L-Lysine Attenuates Hepatic Steatosis in Senescence-Accelerated Mouse Prone 8 Mice

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Summary Non-alcoholic hepatic steatosis is a phenotype of metabolic syndrome, and aging is a risk factor for this condition. Senescence-accelerated mouse prone 8 (SAMP8) is a murine model for studying aging-associated disorders. We here examined the effect of dietary supplementation with L-lysine (Lys) on non-alcoholic hepatic steatosis in SAMP8 mice. Triglyceride (TG) and cholesterol (Chol) accumulated in the liver of Lys-rich diet-fed SAMP8 mice. Plasma alanine aminotransferase activity, an index of liver injury, was decreased by Lys. The mRNA expression levels of peroxisome proliferator-activated receptor gamma coactivator 1-α and carnitine palmitoyltransferase 1a, which regulate β-oxidation, were increased in the livers of SAMP8 mice fed the Lys-rich diet. Taken together, our study suggests dietary intake of Lys prevents hepatic steatosis by stimulating β-oxidation in SAMP8 mice.

Key Words L-lysine, hepatic steatosis, β-oxidation, PGC-1α

Aging-related metabolic disorders such as hepatic steatosis (1), insulin resistance (2) and sarcopenia (3) are increasingly serious problems in aging populations. Non-alcoholic fatty liver disease (NAFLD) is a liver disease characterized by the deposition of lipid droplets and is not due to excessive alcohol intake (4). Aging is a known risk factor for NAFLD (5, 6). The early stage of NAFLD is “simple hepatic steatosis” with relatively good prognosis (6). However, the progression of non-alcoholic hepatic steatosis causes non-alcoholic steatohepatitis (NASH) (6), and eventually induces cirrhosis or hepatocellular carcinoma (7, 8). Therefore, effective approaches for the prevention of hepatic steatosis are needed.

Several amino acids have been reported to show beneficial effects in the treatment of hepatic steatosis (9–11), and L-lysine (Lys) is known to regulate lipid metabolism in the liver (12, 13). However, there is no study which investigated the underlying mechanisms in the effect of Lys on hepatic steatosis. Additionally, the effect of Lys on age-related hepatic steatosis is unknown.

Senescence-accelerated mouse prone 8 (SAMP8) is a murine model for the study of aging-associated disorders (14). It has been reported that hepatic steatosis is induced in SAMP8 mice without the need to feed a high-fat or high-carbohydrate diet (15, 16), indicating that SAMP8 would be an appropriate model for age-associated non-alcoholic hepatic steatosis.

We here examined the effect of dietary supplementation with Lys on hepatic steatosis in SAMP8 mice and investigated the mechanisms underlying the effects of Lys.

MATERIALS AND METHODS

Materials. Antibodies against phospho (Thr308)-Akt, AMP-activated protein kinase (AMPK), phospho (Thr172)-AMPK, acetyl-CoA carboxylase (ACC) and phospho (Ser79)-AMPK, were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against Akt and phospho (Ser473)-Akt were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) antibody, hematoxylin, and eosin Y were purchased from Merck (Darmstadt, Germany). β-Actin antibody was obtained from Novus Biologicals (Littleton, CO). Horse radish peroxidase (HRP)-conjugated anti-rabbit and -mouse IgGs were obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). PrimeScript RT Master Mix and SYBR Premix Ex Taq II were purchased from Takara Bio Inc. (Shiga, Japan). An enzyme-linked immunosorbent assay (ELISA) kit for measuring insulin was purchased from Morinaga Institute of Biological Science (Yokohama, Japan). Oil Red O was purchased from Sigma (St. Louis, MO). All other chemicals were obtained from Wako Pure Chemicals.
Industries, Ltd. (Osaka, Japan).

Animals. Male SAMP8 (15-wk-old, 27–35 g, n = 17) and SAMR1 (15-wk-old, 31–35 g, n = 6) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were individually housed in stainless steel wire cages and were maintained at 22°C and 55% relative humidity with a 12-h light/12-h dark cycle. All animal protocols were approved by the Iwate University Animal Research Committee and were performed in compliance with the Guidelines for Animal Experiments of Iwate University (approval number A201517).

Experiment design. At 28 wk of age, the SAMP8 mice were divided into treatment groups and provided access ad libitum to AIN-93M (17), and thus 3 h after the final feeding, each group was provided with 1.5% Lys (PLK, pH 7.4), 10 mM NaF, 2 mM EDTA, 2.0% Triton-X-100, 2 mM phenylmethylsulfonyl fluoride) followed by centrifugation at 10,000 × g for 10 min at 4°C. The supernatant was used as the SDS-PAGE sample and was separated on a polyacrylamide gel, followed by transfer to a PVDF membrane (Millipore Corp., Billerica, MA). The membrane was blocked for 1 h with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) at room temperature. The membrane was incubated overnight at 4°C with primary antibodies, then the membrane was incubated with HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG in TBS-T. The secondary antibody was detected using an ECL Western blot detection kit (GE Healthcare). The bands were scanned using a luminescent image analyzer (ImageQuant LAS 4000, GE Healthcare) and the relative intensity of each band was estimated using NIH Image software.

qRT-PCR. The mRNA expression levels of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), peroxisome proliferator-activated receptor α (PPARα), carnitine palmitoyltransferase 1a (CPT-1a), acyl-CoA oxidase (ACO), and sterol regulatory element-binding protein-1c (SREBP-1c) were quantified using quantitative reverse transcription PCR (qRT-PCR). Total RNA (500 ng) extracted from liver tissue using the APG method (21) was reverse transcribed into cDNA by incubating it with PrimeScript RT Master Mix at 37°C

Table 1. Composition of experimental diet.

<table>
<thead>
<tr>
<th>Ingredient (g/kg diet)</th>
<th>R</th>
<th>P</th>
<th>PLK</th>
<th>PHK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk casein1</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>t-Lysine (g/kg)</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Sucrose1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cornstarch1</td>
<td>620.7</td>
<td>620.7</td>
<td>605.7</td>
<td>590.7</td>
</tr>
<tr>
<td>Soy bean oil4</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Mineral mixture1</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mixture1</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Cellulose1</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

1 Oriental Yeast Co., Tokyo, Japan.
2 Ajinomoto Co., Tokyo, Japan.
3 Toyo Sugar Refining Co., Tokyo, Japan.
4 Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Measurement of triglyceride (TG) and cholesterol (Chol) in the liver: Briefly, lipid fractions were prepared according to Folch et al. (20). The concentration of TG in the liver lipid extract was measured using a TG test kit (Triglyceride E Test Wako, Wako Pure Chemical Industries, Ltd.) and the concentration of Chol was measured using a Chol test kit (Cholesterol E Test Wako, Wako Pure Chemical Industries, Ltd.), both according to the manufacturer’s protocols.

Measurement of insulin, glucose, TG, and alanine aminotransferase activity (ALT) in plasma: The plasma concentration of insulin was measured using an ELISA kit (Morinaga Institute of Biological Science), that of glucose was measured using a glucose test kit (Glucose C2 Test Wako, Wako Pure Chemical Industries, Ltd.), and that of TG was measured using a TG test kit (Triglyceride E Test Wako, Wako Pure Chemical Industries, Ltd.). The activity of ALT in plasma was measured using an ALT activity assay kit (Transferase CII Test Wako, Wako Pure Chemical Industries, Ltd.). All measurements were conducted according to the manufacturers’ protocols.

Measurement of plasma amino acid concentrations: Plasma was mixed with an equal volume of 3.0% sulfosalicylic acid overnight at 4°C, then the mixture was centrifuged at 18,000 × g for 15 min. The concentration of amino acids in the plasma supernatant treated with sulfosalicylic acid was measured using an amino acid autoanalyzer (JLC-500/V; JEOL, Tokyo, Japan).

Western blotting: Liver tissue was homogenized in 10 volumes of buffer solution (50 mM HEPES-NaOH (pH 7.6), 10 mM Na3VO4, 10 mM sodium pyrophosphate, 100 mM NaF, 2 mM EDTA, 2.0% Triton-X-100, 2 mM phenylmethylsulfonyl fluoride) followed by centrifugation at 10,000 × g for 10 min at 4°C. The supernatant was used as the SDS-PAGE sample and was separated on a polyacrylamide gel, followed by transfer to a PVDF membrane (Millipore Corp., Billerica, MA). The membrane was blocked for 1 h with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) at room temperature. The membrane was incubated overnight at 4°C with primary antibodies, then the membrane was incubated with HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG in TBS-T. The secondary antibody was detected using an ECL Western blot detection kit (GE Healthcare, Tokyo, Japan). The bands were scanned using a luminescent image analyzer (ImageQuant LAS 4000, GE Healthcare) and the relative intensity of each band was estimated using NIH Image software.
for 15 min. qRT-PCR with SYBR Premix Ex Taq II was performed using a Thermal Cycler Dice Real Time System Single TP-850 instrument (Takara Bio Inc.). Each mRNA level was corrected by reference to the level of 18S ribosomal RNA (18S). Primer sequences are shown in Table 2.

### Statistical analysis
Data are expressed as means ± standard errors of the mean. Data analyses were performed using GraphPad Instat Software ver. 3.0a (2001, GraphPad Software, Inc., San Diego, CA). Differences were identified using analysis of variance and Tukey’s post-test in four-group comparisons and were considered significant when \( p < 0.05 \). Pairwise comparisons were performed using unpaired \( t \)-tests, and two-tailed \( p \) values were calculated.

## RESULTS

**Dietary Lys suppressed the accumulation of TG and Chol in the livers of SAMP8 mice**

We first histologically analyzed the effect of Lys on lipid accumulation in the livers of SAMP8 mice. As shown in Fig. 1A and B, there was marked accumulation of lipid droplets in the livers of SAMP8 mice fed the standard diet (P), whereas dietary supplementation with Lys (PLK and PHK) prevented accumulation and significantly decreased the levels of TG and Chol in the liver (Fig. 1C, D). The activity of ALT, an index of liver injury, was increased in the P group compared with...
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The R, PLK or PHK groups (Fig. 1E, PLK: p=0.04 vs. P, PHK: p=0.03 vs. P). On the other hand, body weight and the amount of food intake (Table 3) were not different among the three groups of SAMP8 (P, PLK, PHK). Therefore, the suppressive effect of Lys on the hepatic steatosis in SAMP8 would not be due to a reduction of food intake.

Increases in plasma TG and glucose were attenuated by dietary Lys

The plasma concentration of TG in the P group tended to be higher than that of mice in the R group, and plasma TG was reduced in mice fed diets supplemented with 1.5% Lys (p=0.0081 vs. P) or 3.0% Lys (p=0.0009 vs. P) (Fig. 2A). The plasma glucose concentration in the P group mice was significantly higher than that in the R. Supplementation of Lys tended to decrease the plasma glucose concentration in PLK and PHK groups compared with the P group, and there was no difference in the plasma glucose concentration between the two Lys supplementation groups (PLK and PHK) and the R group (Fig. 2B). The plasma insulin concentration in the P group was lower than that in the R group, probably due to the impairment of insulin secretion, and the insulin concentration did not differ among the P, PLK and PHK groups (Fig. 2C).

Plasma Lys concentration increased in a dose-dependent manner, but the concentrations of other basic and branched-chain amino acids did not change

The plasma Lys concentration did not differ between mice in the P and R groups and increased in mice in the PLK and PHK groups (Fig. 2D). The plasma concentrations of arginine (Arg), histidine (His), leucine (Leu), valine (Val) and isoleucine (Ile) were not different among the four groups (Fig. 2E–I).

Table 3. Body weight change and food intake.

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>P</th>
<th>PLK</th>
<th>PHK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>34.2±0.8a</td>
<td>29.6±1.0b</td>
<td>30.2±1.0b</td>
<td>29.7±1.1b</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>35.9±0.5a</td>
<td>31.5±1.1b</td>
<td>32.2±1.0b</td>
<td>31.6±1.1b</td>
</tr>
<tr>
<td>Average food intake (g/d)</td>
<td>4.4±0.2</td>
<td>4.4±0.1</td>
<td>4.3±0.1</td>
<td>4.4±0.1</td>
</tr>
</tbody>
</table>

Values are means±SE of 5–6 mice per group. Different letter means significant (p<0.05) difference between groups.
PGC-1α and genes related to β-oxidation in the liver of SAMP8 mice were upregulated by Lys

The amount of PGC-1α protein, which regulates the β-oxidation of fatty acid (22, 23), was lower in the livers of mice in the P group compared to mice in the R group (Fig. 3A). However, dietary supplementation with Lys tended to increase the amount of PGC-1α protein in the liver (Fig. 3A, PLK: p = 0.06 vs. P, PHK: p = 0.02 vs. P), although the increase was not significant. The mRNA expression level of PGC-1α in the liver was significantly increased by dietary supplementation with 3.0% Lys (Fig. 3B). We quantified the mRNA level of the β-oxidation related genes PPARα (23, 24), CPT-1a (25, 26), and ACO (27) in the liver using qRT-PCR. The mRNA expression level of PPARα in the P group tended to be lower than that of the R group (p = 0.09 vs. P), showing that dietary supplementation with Lys tended to increase PPARα mRNA levels (Fig. 4A, PLK: p = 0.01 vs. P, PHK: p = 0.05 vs. P), and similarly CPT-1a (Fig. 4B) and ACO mRNA expression levels (Fig. 4C). On the
other hand, the mRNA expression level of the lipogenesis-related gene SREBP-1c was not different among the groups (Fig. 4D).

AMPK/ACC signaling was not activated by Lys whereas the phosphorylation level of Akt was improved by Lys.

The phosphorylated form (active form) of AMPK and its downstream target, ACC (29), were not increased by supplementation with Lys (Fig. 5B, C). The amount of ACC and pACC protein was significantly lower in the livers of mice in the P group compared with that of mice in the R group, whereas levels were restored in the PLK and PHK groups (Fig. 5D, E). The phosphorylation level of Akt, an insulin-signaling molecule (30), was markedly decreased in the livers of the P group mice compared with the R group mice, and was much higher in SAMP8 mice fed the Lys-rich diets (Fig. 5F).

**DISCUSSION**

In the present study, we investigated the effect of dietary supplementation with Lys on aging-related hepatic steatosis in SAMP8 mice. In line with previous reports (15, 16), hepatic steatosis was accompanied by liver injury in SAMP8 mice (Fig. 1). The amounts of TG in the livers of SAMP8 mice fed the control diet (P) tended to be higher than those in SAMR1 mice (R) (Fig. 1C, p=0.08 vs. R), although some lipid droplets accumulated in the livers of both P and R group mice (Fig. 1B). In contrast, dietary supplementation with 1.5% Lys (PLK) or 3.0% Lys (PHK) attenuated hepatic steatosis, TG accumulation, and liver injury (Fig. 1A–E).

To clarify the mechanism underlying the preventive effect of Lys on hepatic steatosis, we evaluated the protein amounts or mRNA expression levels of β-oxidation-related factors in the livers of SAMP8 mice. PGC-1α is a coactivator of various nuclear receptors and transcription factors and reportedly regulates mitochondrial biogenesis, energy metabolism, and mitochondrial β-oxidation (22, 23, 31). We found that the decreased protein amount and mRNA expression level of PGC-1α in the livers of SAMP8 mice were upregulated by Lys (Fig. 3A, B). Additionally, the mRNA expression level of PPARα, a protein which regulates β-oxidation cooperatively with PGC-1α (23), tended to increase in SAMP8 mice fed a Lys-rich diet (Fig. 4A, PLK; p=0.01 vs. P, PHK; p=0.05 vs. P).

We verified that Lys suppresses hepatic steatosis by stimulating β-oxidation in the livers of SAMP8 mice. As expected, the mRNA expression level of CPT-1α, a protein which catalyzes the first step in β-oxidation, was markedly increased by Lys supplementation (Fig. 4B). Nishikata et al. (32) showed that a mixture of ketogenic amino acids suppressed lipogenesis in the livers of mice fed a high-fat diet. However, the mRNA expression level of SREBP-1c, a major regulator of lipogenesis (28), was not suppressed by Lys supplementation (Fig. 4D). Therefore, the preventive effect of dietary Lys towards hepatic steatosis may be due to the stimulation of β-oxidation, and not to the suppression of lipogenesis in SAMP8 mice. The mechanism underlying the effect of Lys on hepatic lipid metabolism may be therefore different from that of a mixture of ketogenic amino acids (32).

AMPK is known to regulate PGC-1α (33); thus the mechanism underlying the effect of Lys on the amount of PGC-1α was investigated in SAMP8 mice by evaluating the phosphorylation levels of AMPK and ACC, the downstream targets of AMPK. The phosphorylation levels of AMPK and ACC were unaffected by Lys (Fig. 5B, C) and thus further studies are needed to demonstrate the mechanisms by which Lys upregulates PGC-1α in the livers of SAMP8 mice.

Reduced insulin sensitivity is considered a key factor in the development of non-alcoholic hepatic steatosis (34), and insulin secretion is known to be impaired by aging (35). It has been reported that SAMP8 mice have reduced insulin secretion (36), and Fujitsuka et al. showed that the plasma insulin concentration in SAMP8 mice is markedly lower than that in SAMR1 mice (37). In this study, the plasma insulin concentration of the P group was lower than that of the R group, while the plasma glucose concentration of the P group was higher than that of the R group, probably due to impairment of insulin secretion (36) (Fig. 2B, C). Dietary supplementation with Lys tended to reduce plasma glucose levels without changing the plasma insulin level (Fig. 2B, C), suggesting that Lys may improve insulin sensitivity in SAMP8 mice. The phosphorylation level of Akt, which is involved in insulin signaling (38), was markedly higher in the livers of SAMP8 mice fed a Lys-rich diet compared to the P group (Fig. 5F). Therefore, the preservation of Akt activity in SAMP8 mice by Lys supplementation may help maintain glucose homeostasis.

It was reported that the mRNA expression level of cationic amino acid transporter (CAT-1) in the liver is increased by intake of Lys in pigs (39), and three basic amino acids (Lys, Arg, and His) are commonly transported via CAT-1 (40). Additionally, His is reported to suppress lipogenesis-related genes in the mouse liver (41). However, the plasma concentrations of Arg and His were not changed in SAMP8 mice fed a Lys-rich diet (Fig. 2E, F). In addition, the plasma concentrations of branched-chain amino acids (BCAA; Leu, Val, and Ile) were not affected by Lys supplementation (Fig. 2G–I), although a mixture of ketogenic amino acids (32) or BCAA (42) is reported to reduce hepatic TG. Therefore, the effect of Lys on hepatic steatosis would not depend on BCAA, including Leu or basic amino acids other than Lys. On the other hand, we did not investigate the effect of the metabolites of Lys on hepatic steatosis in this study. For example, carnitine, which is known to stimulate β-oxidation is synthesized from Lys and methionine (43, 44). Further studies are needed to measure the concentrations of some of Lys’s metabolites including carnitine and clarify the possibility that Lys metabolites play a central role in the suppressive effect of Lys on hepatic steatosis. We focused on hepatic steatosis in SAMP8 mice and investigated the effect of Lys supplementation on hepatic lipid metabolism in this study. However, non-alcoholic hepatic steatosis is known to be associated with the progression of age-associated loss of skeletal muscle (sarcopenia) (45–49), and we previously
showed that dietary supplementation with Lys attenuates sarcopenia in SAMP8 mice (18). Therefore, it is possible that hepatic steatosis causes the progression of sarcopenia in SAMP8 mice, and Lys may affect sarcopenia in SAMP8 mice by improving hepatic steatosis.

In conclusion, it was demonstrated that the dietary intake of Lys attenuates hepatic steatosis in SAMP8 mice. Lys exerts this effect by stimulating β-oxidation via PGC-1α and/or PPARα. Our findings may contribute to the prevention of age-related hepatic steatosis and thus improve the quality of life of the elderly. We additionally found that Lys suppresses the accumulation of Chol in the liver of SAMP8 (Fig. 1D) in this study. Therefore, further study which investigates the underlying mechanisms in the effect of Lys on the Chol metabolism is needed.

Author contributions

Study conception and design: Sato, Ito, and Nagasawa. Acquisition of data: Sato and Muramatsu. Analysis and interpretation of data: Sato, Muramatsu, Ito, Yamamoto, and Nagasawa. Drafting of manuscript: Sato, Muramatsu, Ito, and Nagasawa. Critical revision: Sato.

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