Gene Expression and Histochemical Analyses in the Fatty Livers of Rats Fed a Histidine-Excess Diet

Takahiko J. Fujimi1, Mikako Sate1, Minori Tsuya1, Mayuko Hiro1, Riku Asahi4, Ryuta Suzuki1, Shigeru Nakajima1, Hiroshi Yokoyama2, Tomokazu Matsuura2 and Nobuyuki Kanzawa3

1 Department of Registered Dietitians, Faculty of Health and Nutrition, Bunkyo University, 1100, Namegaya, Chigasaki, Kanagawa 253–8550, Japan
2 Department of Laboratory Medicine, The Jikei University School of Medicine, 3–25–8, Nishi-shinbasho, Minato-ku, Tokyo 105–8461, Japan
3 Department of Material and Life Science, Faculty of Science and Technology, Sophia University, 7–1, Kioi-cho, Chiyoda-ku, Tokyo 102–8554, Japan

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Summary Triglyceride (TG) and cholesterol accumulation are known to occur in the liver of rats fed a histidine-excess (5%) diet, but there are few studies reporting histochemical and molecular biological analyses of the rat liver. The aim of this study was to elucidate the molecular basis of this lipid-accumulation mechanism. Lipid accumulations, tissue section images, and gene expression levels were compared in the livers of rats fed a control or histidine-excess diet for 5 wk (n = 8/group). Serum levels of TGs, free fatty acids, total cholesterol, high-density lipoprotein cholesterol, glucose, albumin, and the enzyme activities of aspartate aminotransferase and alanine aminotransferase were also analyzed. In the livers of rats fed a histidine-excess diet, histochemical analyses showed what appeared to be a preliminary stage of nonalcoholic fatty liver, characterized by lipid accumulation around the central vein area and minor fibrosis. However, there were no changes in serum TG or free fatty acid levels. Quantitative PCR analyses showed the up-regulation of FAT/CD36, which is related to the uptake of fatty acids into cells, and the downregulation of two apolipoprotein genes, ApoC3 and ApoE. The mRNA levels of PPARγ, LXRα, and AMPKα in the liver were also reduced by excess histidine intake. The results of this study suggest that steatosis caused by excess histidine intake may be the result of an imbalance between lipid transport from the liver and the uptake of free fatty acids into hepatocytes.

Key Words amino acid, steatosis, obesity, apolipoproteins, lipids

The importance of amino acids for living organisms lies in the diversity of their metabolites. Amino acids are not only a nitrogen source but are also the building blocks of body composition and precursors of physiologically active substances, such as neurotransmitters, hormones and vitamins. Each amino acid undergoes a characteristic metabolic pathway depending on its structure. To ensure adequate amino acid intake, the appropriate intake of dietary protein is important to maintain for animal health. There are numerous reports that the inadequate intake or a deficiency in amino acids has physiological effects. Growth, wasting and whole-body metabolism have been shown to be influenced by disturbances in the optimized balance of essential and nonessential amino acids in the diet (1). On the other hand, oral administration of certain amino acids, i.e. branched-chain amino acids, can have health benefits (2, 3).

We have previously reported the beneficial effects of histidine intake on obesity and food intake. The consumption of a histidine-excess diet has been shown to suppress food intake and reduce adipose tissue weight (4–6). However, the effects of excess-intake of histidine in rat liver and serum lipids were also reported by Aoyama et al. (7). They showed that liver triglyceride (TG) and cholesterol levels increased after feeding rats a histidine-excess (5%) diet more than 21 d, but serum TG levels decreased under these conditions. We have previously reported the effects of short-term excess histidine consumption on the suppression of food intake and fat accumulation in rats (5% histidine for 8 d) (4). In the report, fat accumulation in liver was also briefly described. In this way, as a phenomenon, fatty liver by histidine-excess intake have been reported, but histochemical and molecular biological analysis of fat accu-
mulation in rat liver by feeding of histidine-excess diet have not been studied well.

Understanding of the benefits and harms of histidine intake is crucial in considering its use for obesity. An increased understanding of mechanism about the histidine-induced fatty liver may also provide new insights into human nutrient intake and lipid synthesis. For that reasons, we examined the effects on the liver after feeding rats a histidine-excess (5%) diet for 5 wk. in the present study. To elucidate the molecular basis of these effects, gene expression in the liver was analyzed by quantitative PCR (qPCR).

**MATERIALS AND METHODS**

*Animal experimental procedures.* The experimental diets used in this study were prepared according to a previous report. Those were consisted of either 25% casein (control diet: Cont) or 20% casein plus 5% histidine (histidine-excess diet: His), contained enough methionine. Mineral mixture, and vitamin mixture with choline are AIN-76 compliant products (Table 1). Four-week-old male Wistar rats were purchased from CLEA JAPAN, Incorporation (Tokyo, Japan). The animals were housed in individual cages at a constant temperature (23 ± 2°C) in a light-controlled room (lights on from 6:00 am to 6:00 pm). The rats were acclimated to the experimental conditions and given free access to the Cont diet and water for 7 d. After acclimation, rats on the Cont diet, they were divided into two or three groups (n = 8) based on body weight. There were no significant differences in the average initial body weight of each group (Welch’s t-test, or one-way analysis of variance [ANOVA]). One of the following three feeding conditions was used: free access to the Cont diet (Cont FA), free access to the His diet (His FA) or feed restricted Cont diet (Cont R). Feed restriction was performed as follows. The amount of feed given on the first day (day 8) was the average amount of feed consumed on the last day of acclimation (17 g/rat). Feeding was then gradually reduced over 7 d to 12 g/rat on day 14. Thereafter, feeding was equal to the average intake of the His FA group on the previous day. All rats were allowed free access to water throughout the experimental periods. Food intake and body weight were recorded daily. One day before sacrifice, the rats were fasted. At the end of the experimental period, rats were anesthetized by Somnopentyl (Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) injection.

After sacrifice, the rats were fasted. At the end of the experimental period, rats were anesthetized by Somnopentyl (Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) injection. Whole blood was collected from the heart and the liver (Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) injection. After separation, tests of aspartate (AST) and alanine aminotransferase (ALT) activity were immediately performed using a Transaminase CII-Test Wako Kit (Tokyo, Japan), according to the manufacturer’s instructions. The thermal reaction and data analyses were performed using a MyGOpro real-time PCR system (IT-IS Life Science Ltd., Dublin, Republic of Ireland) and its associated analysis software. The data were analyzed using the 2^-ΔΔCt method, using the β-actin (Actb) mRNA level as an internal control. The primer sets used in this study are shown in Table 2.

**Biochemical analysis**. Serum was immediately separated from collected whole blood samples using VENOJECT™II blood collection tubes (Terumo, Tokyo, Japan), according to the manufacturer’s instructions. After serum separation, tests of aspartate (AST) and alanine aminotransferase (ALT) activity were immediately performed using a Transaminase CII-Test Wako Kit. Other serum components were assayed using a Glucose CII-Test Wako Kit (glucose), an A/G B-test Wako Kit (albumin and globulin), a Cholesterol E-Test Wako Kit (total cholesterol; Total-Cho), an HDL-cholesterol E-Test Wako Kit (high-density lipoprotein cholesterol; HDL-Cho), a Triglyceride E-Test Kit Wako (triglycerides; TGs) and NEFA C-Test Kit Wako (free fatty acids), using serum samples stored at −20°C. All serum test kits were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

**Lipid extraction from liver tissue**. Liver TG extraction was performed using the method of Folch et al. (8) with some modifications. Frozen liver tissue (~0.25 g) was homogenized in 2 mL saline for 3 min using an ULTRA TURRAX® T25 basic (IKA LABORTECHNIK, Staufen, Germany). After adding an additional 2 mL of saline, the lipid component was extracted with 15 mL of methanol/chloroform (1 : 2) by shaking at 180 rpm for 30 min using a DOUBL SHAKER NR-3 (TAITEC Corporation, Saitama, Japan). The lower phase (9.5 mL) was transferred to a new tube after phase separation by centrifugation at 3,000 rpm, for 10 min at 4°C. One milliliter of the recovered lipid phase was dispensed into a new test tube and the liquid components were vaporized at room temperature for 2 d. The solid components of the lipid fraction were dissolved in isopropanol/Triton® X-100 (9 : 1). The TG concentration was mea-
**RESULTS**

**Fatty liver induced by a histidine-excess diet was different from hypotrophic fatty live**

As shown in our previous reports, a histidine-excess diet results in the suppression of food intake. To determine whether liver TG accumulation in response to a histidine-excess diet is due to a hypotrophic condition, Cont R diet group was compared to Cont FA and His FA diet groups. Comparisons of body weight changes and food intake between the three groups (Cont FA, Cont R, and His FA) are shown in Fig. 1A and B. These results indicated that there was no significant difference in total caloric intake during the period or body weight at the endpoint, between the Cont R and His FA diet groups. At the endpoint analysis, liver weight (Fig. 1C) and lipid (TG and total cholesterol) content (Fig. 1D and E) increased only in the His FA diet group and there was no significant difference between the Cont FA and Cont R diet groups. In contrast, posterior abdominal wall fat weight was reduced in the Cont R and His FA diet groups (Fig. 1F). Therefore, the decrease in posterior abdominal wall fat due to excess histidine intake was considered to be caused by the decrease in caloric intake due to the food intake suppression effect and the accumulation of liver fat was due to the function of histidine.

**Body weight, liver weight, and liver lipid content after longer-term rearing of animals on a His FA diet**

To analyze the effects of a histidine-excess diet, longer-term (5 wk) rearing was performed in the Cont FA and the His FA diet groups. A comparison of the body weight change between the two groups is shown in Fig. 2A. One week after dividing the rats into groups according to diet (day 14), there was a significant difference in body weight between the two groups. Subsequently, significantly lower body weight was observed in rats on a His FA diet compared to those on a Cont FA diet, throughout the feeding period. Tissue weight gain and lipid accumulation in the livers of the His FA group at the end of the experimental period gave the same results as those presumed in Fig. 1 (Fig. 2B–D). These results shown in Fig. 2 are consistent with the previously reported results of both short-term (7–8 d) and long-term (42 d) experiments.

**Serum biochemical analyses of His FA diet animals after longer-term rearing**

Serum lipid levels were then tested to analyze lipid metabolism. A comparison of serum lipid levels in the two groups is shown in Fig. 3A–D. Although changes in TG accumulation (increase in liver fat) were observed in rats fed a His FA diet (Fig. 2), serum TG and free fatty acid levels did not differ between rats fed Cont FA or His FA diets (Fig. 3A and B). However, total cholesterol and HDL-cholesterol levels were significantly higher in rats fed a His FA diet than those fed a Cont FA diet (Fig. 3C and D). To evaluate the condition of the livers in the two groups, serum indices of liver function were tested.
Although, albumin production and blood glucose levels (Fig. 3E and F) did not differ between the two groups, the activities of two enzymes (Fig. 3G: AST and 3H: ALT) that are biomarkers of cell damage were higher in rats on a His FA diet than those on a Cont FA diet. These results suggest that rats fed a His FA diet had fatty and slightly damaged livers.

Histochemical analysis

To visualize the conditions of the liver, paraffin-embedded liver sections were stained (Fig. 4A, B, F and G: H-E staining; Fig. 4C, D, E, H, I and J: Azan staining). While fat accumulation was not observed in the livers of rats on a Cont FA diet (Fig. 4A and B), H-E staining showed sponge-like white spaces in the pink eosin stain, which indicated the accumulation of lipid droplet(s), scattered in the livers of rats fed a His FA diet (Fig. 4F). Lipid accumulation in rats fed a His FA diet was characterized by macrovesicular lipid droplets, and the lipid droplets were frequently observed around central veins. An analysis of higher-magnification images indicated
that the accumulated lipid droplets occurred in various sizes (Fig. 4G). Azan staining of adjacent sections showed that some blue-stained collagen fibers were slightly observed between cells in the region where lipid accumulation was observed (Fig. 4I and J). Neither balloon-like degeneration of hepatocytes (a swelling of cells without the accumulation of lipid droplets) nor distinct inflammatory cell infiltration were observed.

Quantitative analyses of transcription

qPCR was performed to elucidate the molecular basis of lipid accumulation in the livers of rats fed a His FA diet (Fig. 5). Firstly, the transcription levels of several genes related to fatty acid metabolism (ACC2, FAT/CD36 and L-FABP) were assessed (Fig. 5). There were no significant differences in the gene expression levels of intracellular fatty acid trafficking protein (L-FABP) or the rate-limiting enzyme for fatty acid synthesis (ACC2) between groups. In contrast, the fatty acid uptake factor gene (FAT/CD36) was markedly upregulated (more than 3-fold) in the livers of rats fed a His FA diet. The expression levels of the rate-limiting enzyme for cholesterol synthesis (HMGCR) also showed no significant change in the livers of rats fed a His FA diet. Next, the transcription levels of three apolipoprotein genes (ApoB-100, ApoC3, and ApoE) were measured. These apolipoproteins are abundant in TG-rich lipoproteins that transport lipids from the liver to peripheral tissues (very low-density lipoprotein and low-density lipoprotein). The mRNA levels of two apolipoproteins (ApoC3 and ApoE) were significantly reduced in the livers of rats fed a His FA diet (0.6- and 0.8-fold, respectively). Several transcription factor genes involved in lipid metabolism (Lpin1, SREBP-1c, PPARα, PPARγ, PGC-1α, and LXRα) were also evaluated. No significant differences were observed in the mRNA levels of SREBP-1c, which is known as a master regulator of TG synthesis; PPARα; PGC-1α; and Lpin1 between the groups. However, a significant decrease in the mRNA levels of PPARγ and LXRα was observed in the livers of rats fed a His FA diet. PPARγ mRNA levels in rats fed a His FA diet were less than one-third of the control group and showed the largest decrease among the genes examined. Finally, the levels of the energy-sensing enzyme in liver cells, AMP-activated protein kinase α subunit gene (AMPKα) were measured. A significant decrease in AMPKα mRNA levels were observed in the livers of rats fed a His FA diet.
DISCUSSION

In the present study, two groups of rats fed different diets were compared. The results of body weight (low), food intake (low), adipose tissue weight (low), liver weight (high), and serum cholesterol level (high) measurements in rats fed a histidine-excess diet in this study were consistent with the results of many previous reports (4, 6, 7, 9–11). There have been discrepancies among previous reports regarding TG accumulation in the liver, associated with liver weight gain. Ohmura et al. (10) showed no hepatic TG accumulation, but other reports have shown an accumulation of TGs in the liver associated with the increase in liver weight caused by a histidine-excess diet (4, 7). These discrepancies may be due to differences in the composition of the experimental diets used. Furthermore, it has also been reported that serum and liver TG levels in rats fed a histidine-excess diet are influenced by differences between rat strains and diets (11). These previous reports are consistent with recent understanding that hepatic fat accumulation is affected by both genetic and environmental factors (12–15). It seems that consistent experimental conditions, dietary composition, and animal strains in a series of continuous studies are important for the analysis of hepatic fat accumulation. Even in that context, the results of the present study agreed well with those of our previous studies using the same dietary composition and rat strain (4, 6).

One of the novel results that differentiated this study from previous studies is the histological analysis of livers from rats fed a His FA diet (Fig. 4). H-E staining clearly showed lipid droplets in the livers of these rats (Fig. 4F). Lipid accumulation was frequently observed around the central vein. In other words, lipid accumulation was unevenly distributed. In general, the progression of nonalcoholic fatty liver pathology is associated with increased serum AST and ALT activities. The minor fibrosis observed with Azan staining seemed to support the serum AST (significance increase [1.4-fold] in the His FA condition, Fig. 3G) and ALT (tendency to increase in the His FA condition, Fig. 3H) activity results. These observations indicated that the intake of excess histidine appeared to produce a phenotype similar to the preliminary stage of nonalcoholic fatty liver, which also shows the accumulation of large lipid droplets mainly around the central vein, minor fibrosis, and cell damage. In the present study, histidine was admin-
Fig. 4. Representative images of hematoxylin-eosin and Azan staining of liver tissue from rats fed two different diets. (A–E) Livers of rats fed the control diet. (F–J) Livers of rats fed the histidine-excess diet. H-E staining images are shown in panels A, B, F, and G. Azan staining images of adjacent sections are shown in panels C, D, E, H, I, and J. Lower magnification images (×25) are panels of A, C, F, and H. Scale bars shown in the lower magnification images (panels A and C) indicate 500 μm. Higher magnification images (×200) of the areas indicated by the yellow rectangles (in A, C, F, and H) are panels B, D, G, and I. Scale bars shown in the higher magnification images (panels B and D) indicate 50 μm. Panels E and J show the digitally magnified images of the area indicated by the yellow rectangles in panels D and I. Yellow arrows indicate the signs of fibrosis. CV: central vein.
Illustrated for 5 wk, but with a longer treatment period, the pathological progression to nonalcoholic steatohepatitis may be observed.

Another novel component of the present study was the analysis of gene expression in rats fed excess histidine (Fig. 5). No changes were observed in the transcriptional levels of genes encoding key factors for lipid synthesis (SREBP-1c, ACC2, and HMGCR) or fatty acid oxidation (PPARα). Among the tested genes, greatest change observed in the His FA condition was an increase (3.4-fold) in the mRNA level of FAT/CD36, which is related to the uptake of fatty acids into hepatocytes. The levels of two apolipoprotein genes (ApoC3 and ApoE) were also downregulated in the livers of rats fed a His FA diet. These results indicated that there was an increase in fatty acid uptake into the liver and a decrease in lipid transport from the liver to peripheral tissues. Therefore, at the transcriptional level, it was proposed that steatosis caused by excess histidine consumption may be a result of an imbalance between lipid transport from the liver to the periphery and fatty acid uptake into the liver, rather than a decrease in fatty acid oxidation or an increase in intracellular lipid synthesis.

Another noteworthy change observed in this study was

![Fig. 5. Quantitative analyses of mRNA levels. Encoded protein names of analyzed genes are shown on the top of the graphs. The value of the Cont FA group was set as a reference. The value on the vertical axis indicates the relative amounts (R.A.) of mRNA. Black bars indicate rat fed the Cont FA diet, and white bars indicate rats fed the His FA diet. Data were analyzed using Welch’s t-test (** p<0.01). Error bars indicate standard deviation.](image-url)
the decrease in of PPARγ mRNA levels (less than one-third). In some animal models, PPARγ, which controls the enzymes involved in lipid metabolism, is upregulated in fatty livers (16–18). While promoting fat accumulation, it has been reported that strong inhibition of PPARγ (administration of a PPARγ antagonist in heterozygous PPARγ-deficient mice) causes TG accumulation in the liver (19). Furthermore, inhibition of PPARγ has been reported to promote liver fibrosis by suppressing hepatic stellate cell proliferation and activation (20–22). The significant reduction in PPARγ mRNA levels observed in the livers of rats fed excess histidine (Fig. 5K) may be supported the results of steatosis with slight fibrosis (Fig. 4F–I). However, seemingly contradictory results were observed in this study, compared with the current knowledge of transcriptional regulation. For example, it is known that LXRα strongly activates SREBP-1 and accelerates fatty acid synthesis (23, 24). The data presented in Fig. 5 show that LXRα mRNA levels decreased, but no significant change was seen in SREBP-1c mRNA levels. It is also known that FAT/CD36 is a target gene of both PPARγ and LXRα in the liver (25). However, in the present study, FAT/CD36 gene transcription was upregulated, despite the downregulation of PPARγ and LXRα in the livers of rats fed a histidine-excess diet (Fig. 5). Therefore, it is difficult to clearly explain the relationships between the transcription levels of genes tested in this study, without inconsistencies.

To elucidate the molecular mechanisms for histidine-induced steatosis, it is important to understand the relevant signaling pathways. One of the pathways that responds to nutrient or energy levels is the mechanical target of rapamycin (mTOR) pathway. There are two mTOR (serine/threonine protein kinase) complexes, known as mTORC1 and mTORC2. The key outputs of this pathway through mTORC1 include lipid synthesis and energy metabolism. We hypothesized that histidine may affect mTORC1 activity through 5′-AMP-activated protein kinase (AMPK) and/or vacuolar-type H+-ATPase (v-ATPase). AMPK is known as a sensor of intracellular energy and a regulator of whole-body metabolism, and activated AMPK inhibits mTORC1 (26, 27). Histidine decarboxylase converts histidine to histamine, which is one of the activators of AMPK (28, 29). Amino acids also activate mTORC1 through Rag GTPases and v-ATPase (30). Excess histidine accumulation in lysosomes via the histidine transporter (solute carrier family 15A4; SLC15A4) may interfere with v-ATPase function due to pH-related effects, through the buffering capacity of histidine (31, 32). Some of the genes tested (PPARα, PPARγ, SREBP-1c, and Lipin1) in this study are included in the downstream pathways of mTORC1. They are regulated under the mTORC1 at the levels of transcription or protein (30). Furthermore, ACC and HMGCR are substrates for AMPK, and their phosphorylated proteins are inactivated (33–35). The evaluation of protein levels and activities of AMPK or v-ATPase may be required to determine the precise mechanism, although downregulation of AMPKα was observed in rats fed a histidine-excess diet, which shows initial support for the aforementioned hypothesis (Fig. 5N). Further understanding of the above signaling pathways in the livers of rats fed excess histidine will be the focus of future research.

Authorship
Research conception and design: TJF and SN; experiments: TJF, MS, MT, RS, MH, RA and HY; statistical analysis of the data: MS and TJF; interpretation of the data: HY, TM, NK, and RA; writing of the manuscript: TJF.

Disclosure of state of COI
No conflicts of interest to be declared.

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