Research, Research Support Center, Juntendo University Graduate School of Medicine, Tokyo, Japan) for technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research (24590995 to K. K., 24590996 and 15 K09023 to K.I., and 24390191 to S. W.) and a High Technology Research Center Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1 Chalasani N, Younossi Z, Lavine JE *et al.* The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the

American Gastroenterological Association. *Am J Gastroenterol* 2012; **107**: 811–26.
2 Michelotti GA, Machado MV, Diehl AM. NAFLD, NASH and liver cancer. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 656–65.
3 Zezos P, Renner EL. Liver transplantation and non-alcoholic fatty liver disease. *World J Gastroenterol* 2014; **20**: 15532–8.
4 Watanabe S, Hashimoto E, Ikejima K *et al.* Evidence-based clinical practice guidelines for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *Hepatol Res* 2015; **45**: 363–77.
5 Watanabe S, Hashimoto E, Ikejima K *et al.* Evidence-based clinical practice guidelines for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *J Gastroenterol* 2015; **50**: 364–77.
6 Hashimoto E, Taniai M, Tokushige K. Characteristics and diagnosis of NAFLD/NASH. *J Gastroenterol Hepatol* 2013; **28** (Suppl 4): 64–70.
7 Tanphaichitr V, Leelahagul P. Carnitine metabolism and human carnitine deficiency. *Nutrition* 1993; **9**: 246–54.
8 Magoulas PL, El-Hattab AW. Systemic primary carnitine deficiency: an overview of clinical manifestations, diagnosis, and management. *Orphanet J Rare Dis* 2012; **7**: 68.
9 Flanagan JL, Simmons PA, Vehige J, Willcox MD, Garrett Q. Role of carnitine in disease. *Nutr Metab (Lond)* 2010; **7**: 30.
10 Guarnieri G. Carnitine in maintenance hemodialysis patients. *J Ren Nutr* 2015; **25**: 169–75.
11 Nakamura M, Nagamine T. The effect of carnitine supplementation on hyperammonemia and carnitine deficiency treated with valproic acid in a psychiatric setting. *Innov Clin Neurosci* 2015; **12**: 18–24.
12 Somi MH, Fatahi E, Panahi J, Havasian MR, Judaki A. Data from a randomized and controlled trial of L-carnitine prescription for the treatment for non- alcoholic fatty liver disease. *Bioinformation* 2014; **10**: 575–9.
13 Malaguarnera M, Gargante MP, Russo C *et al.* L-carnitine supplementation to diet: a new tool in treatment of nonalcoholic steatohepatitis – a randomized and controlled clinical trial. *Am J Gastroenterol* 2010; **105**: 1338–45.
14 Ishikawa H, Takaki A, Tsuzaki R *et al.* L-carnitine prevents progression of non-alcoholic steatohepatitis in a mouse model with upregulation of mitochondrial pathway. *PLoS One* 2014; **9**: .e100627
15 Ikejima K, Okumura K, Kon K, Takei Y, Sato N. Role of adipocytokines in hepatic fibrogenesis. *J Gastroenterol Hepatol* 2007; **22**(Suppl 1): S87–92.
16 Okumura K, Ikejima K, Kon K *et al.* Exacerbation of dietary steatohepatitis and fibrosis in obese, diabetic KK-A(y) mice. *Hepatol Res* 2006; **36**: 217–28.
17 Kon K, Ikejima K, Okumura K, Arai K, Aoyama T, Watanabe S. Diabetic KK-A(y) mice are highly susceptible to oxidative hepatocellular damage induced by acetaminophen. *Am J Physiol Gastrointest Liver Physiol* 2010; **299**: G329–37.
18 Yamagata H, Ikejima K, Takeda K *et al.* Altered expression and function of hepatic natural killer T cells in obese and diabetic KK-A(y) mice. *Hepatol Res* 2013; **43**: 276–88.
19 Aoyama T, Ikejima K, Kon K, Okumura K, Arai K, Watanabe S. Pioglitazone promotes survival and prevents hepatic regeneration failure after partial hepatectomy in obese and diabetic KK-A(y) mice. *Hepatology* 2009; **49**: 1636–44.
20 Ikarashi N, Toda T, Okaniwa T, Ito K, Ochiai W, Sugiyama K. Anti-obesity and anti-diabetic effects of acacia polyphenol in obese diabetic KKAy mice fed high-fat diet. *Evid Based Complement Alternat Med* 2011; **2011**: 952031.
21 Wakutsu M, Tsunoda N, Mochi Y *et al.* Improvement in the high-fat diet-induced dyslipidemia and adiponectin levels by fish oil feeding combined with food restriction in obese KKAy mice. *Biosci Biotechnol Biochem* 2012; **76**: 1011–4.
22 Morinaga M, Kon K, Saito H *et al.* Sodium 4-phenylbutyrate prevents murine dietary steatohepatitis caused by trans-fatty acid plus fructose. *J Clin Biochem Nutr* 2015; **57**: 183–91.
23 Fukuo Y, Yamashina S, Sonoue H *et al.* Abnormality of autophagic function and cathepsin expression in the liver from patients with non-alcoholic fatty liver disease. *Hepatol Res* 2014; **44**: 1026–36.
24 Hosoya S, Ikejima K, Takeda K *et al.* Innate immune responses involving natural killer and natural killer T cells promote liver regeneration after partial hepatectomy in mice. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G293–9.
25 Britton CH, Mackey DW, Esser V *et al.* Fine chromosome mapping of the genes for human liver and muscle carnitine palmitoyltransferase I (CPT1A and CPT1B). *Genomics* 1997; **40**: 209–11.
26 Ribas GS, Vargas CR, Wajner M. L-carnitine supplementation as a potential antioxidant therapy for inherited neurometabolic disorders. *Gene* 2014; **533**: 469–76.
27 Strijbis K, Vaz FM, Distel B. Enzymology of the carnitine biosynthesis pathway. *IUBMB Life* 2010; **62**: 357–62.
28 Czech MP, Tencerova M, Pedersen DJ, Aouadi M. Insulin signalling mechanisms for triacylglycerol storage. *Diabetologia* 2013; **56**: 949–64.
29 Taniguchi CM, Ueki K, Kahn R. Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *J Clin Invest* 2005; **115**: 718–27.
30 Malik R, Chang M, Bhaskar K *et al.* The clinical utility of biomarkers and the nonalcoholic steatohepatitis CRN liver biopsy scoring system in patients with nonalcoholic fatty liver disease. *J Gastroenterol Hepatol* 2009; **24**: 564–8.
31 Alkhouri N, Dixon LJ, Feldstein AE. Lipotoxicity in nonalcoholic fatty liver disease: not all lipids are created equal. *Expert Rev Gastroenterol Hepatol* 2009; **3**: 445–51.
32 Jun DW, Cho WK, Jun JH *et al.* Prevention of free fatty acid-induced hepatic lipotoxicity by carnitine via reversal of mitochondrial dysfunction. *Liver Int* 2011; **31**: 1315–24.
33 Li Y, Zhang X, Tong N. Pioglitazone ameliorates palmitate induced impairment of mitochondrial morphology and function and restores insulin level in beta cells. *Pharmazie* 2013; **68**: 270–3.
34 Jang A, Kim D, Sung KS, Jung S, Kim HJ, Jo C. The effect of dietary alpha-lipoic acid, betaine, L-carnitine, and swimming

on the obesity of mice induced by a high-fat diet. *Food Funct* 2014; **5**: 1966–74.
35 Wu T, Guo A, Shu Q *et al.* L-carnitine intake prevents irregular feeding-induced obesity and lipid metabolism disorder. *Gene* 2015; **554**: 148–54.
36 Zhang JJ, Wu ZB, Cai YJ *et al.* L-carnitine ameliorated fasting-induced fatigue, hunger, and metabolic abnormalities in patients with metabolic syndrome: a randomized controlled study. *Nutr J* 2014; **13**: 110.
37 Samimi M, Jamilian M, Afshar Ebrahimi F, Rahimi M, Tajbakhsh B, Asemi Z. Oral carnitine supplementation reduces body weight and insulin resistance in women with polycystic ovary syndrome: a randomized, double-blind, placebo-controlled trial. *Clin Endocrinol (Oxf)*. (in press).
38 Reid AE. Nonalcoholic steatohepatitis. *Gastroenterology* 2001; **121**: 710–23.
39 Fan JG, Saibara T, Chitturi S, Kim BI, Sung JJ, Chutaputti A. What are the risk factors and settings for non-alcoholic fatty liver disease in Asia-Pacific? *J Gastroenterol Hepatol* 2007; **22**: 794–800.
40 Hong ES, Kim EK, Kang SM *et al.* Effect of carnitine–orotate complex on glucose metabolism and fatty liver: a double-blind, placebo-controlled study. *J Gastroenterol Hepatol* 2014; **29**: 1449–57.
41 Kanety H, Feinstein R, Papa MZ, Hemi R, Karasik A. Tumor necrosis factor α-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. *J Biol Chem* 1995; **270**: 23780–4.
42 Gao Z, Zhang X, Zuberi A *et al.* Inhibition of insulin sensitivity by free fatty acids requires activation of multiple serine kinases in 3 T3-L1 adipocytes. *Mol Endocrinol* 2004; **18**: 2024–34.
43 Herschkovitz A, Liu YF, Ilan E, Ronen D, Boura-Halfon S, Zick Y. Common inhibitory serine sites phosphorylated by IRS-1 kinases, triggered by insulin and inducers of insulin resistance. *J Biol Chem* 2007; **282**: 18018–27.
44 Tanti JF, Gual P, Gremeaux T, Gonzalez T, Barres R, Le Marchand-Brustel Y. Alteration in insulin action: role of IRS-1 serine phosphorylation in the retroregulation of insulin signalling. *Ann Endocrinol (Paris)* 2004; **65**: 43–8.