Suppressive Effects of Bunashimeji (*Hypsizigus marmoreus*) on Triacylglycerol Accumulation in Orotic Acid-treated Growing Rats

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本研究では、現代の日本人の摂取脂質エネルギー量増加に伴う肥満に注目し、脂肪肝を誘発させるオロチン酸（OA）とプナシメジの添加がスプラグダウェリー（SD）ラットの肝臓における脂質代謝に及ぼす影響について検討した。3週間のSD系雄性ラットを購入し、市販飼料で1週間予備飼育後、(1) 15% cellulose powder (control), (2) 15% Bunashimeji powder (Buna), (3) 1% orotic acid and 15% cellulose powder (OA-control), および (4) 1% orotic acid and 15% Bunashimeji powder (OA-Buna) の4群に分け、各々脂質代謝に関するプロフィールを測定した。その結果、control と OA-cont のセルロース添加群間で、肝臓重量、肝臓総脂質含量ならびに肝臓TGにおいて顕著な差が認められた。また、control と Buna ならびに OA-control と OA-Buna の群間で肝臓重量に有意な差は認められなかったが、肝臓総脂質含量と肝臓TG は、プナシメジ添加で有意な低下が認められた。そこで、プナシメジ添加が脂質代謝酵素のmRNA発現に及ぼす影響について調べたところ、脂肪酸合成に関与する酵素であるACC、肝臓でのTG合成に関わるDGATの発現に有意な差が認められた。
オロチン酸処理ラットにおけるブナシメジの肝酔脂質蓄積抑制作用

これらにより、ブナシメジを摂取することで、肝臓での脂肪酸合成を抑えると同時に、TG合成を防げ、肝臓脂肪蓄積を抑制させる機序が示唆された。これらの研究結果は、ブナシメジが肝臓の脂質代謝改善に資する優れた食品素材であることを示すものである。

Abbreviations: OA, Orotic acid; SD rats, Sprague-Dawley rats; control, 15% cellulose powder diet; Buna, 15% Bunashimeji powder diet; OA-control, 1% orotic acid-supplemented 15% cellulose powder diet; OA-Buna, 1% orotic acid-supplemented 15% Bunashimeji powder diet; TG, Triacylglycerol; TC, Total cholesterol; PL, Phospholipids; HDL-C, High-density lipoprotein cholesterol; ACC, Acetyl-CoA carboxylase; Malic, malic enzyme; DGAT, Acyl-CoA diacylglycerol acyltransferase; G6PD, Glucose-6-phosphate dehydrogenase.

1. Introduction

It is well known that nonalcoholic fatty liver disease (NAFLD) is a public health problem, especially in developed countries, and has increased at an alarming rate in recent years. NAFLD results from an imbalance between energy intake and energy expenditure. NAFLD generally accompanies serious triacylglycerol (TG) accumulation within the liver cells, and it may cause liver damage, ranging from hepatic steatosis to steatohepatitis, liver fibrosis, and cirrhosis.

Many studies have reported that various species of mushrooms have a wide range of beneficial effects that can prevent lifestyle-related diseases. Among them, Bunashimeji (Hypsizigus marmoreus), a popular edible mushroom in Japan, has been shown to exhibit many biological activities, such as anti-tumor[1-3] and antioxidant[4-5] effects. However, there have been few reports on the effects of Bunashimeji on lipid metabolism.

In fundamental studies, we have shown that the Bunashimeji has slowed weight gain and suppressed hepatic TG accumulation in animals that consume a high-fat diet[6]. However, further detailed studies must be conducted in various animal models. For example, in certain rodents, the intake of a high-fat diet or an OA diet was shown to generally cause obesity. The present study was carried out to investigate whether Bunashimeji is effective in various animal models, excluding those of a high-fat diet.

Orotic acid (OA), a precursor in pyrimidine nucleotide biosynthesis, is found in large quantities in cow’s milk[7,8]. OA is known to induce a specific defect in the secretion of very low density lipoprotein, specifically in rodents[9,10]. Accordingly, this defect results in the formation of fatty liver by the accumulation of TG, and this change is facilitated generally by a high-sucrose diet[11]. The accumulation of lipid droplets in the hepatocytes results in hepatomegaly accompanied by an increase in liver weight. Furthermore, it is reported that enhanced TG synthesis mediated by changes in liver phosphatidate phosphohydrolase (PAP) activity might be involved in the development of a fatty liver induced by OA administration[12].

In the present study, we showed, analyzed, and described a marked suppressive action of Bunashimeji on triacylglycerol accumulation in the livers of the rats administered orotic acid. These findings may provide clues that will lead to further elucidation of the biological mechanisms of edible mushrooms.

2. Material and Methods

2.1. Mushroom powder

Using an automatic air-drying apparatus, Bunashimeji fruit bodies were air-dried for 24 h by maintaining the temperature between 40 and 50°C. Dry powder of Bunashimeji fruit bodies were provided by the Mie Prefecture Forestry Research Institute (Mie, Japan).

2.2. Animals and diets

Three-week-old male Sprague-Dawley (SD) rats weighing 40-60 g were obtained from Japan SLC (Hamamatsu, Japan). They were housed individually in stainless steel wire cages in a room maintained at 22 ± 2°C with a 12-h light-dark cycle (light period: 08:00-20:00) and fed a commercial diet (Type MF, Oriental Yeast Co.). After 7 days of acclimation, the rats were divided into 4 groups with the same mean body weights and were freely fed the fol-

<table>
<thead>
<tr>
<th>Table 1 Compositions of experimental diets (wt %)</th>
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<tr>
<td>Ingredient</td>
</tr>
<tr>
<td>Casein</td>
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<tr>
<td>Corn oil</td>
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<tr>
<td>DL-Methionine</td>
</tr>
<tr>
<td>Vitamin mixture (AIN-76)</td>
</tr>
<tr>
<td>Mineral mixture (AIN-76)</td>
</tr>
<tr>
<td>Choline bitartrate</td>
</tr>
<tr>
<td>Cellulose powder</td>
</tr>
<tr>
<td>Bunashimeji powder</td>
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<tr>
<td>Orotic acid</td>
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<tr>
<td>Sucrose</td>
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</table>

Composition of all ingredients is given in grams per 100 g diet.

AIN-76, formula for purified diets[13].

(21) 21
lowing 4 purified experimental diets: (1) 15% cellulose powder (control), (2) 15% Bunashimeji powder (Buna), (3) 1% orotic acid and 15% cellulose powder (OA-control), and (4) 1% orotic acid and 15% Bunashimeji powder (OA-Buna) (Table 1). In the studies on the nutritional functions of Bunashimeji, we mix it at 10% level in the experimental diets\(^6\). In the present study, however, a higher level, 15%, was used to induce clearly a lower accumulation of hepatic TG. These experimental diets were prepared according to the AIN-76 formulation\(^10\) and contained (in weight, %) casein, 20; fat (corn oil), 10; DL-methionine; 0.3; vitamin mixture (AIN-76\(^{wa}\)), 1; mineral mixture (AIN-76\(^{wa}\)), 4; choline bitartrate, 0.2; cellulose, 15; and sucrose, 49.5. The OA diets were prepared by the supplementation of 1.0% OA to the basal diet at the expense of sucrose. Food intake and body weights were recorded every alternate day.

The animal experiments were conducted in accordance with the guidelines for the Animal Research Committee, Mie University.

### 2.3. Blood sampling and analytical method for lipids

After the feeding period, the rats were anesthetized using diethyl ether and blood was withdrawn from the abdominal aorta. Serum was prepared by centrifugation of blood at 1700 × g for 20 min at 20°C and stored at −20°C until analysis. The concentrations of triacylglycerols (TG), total cholesterol (TC), phospholipids (PL), and HDL-cholesterol (HDL-C) in the serum were determined by commercial analytical kits ("Triglyceride E-test," "Cholesterol E-test," and "Phospholipids C-test," (Wako Pure Chemical Industries, Ltd., Osaka, Japan), [("HDL-C2 Daiichi" (Daichi Pure Chemicals Co., Tokyo, Japan)].

### 2.4. Tissue sampling and hepatic lipid analytical method

After the abdominal withdrawal of blood, the whole liver and perirenal adipose tissue were quickly removed, rinsed in ice-cold saline, blotted on filter paper, and weighed. Hepatic total lipid was extracted by the method of Foch et al\(^6\). Hepatic TG, TC, and PL were determined by using the same commercial analytical kits as those used for the blood lipid analyses.

### 2.5. Fecal sampling and analytical method

Fecal samples were collected 4 times on a per-cage basis for 24 h after 2 weeks of feeding, and stored at −20°C until analysis. These samples were freeze-dried, ground, and then analyzed for bile acids, according to the procedure described by Eaton and Klaassen\(^5\).

### 2.6. Northern blot analysis

The rat acetyl-CoA carboxylase (ACC) probe was a gift from Dr. T. Ide (National Food Research Institute, Japan). cDNA probes of Glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (Malic), and Acyl-CoA: diacylglycerol acyltransferase (DGAT) were synthesized from the rat liver total RNA by RT-PCR with sense primer: 5′-ATGACCGTGGGAGGATGA-3′ and antisense primer: 5′-ACAAACACACTTCCCCGTA-3′ corresponding to the positions 1078-1097 and 2010-2029 of rat G6PD cDNA, sense primer: 5′-GAGGCAGCGTCTTCCAAATA-3′ and antisense primer: 5′-CCTGATTGTTTCTGCGCC-3′ corresponding to the positions 687–706 and 1659–1678 of rat malic cDNA and with sense primer: 5′-GCTGT-GGCCCTTACTGTTGGA-3′ and antisense primer: 5′-ACGTGCTCAACTATGATGCC-3′ corresponding to the positions 603–622 and 1498–1517 of rat DGAT cDNA, respectively. Amplified fragments of G6PD, malic, ACC, and DGAT cDNA were subcloned into pCRTM2.1 plasmid vector (Invitrogen, San Diego, CA) and those were sequenced by the dideoxyribonucleotide chain termination method\(^10\).

Total RNA was extracted using guanidinium-thiocyanate with acid phenol-chloroform, according to the method described by Chomczynski and Sacchi\(^7\). Total RNA (20 μg) from rat liver was separated by electrophoresis in 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (NYTRAN, Schleicher & Schuell, Keene, NH, USA). The membrane was prehybridized with salmon sperm DNA (200 μg/mL) at 42°C for 2 h and then separately hybridized with the radiolabeled rat G6PD, malic enzyme, ACC, and DGAT cDNA probe (TOYOBO Co., Ltd., Osaka, Japan), as an internal control, in 50% formamide, 5 × SSPE (1 × SSPE: 0.18 M NaCl, 10 mM NaH2PO4 and 1 mM EDTA, pH 7.4), and 0.1% SDS at 42°C for 16 h, and followed by sequential washing with 2 × SSC (1 × SSC: 0.15 M NaCl and 15 mM sodium citrate) containing 0.1% SDS at 65°C for 30 min, 1 × SSC containing 0.1% SDS at 65°C for 30 min, and 0.5 × SSC containing 0.1% SDS at 65°C for 30 min. After washing, the membrane was subjected to autoradiography. The amount of the each G6PD, malic enzyme, ACC, and DGAT mRNA hybridized with the corresponding probe was analyzed by a Bio Imaging Analyzer BAS 1000 system (Fuji Photo Co., Tokyo, Japan).

### 2.7. Statistical analyses

All values are expressed as means ± standard error. Statistical comparisons among tasks were performed by an ANOVA with repeated-measures and a Tukey-Kramer multiple comparisons test (Statview ver. 5.0, SAS Institute Inc., USA). \( p < 0.05 \) was considered statistically significant.
3. Results

3.1. Food intake, growth, and liver weight

Growth performance and liver weights are shown in Table 2. Food intake in the group fed Buna was significantly lower than that in the group fed control, but no significant difference was observed in food intake between OA-control and OA-Buna groups. The body weight in rats fed OA-Buna was significantly higher than that of rats fed OA-control after 3 weeks of feeding (p < 0.05). Liver weight was significantly lower in rats fed OA-Buna than in those fed OA-control.

3.2. Serum and hepatic lipid levels

The concentrations of serum lipids are shown in Fig. 1. No statistical differences in TG concentrations were observed among the 4 groups. Serum TC, HDL-C, and PL concentrations were all significantly lower in the rats fed Buna than in the rats fed control; these concentrations were also lower in the rats fed OA-control than in the rats fed OA-Buna. On the other hand, as shown in Fig. 2, in both the OA-free and enriched groups, hepatic TL, TG, and TC contents of the Bunashimeji group were significantly lower than those of the control group. Although, significant differences in Hepatic PL contents were not observed between the rats fed Buna and control, the content were significantly lower in the rats fed OA-Buna than in the rats fed OA-control.

3.3. Messenger RNA levels of liver enzymes

The mRNA abundances of ACC and DGAT of the Buna were significantly lower than those of the control, whereas no significant difference in hepatic mRNA levels of G6PD and malic was observed between the Buna and the control groups.

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**Table 2** Growth parameters and liver weights of rats

<table>
<thead>
<tr>
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<th>control</th>
<th>Buna</th>
<th>OA-control</th>
<th>OA-Buna</th>
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<tr>
<td>Initial B.W. (g)</td>
<td>113.2 ± 2.3</td>
<td>113.0 ± 3.2</td>
<td>113.0 ± 2.3</td>
<td>113.2 ± 2.4</td>
</tr>
<tr>
<td>B.W. gain (g)</td>
<td>104.2 ± 1.7*</td>
<td>96.8 ± 3.2*</td>
<td>79.4 ± 2.3*</td>
<td>103.0 ± 4.1*</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>17.0 ± 0.2*</td>
<td>14.1 ± 0.4*</td>
<td>15.4 ± 0.2*</td>
<td>15.5 ± 0.5*</td>
</tr>
<tr>
<td>Total food intake (g) / B.W. gain (g)</td>
<td>2.46 ± 0.03*</td>
<td>2.19 ± 0.06*</td>
<td>2.92 ± 0.09*</td>
<td>2.26 ± 0.03*</td>
</tr>
<tr>
<td>Liver weight (g/100g B.W.)</td>
<td>4.03 ± 0.14*</td>
<td>3.75 ± 0.06*</td>
<td>7.15 ± 0.40*</td>
<td>5.52 ± 0.08*</td>
</tr>
</tbody>
</table>

**Fig. 1** Concentration of triacylglycerols (TG), total cholesterol (TC), phospholipids (PL) and high density lipoprotein cholesterol (HDL-C) in the serum of SD rats fed with different experimental diets for 3 weeks. Values are mean ± SEM (n = 5). The a, b, and c values without common superscript letters denote significant difference (p < 0.05).
control groups. On the OA-enriched diets, the mRNA abundances of malic, a key enzyme of fatty acid synthesis, of the OA–Buna was significantly lower than those of the OA control. OA–Buna, in addition, tended to lower G6PD and ACC mRNA expression compared to the OA control (Fig. 3).

3. 4. Fecal dry weight and fecal total bile acids

In both the OA-free and enriched groups, fecal dry weights of the Bunashimeji group were significantly lower than that in the control group. Bile acid excretion was significantly higher in the rats fed OA–Buna than in the rats fed OA–control, but no significant difference was observed in bile acid between the control and the Buna (Fig. 4).

4. Discussion

In previous studies, we have demonstrated positive effects of Bunashimeji (Hypsisizus marmoreus) on lipid metabolism however, the detailed mechanisms of these effects have not been defined. The aim of this study was to examine the suppressive action of Bunashimeji on triacylglycerol accumulation in the livers of the rats given orotic acid.

It is known that orotic acid is a common component of milk and that OA administration induces fatty liver Standerfer and Handler first demonstrated that dietary orotic acid caused the accumulation of lipids in the livers of rats. Although the mechanism for this accumulation has not been fully characterized, it is reported that enhanced TG synthesis mediated by changes in liver phosphatidate phosphohydrolase (PAP) activity might be involved in the development of fatty liver induced by OA administration. Furthermore, Serum TG, TC, PL, and HDL-C of the OA-control showed a lower value than the OA–Buna. It has been suggested that hypolipidemia was induced by the impairment of secretion of very low density lipoproteins from the liver with inhibiting microsomal triglyceride transfer protein (MTP) activity. In fact, liver weight and hepatic lipid contents of the rats increased, although the serum lipids concentrations decreased, compared with OA–free group. As a result, serum lipid concentrations and hepatic lipid contents suggest that OA-induced fatty liver is induced by inhibiting MTP activity. Furthermore, in a comparison between the control and the OA–control, the retardation of body-weight gain, liver enlargement, and fat accumulation the liver caused by consumption of an OA diet that have previously been reported were observed.

The final liver weight was lower in the OA–Buna than in the OA–control, although no significant difference in food intake between groups was observed. In contrast, the food intake in the Buna group was significantly lower than that in the control group; however, no significant difference in liver weight between groups was observed.

Kawagishi et al. identified a lectin as a food-intake-suppressing substance in Hiratake (Pleurotus ostreatus).
However, there have been no reports of such a substance in Bunashimeji. Accordingly, it is uncertain that whether such a material may have contributed to the results of the present study.

On the other hand, in both the OA-free groups and enriched groups, hepatic TL and TG contents of the Bunashimeji group were significantly lower than those of the control group.

To confirm the result obtained with hepatic TG level, we analyzed the mRNA levels of various fatty acid synthesis enzymes by northern-blot hybridization using specific cDNA probes.

The mRNA abundances of Malic were significantly increased in rats fed OA. G6PD also tended to be enhanced by supplementation with OA. However, feeding of Bunashimeji eliminated these OA-induced increases. In the OA-free group, the mRNA abundances of ACC and DGAT of the Buna were significantly lower than those of the control.

In the present study, dietary Bunashimeji counteracted both the hepatomegaly and hepatic steatosis that occurred in the control and the OA-control rats. In the OA-free group, the alleviation of hepatic steatosis in Bunashimeji-fed rats may be due in part to the suppression of ACC mRNA expression, a key enzyme of fatty acid synthesis, and DGAT mRNA expression, an important factor for lipoprotein secretion, in the liver. On the other hand, in the OA-enriched group, the alleviation of hepatic steatosis in Bunashimeji-fed rats may be due in part to the suppression of Malic mRNA expression and tendency to suppression of G6PD and ACC mRNA expression, key enzymes of fatty acid synthesis, in the liver.

In this study, we demonstrated that a lipid metabolism pathway of Bunashimeji by a meal showed a significant difference. These results indicated that the regulation and accumulation of hepatic lipid synthesis, mainly triacylglycerols, strongly suggests that Bunashimeji alleviated the onset of fatty liver, and also suppressed the onset of OA-induced fatty liver.

In addition, in OA-free group, serum lipid decreased significantly in the Bunasimeji group. It is well known that dietary fiber obstructs absorption of cholesterol and bile acid in the small intestine. However, bile acid excretion in the Bunashimeji group was shown to be lower. Thus, in this context, it is possible, as was concluded in a report by Fushima et al., to assume that mushroom fiber increased LDL receptor mRNA expression in rats.

Our study provides evidence that Bunashimeji, a popular edible mushroom in Japan, is effective for lipid metabolism improvement.

Acknowledgment

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References

1) Matsuzawa T, Sano M, Tomita I, Saitoh H, Ikekawa T. Stud-


