Note

Effects of Dietary Supplementation with Betaine on a Nonalcoholic Steatohepatitis (NASH) Mouse Model

Sakura KAWAKAMI1, Kyu-Ho HAN1, Yumi NAKAMURA1, Ken-ichiro SHIMADA1, Tomoko KITANO1, Tsutomu ARITSUKA2, Taizo NAGURA2, Kiyoshi OHR3, Kimihide NAKAMURA4 and Michihiro FUKUSHIMA1,∗

1 Department of Food Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan
2 Research Center, Nippon Beet Sugar MFG, Co., Ltd., Obihiro, Hokkaido 080–0831, Japan
3 Hokkaido Tokachi Area Regional Food Processing Technology Center, Obihiro, Hokkaido 080–2462, Japan
4 Health Care Administration Center, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan

(Received April 17, 2012)

Summary

The effects of betaine supplementation on non-alcoholic steatohepatitis (NASH) model mice were examined by measuring the accumulation of fat in the livers of NASH model mice compared to a control. Betaine from sugar beets was provided to the model mice as a dietary supplement. After 3 wk of dietary supplementation, there were no significant differences in body weight or liver weight between the groups. However, the liver to body weight ratio in the high-fat diet with betaine (HFB) group was significantly (p<0.05) higher than that in the high-fat diet (HF) group. There were no differences in serum triglyceride (TG) concentrations, AST and ALT activities, or hepatic glutathione concentrations between the groups. Hepatic TG level in the HFB group was significantly (p<0.05) lower than that in the HF group. Hepatic cells obtained from the HF group showed increased occurrence of explosive puff and necrosis as compared with those in the HFB group. Betaine supplementation had an inhibitory effect on fat accumulation in the liver: the Oil red-positive area in the HFB group (0.82±0.85%) was significantly (p<0.001) smaller than that in the HF group (9.06±2.24%). These results indicate the potential of betaine to serve as an agent for amelioration of hepatic steatosis in NASH model mice.

Key Words betaine, nonalcoholic steatohepatitis (NASH) model mice, fat accumulation in liver, hepatic steatosis

Materials and Methods

Material. Betaine, which was derived as a byproduct from sugar beet molasses during sucrose production, was a kind gift of Nippon Beet Sugar (Japan).

Animals and diets. Four-week-old male C57BL/6J-NASH model mice (STAM™) were purchased from Stelic Institute & Co. (Tokyo, Japan). Groups of two or three mice each were housed in plastic cages with a controlled environment.

Betaine is a nontoxic amino acid derivative obtained from dietary sources or synthesized from choline. High levels of betaine can be found in animal products, especially shellfish, and some plants such as members of the beet family (e.g., beetroot, spinach). A previous study demonstrated that betaine was effective at reducing lipid accumulation in tissues of patients with fatty liver, coronary atherosclerosis, and hyperlipidemia, and it is therefore considered to be a lipotropic substance (1).

Nonalcoholic fatty liver disease (NAFLD) encompasses a broad spectrum of liver abnormalities ranging from simple accumulation of triglycerides (TG) in hepatocytes to nonalcoholic steatohepatitis (NASH), which is sometimes followed by progression to fibrosis and cirrhosis (2). Because of its high prevalence in obesity, diabetes and insulin resistance, NAFLD is increasingly appreciated as a hepatic manifestation of the metabolic syndrome (3, 4). The mechanism involved in the development of NAFLD is poorly understood, although several hypotheses have been proposed. One generally accepted theory is the “two-hit” hypothesis, wherein the first hit involves the simple accumulation of fat, rendering the liver more sensitive to a second, undefined insult that results in more severe liver damage (5).

The current study was undertaken to examine the effects of dietary supplementation with betaine from sugar beets on the accumulation of fat in the liver of NASH model mice at the “first hit” stage of NAFLD.
12-h light/dark cycle. Room temperature and relative humidity were kept at 23 ± 1°C and 60 ± 5%, respectively. The animals were acclimated to the facility for 7 d and given free access to water and high-fat diet 32 (CLEA Japan Inc., Tokyo, Japan). Free access to food and water continued throughout each experiment. Ten mice were randomly divided into 2 groups: HF, with high-fat diet 32, and HFB, given high-fat diet 32 supplemented with betaine. There were no significant differences in body weight between the study groups at the beginning of the experiment. The composition of high-fat diet 32 was as follows: milk casein, 24.5%; albumen powder, 5.0%; l-cystine, 0.43%; beef tallow, 15.88%; high-oleic safflower oil, 20.0%; cellulose powder, 5.5%; maltodextrin, 8.25%; lactose, 6.928%; sucrose, 6.75%; vitamin mixture, 1.4% (AIN-93); mineral mixture, 5.0% (AIN-93G); choline bitartrate, 0.36%; and tert-butyl hydroquinone, 0.002%. Betaine was dissolved at 50 mg/mL in distilled water. Betaine-treated mice (5 wk old) were administered a dose of 10 mL/kg body weight orally two times per day for 3 wk by intragastric gavage. The dosages of betaine used in the present study was based on previous report by Ji and Kaplowitz (6) showing that betaine in the drinking water at a concentration of 0.5% (w/v) (25–35 mg/25–27 g mice/d, which was calculated from 5–7 mL as daily normal drinking water), had protective effects on alcohol-induced fatty liver and liver injury in mice. The control mice (5 wk old) were given 10 mL of distilled water/kg body weight orally two times per day for 3 wk by intragastral gavage. Body weight and food consumption were recorded weekly and daily, respectively, throughout the study period. At the completion of the 3-wk feeding period, the mice were anesthetized by diethyl ether without fasting and blood samples were collected by cardiac puncture. The livers were surgically removed and washed with cold saline (9 g of NaCl/L deionized water), blotted dry on filter paper and weighed. Samples of the liver were fixed in 10% formalin or flash frozen for histological examination as described below and the rest of the liver was frozen at −20°C for later analysis. The experimental design was approved by the Animal Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine, and adhered to the standard principles described in the Guide for the Care and Use of Laboratory Animals (7).

Biochemical analysis in serum. The TG concentration, and ALT and AST activities were determined enzymatically using commercially available assay kits (Fuji Dri-Chem system; Fuji Film Corporation, Tokyo, Japan) run with a FUJI Dri-Chem 7000 (Fuji Film Corporation).

Hepatic TG and glutathione (GSH) concentrations. Hepatic triglyceride levels in the total lipid dissolved with isopropanol were colorimetrically measured using a commercially available kit (Triglyceride-E-test; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Hepatic GSH levels were determined according to the method of Cohn and Lyle (8) using a fluorescence spectrophotometer (excitation wavelength of 342 nm, emission wavelength of 428 nm).

Histological examinations of liver. Liver portions that had been fixed in 10% buffered formalin were embedded in paraffin and then 4 μm sections were deparaffinized in xylene. These sections were then rehydrated through a series of decreasing concentrations of ethanol and stained with hematoxylin-eosin (HE). Alternatively, portions of fresh liver were flash frozen and cryostat sections were cut and prepared for staining with Oil Red O.

Statistics analysis. Data are presented as means and standard deviations. The significance of differences between the HF group and HFB group was determined by Student’s t-test, with a predetermined significance value set at p < 0.05.

Results

Table 1 shows the final body weights, liver weights, serum TG concentrations, serum AST and ALT activities, and hepatic TG and GSH concentrations of NASH model mice fed high-fat diet 32 or high-fat diet 32 supplemented with betaine. There were no significant differences between the final body weights or the liver weights of the groups. However, the liver-to-body weight ratio in the HFB group was significantly (p < 0.05) higher than that in the HF group. The hepatic TG level in the HFB group was significantly (p < 0.05) lower than that in the HF group. Although serum TG concentration, AST and ALT activities, and hepatic GSH concentration were all higher in the HF group, none of these differences were significant.

Histopathological examinations of the liver are shown in Figs. 1 and 2. The explosive puff and necrosis of hepatic cells in the HF group were increased compared with those in the HFB group (Fig. 1, HE stain). Fat accumulation in the liver was greater in the HF group than in the HFB group, indicating a beneficial effect of betaine supplementation (Fig. 2, Oil red stain). The Oil

| Dietary group | HF       | HFB      |
|---------------|----------|----------|
| Body weight (g) | 19.0±1.1 | 17.9±0.5 |
| Body weight gain (g/3 wk) | 1.96±1.25 | 1.44±0.90 |
| Liver weight (g/mouse) | 1.22±0.07 | 1.35±0.15 |
| Liver weight/ body weight ratio (%) | 6.45±0.51 | 7.54±0.76* |
| Serum AST (IU/L) | 208±93 | 155±51 |
| ALT (IU/L) | 79.8±31.1 | 65.2±25.1 |
| Triglyceride (mg/dL) | 324±158 | 288±40 |
| Liver | | |
| Triglyceride (mg/g) | 60.3±6.4 | 45.9±4.2* |
| Glutathione (μmol/g) | 7.48±1.04 | 6.28±0.54* |

Values are means±standard deviations for 5 rats. HF, high-fat diet 32; HFB, high-fat diet 32 with betaine. *p < 0.05 versus HF group by Student’s t-test.

Table 1. Body weight, liver weight, and serum triglyceride concentration, AST and ALT activities, and hepatic triglyceride and glutathione concentrations in NASH model mice fed HF or HFB for 3 wk.
Fig. 1. Hepatic cells from NASH model mice fed high-fat diet 32 (HF) or high-fat diet 32 with betaine supplementation (HFB) for 3 wk. The liver samples were stained with hematoxylin-eosin for histopathological examination.

Fig. 2. Hepatic lipid accumulation in NASH model mice fed high-fat diet 32 (HF) or high-fat diet 32 with betaine supplementation (HFB) for 3 wk. The liver samples were stained with Oil red O for histopathological examination.
been studied (10). The content of hepatic TG in normal mice represents approximately 8 mg per g of liver (Stelic Institute & Co), whereas the results of this study showed dramatically high elevation of serum TG levels, on average 60.3 and 45.9 mg per g of liver in the HF and HFB groups, respectively. Betaine supplementation significantly reduced the accumulation of fat in the livers of mice fed a high-fat diet for 3 wk. as compared to mice on a high-fat diet that did not receive betaine supplementation.

On the other hand, generally normal mice exhibit ALT activity as approximately 30 IU per L of serum (Stelic Institute & Co), and our data showed an average of 80 IU per L of serum in the HF group. The serum AST and ALT activities in the HFB group were lower than those in the HF group, but these differences were not significant. In a previous study, rats with induced liver injury showed marked increases in serum AST and ALT activities, but those receiving dietary supplementation with 1% betaine showed AST and ALT activity levels similar to those of a control group without liver injury (10). Furthermore, administration of betaine also increased both hepatic and serum GSH levels, even following D-galactosamine (D-GalN) injection (10). However, there was no significant difference in the hepatic GSH levels between the groups in this study. It is unclear as to why the results of these two studies vary. One explanation may be that there are inherent differences between liver injury due to D-GalN and liver injury induced by a high-fat diet. In fact, Kwon et al. (11) also reported no significant difference in serum ALT activity between NAFLD rats on a high-fat liquid diet and NAFLD rats on a high-fat liquid diet supplemented with betaine.

The liver structure of the HF group in this study was damaged after the 3 wk feeding period, and various degrees of diffuse hepatic steatosis and necrosis of hepatic cells were observed. Compared with the HF group, the degrees of hepatic steatosis and necrosis were greatly reduced in the HFB group. Shi et al. (12) have reported that betaine can effectively prevent hepatic steatosis and alcohol-induced liver injury, as well as improve liver function. Kwon et al. (11) have also indicated that the function of sulfur-containing substances is significantly disturbed by a high-fat liquid diet, suggesting a causal role of impairment of hepatic transsulfuration reactions in NAFLD. The effect of betaine supplementation on hepatic oxidative stress and transsulfuration reactions in animal models of fatty liver has been studied (13). As the liver is a central organ in the metabolism of sulfur-amino acids in mammals (14), the first step in the transsulfuration reaction chain in the liver is the formation of S-adenosylmethionine (SAM) from methionine and ATP, which is catalyzed by methionine adenosyltransferase (MAT). SAM is the principle biological methyl donor, the precursor of aminopropyl groups used in polyamine synthesis, and a provider of cysteine for synthesis of GSH (15). Both SAM and GSH are scavengers of hydroxyl radicals, with SAM being the most effective (16). Recently, Kathiryel et al. (17) reported that betaine improves NAFLD by decreasing serum homocysteine concentration and increasing hepatic SAM concentration in mice. We previously observed that, in rats with D-GalN-induced liver injury, the hepatic SAM and cysteine levels were significantly elevated for a group receiving 1% betaine supplementation compared with a control group (10). Therefore, it is possible that the results of the current study might be explained in part by betaine-induced increases in the hepatic levels of SAM and cysteine, which help to reduce the degree of hepatic steatosis of NASH model mice during the “first hit” stage of NAFLD.

Even though the pathogenesis of hepatic fat accumulation in NAFLD is not fully understood, suggested mechanisms include 1) higher fat intake, 2) induced synthesis and secretion of VLDL particles by the liver as a consequence of the increase in de novo fatty acid synthesis, and 3) continuous delivery of free fatty acid in lipolytic adipose to the liver. It has been reported that betaine supplementation increases VLDL synthesis by increasing the availability of phosphatidylcholine, an essential component of VLDL (18). Furthermore, it has been reported that betaine increases phosphatidylcholine synthesis by elevating hepatic levels of SAM, which donates a methyl group to phosphatidylethanolamine in the formation of phosphatidylcholine (19). Therefore, the modulated hepatic steatosis effect of betaine in this study for NASH model mice might be explained in part by an increase in the formation of VLDL particles and a subsequent increase in the export of lipids from the liver (20). However, in the present study, dietary betaine lessened hepatic steatosis, with no effect on serum triglyceride level in NASH model mice. Recently, Kathiryel et al. (17) reported that betaine treatment of high-fat mice model slightly reduced body weight but significantly reduced the visceral fat mass. Therefore, it is possible that the modulated hepatic steatosis induced by a high-fat diet in NASH model mice might be due to an effect from the lower release of free fatty acid derived from adipocytes owing to a decrease in the fat mass rather than one by synthesis and secretion of VLDL from the liver.

In conclusion, this study indicates the potential of betaine to serve as an ameliorative agent for hepatic steatosis in NASH model mice at the “first hit” stage of NAFLD. Moreover, these findings highlight the importance of future research to determine the detailed mechanism of hepatic steatosis in NAFLD patients.
Acknowledgments
This research was supported by a grant from the Regional Innovation Cluster Program (City Area Type) of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES
1) Schwahn BC, Wang XL, Mikael LG, Wu Q, Cohn J, Jiung H, Maclean KN, Rozen R. 2007. Betaine supplementation improves the atherogenic risk factor profile in a transgenic mouse model of hyperhomocysteinemia. *Atherosclerosis* **195**: e100–e107.
2) Duvnjak M, Lerotic I, Barsić N, Tomasić V, Virović L, Velagić V. 2007. Pathogenesis and management issues for non-alcoholic fatty liver disease. *World J Gastroenterol* **13**: 4539–4550.
3) Yeh MM, Brunt EM. 2007. Pathology of nonalcoholic fatty liver disease. *Am J Clin Pathol* **128**: 834–847.
4) Farrell GC, Larter CZ. 2006. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology* **43**(Suppl 1): S99–112.
5) Day CP, James OF. 1998. Steatohepatitis: a tale of two “hits”? *Gastroenterology* **114**: 842–845.
6) Ji Č, Kaplowitz N. 2003. Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. *Gastroenterology* **124**: 1488–1499.
7) National Research Council. 1985. *Guide for the Care and Use of Laboratory Animals*. National Institutes of Health Publication no. 85-23, revised ed. National Academy of Sciences, Washington, DC.
8) Cohn VH, Lyle JA. 1966. Fluorometric assay for glutathione. *Anal Biochem* **14**: 434–440.
9) Fujii M, Shibazaki Y, Wakamatsu K, Honma T, Hashiguchi T, Furuuchi M, Nishimura N, Suzuki K, Ichida T, Yoneyama H. 2012. A novel murine model recapitulates the pathogenesis of human non-alcoholic steatohepatitis (NASH) and NASH-related hepatocellular carcinoma. *Digestive Disease Week Poster Su1958*, program p. 347.
10) Okada T, Kawakami S, Nakamura Y, Han KH, Obha K, Aritsuka T, Uchino H, Shimada K, Sekikawa M, Ishii H, Fukushima M. 2011. Amelioration of α-galactosamine-induced acute liver injury in rats by dietary supplementation with betaine derived from sugar beet molasses. *Biosci Biotechnol Biochem* **75**: 1335–1341.
11) Kwon DY, Jung YS, Kim SJ, Park HK, Park JH, Kim YC. 2009. Impaired sulfur-amino acid metabolism and oxidative stress in nonalcoholic fatty liver are alleviated by betaine supplementation in rats. *J Nutr* **139**: 63–68.
12) Shi QZ, Wang LW, Zhang W, Gong ZJ. 2010. Betaine inhibits Toll-like receptor 4 expression in rats with ethanol-induced liver injury. *World J Gastroenterol* **16**: 897–903.
13) Kim SK, Kim YC. 2005. Effects of betaine supplementation on hepatic metabolism of sulfur-containing amino acids in mice. *J Hepatol* **42**: 907–913.
14) Mudd SH, Poole JR. 1975. Labile methyl balances for normal humans on various dietary regiments. *Metabolism* **24**: 721–735.
15) Avila MA, Berasain C, Torres L, Martín-Duce A, Corrales FJ, Yang H, Prieto J, Lu SC, Caballera J, Rodés J, Mato JM. 2000. Reduced abundance of the main enzymes involved in methionine metabolism in human liver cirrhosis and hepatocellular carcinoma. *J Hepatol* **33**: 907–914.
16) Evans PJ, Whiteman M, Tredger JM, Halliwell B. 1997. Antioxidant properties of S-adenosyl-l-methionine: a proposed addition to organ storage fluids. *Free Radic Biol Med* **23**: 1002–1008.
17) Kathirvel E, Morgan K, Nandgiri G, Sandoval BC, Caudill MA, Bottiglieri T, French SW, Morgan TR. 2010. Betaine improves nonalcoholic fatty liver and associated hepatic insulin resistance: a potential mechanism for hepatoprotection by betaine. *Am J Physiol Gastrointest Liver Physiol* **299**: G1068–1077.
18) Yao ZM, Vance DE. 1988. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem* **263**: 2998–3004.
19) Bidgway ND, Vance DE. 1988. Kinetic mechanism of phosphatidylethanolamine N-methyltransferase. *J Biol Chem* **263**: 16864–16871.
20) Olthof MR, Vliet TV, Verhoef P, Zock PL, Katan MB. 2005. Effect of homocysteine-lowering nutrients on blood lipids: results from four randomized, placebo-controlled studies in healthy humans. *PLoS Med* **2**: 446–456.