Type 2 diabetes mellitus is characterized by a chronic hyperglycemic state due to decreased insulin sensitivity in target tissues, including skeletal muscle, adipocytes and the liver, and/or impairment of insulin secretion (1, 2). Obesity is a global pandemic and, as a pathological state, is responsible for type 2 diabetes mellitus, hyperlipidemia and hypertension (3). Obesity exacerbates hyperglycemia through peripheral insulin resistance, which is induced by fat accumulation in visceral adipose tissue, the liver and skeletal muscles (4). These adipose tissues or adipose tissue-infiltrating macrophages, are known to secrete cytokines, such as tumor necrosis factor α and monocyte chemoattractant protein 1, as compared with vehicle treatment. The expressions of uncoupling protein 3 and peroxisome proliferator-activated receptor gamma coactivator 1α in the soleus muscles of EHM treatment groups were significantly elevated as compared to those in vehicle-treated muscle tissues. These results raise the possibility that EHM can regulate both obesity and insulin resistance.

**Key Words**  Hypyszigus marmoreus, fat deposition, pro-inflammatory adipokines

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Influence of Treatment with Extracts of *Hypsyzigus marmoreus* Mushroom on Body Composition during Obesity Development in KK-A’ Mice

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**Summary**  Hypyszigus marmoreus (HM), an edible mushroom, has several effects, including antitumor, antioxidant and anti-allergy properties. On the other hand, the possibly useful effect of HM in diabetic mice has not as yet been elucidated. In this study, we showed treatment with a water soluble extract from HM (EHM) to reduce fat deposits without affecting body weight loss in KK-A’ mice. EHM treatment also abolished the expressions of pro-inflammatory adipokines, such as tumor necrosis factor α and monocyte chemoattractant protein 1, as compared with vehicle treatment. The expressions of uncoupling protein 3 and peroxisome proliferator-activated receptor gamma coactivator 1α in the soleus muscles of EHM treatment groups were significantly elevated as compared to those in vehicle-treated muscle tissues. These results raise the possibility that EHM can regulate both obesity and insulin resistance.

**Materials and Methods**

**Reagents.** Glucose and insulin were purchased from Wako Pure Chemical Industries, Ltd.(Osaka, Japan). All other chemicals were of analytical grade.

**Preparation of water soluble extract from HM.** At the Agricultural Technology Institute of the Nagano Farmers’ Federation, 5 kg of the HM fruiting body were added to 15 L of deionized water followed by boiling for 3 h. The extracted solution was filtered through a 100 mesh size sieve and then concentrated with boiling and lyophilized at –80°C. Finally, 148.7 g of extracts, consisting of about 2.97% of the desired product was acquired. Extracts were resolved individually with phosphate buffered saline (PBS) and used for in vivo animal experiments. The chemical composition of HM (moisture: 5.2 g, crude protein: 21.0 g, lipid: 0.2 g, sugar: 27.1 g, ash: 20.3 g, nitrogen free extract: 26.2 g) per 100 g before water extraction was determined according to the AOAC method (14).

**Animals.** Five-week-old KK-A’ mice (n=16) (Tokyo Laboratory Animal Science, Tokyo, Japan) were randomly assigned to the water soluble extract from HM...
(EHM) treatment group (n=8) or to the PBS control treatment group (n=8). Mice were maintained under specific pathogen-free conditions with a 12-h light:dark cycle at 25±2°C and 55±10% relative humidity. The mice were given a normal chow diet (CE-2, CLEA Japan, Inc., Tokyo, Japan) and water ad libitum. Mice were orally given 10 mg per 20 g body weight EHM resolved with 100 µL of PBS solution or 100 µL of PBS per 20 g body weight as a control at 10:00 AM once per day for 12 wk. Food intakes and body weights were measured and blood samples were collected weekly after an overnight fast from the tip of the tail vein at 10:00 AM. Food intakes and body weights were measured by cardiac puncture using heparinized syringes and to later by cervical dislocation to collect blood samples. Blood samples were immediately centrifuged to collect the serum supernatant. Serum samples were stored at −80°C until needed for measurement of metabolic parameters. Mice fasted overnight were sacrificed 12 wk later by cervical dislocation to collect blood samples by cardiac puncture using heparinized syringes and to obtain tissue samples of the liver, soleus muscle and epididymal fat. The tissues were immediately frozen in liquid nitrogen and stored at −80°C until they were used for RNA preparation. All studies were performed in accordance with the ethical guidelines for animal experimentation of Saitama Medical University and were approved by the institutional review board of the animal ethics committee.

Biological analyses of plasma. Blood sugar was measured with a Glutest Neo Super device (Sanwakagaku-kenkyusho, Tokyo, Japan) by the FAD-glucose dehydrogenase method. Plasma high density lipoprotein (HDL), triglyceride and total cholesterol levels were determined by enzymatic methods using commercial assay kits (HDL E-Test, Triglyceride E-Test, Cholesterol E-Test: Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer’s protocols. Plasma insulin levels were measured by immunoassays using ELISA kits (Shibayagi, Shibukawa, Japan) according to the manufacturer’s protocols. In all analyses, the average of three measurements for each mouse served as the data.

Quantitative real-time RT-PCR. Total RNA was extracted from the soleus muscle and epididymal fat using TRizol Reagent (Invitrogen, Carlsbad, CA) or RNesy Mini Kit (Qiagen, Waltham, MA). Then, total RNAs were removed to determine the genome DNA by Recombinant DNase I (TaKaRa, Kyoto, Japan) and were then reverse-transcribed using a TaKaRa PrimeScript RT reagent kit. Quantitative real-time PCR was performed with the LightCycler system (Roche Diagnostics, Basel, Switzerland) using TaKaRa SYBR Premix Taq II (TaKaRa). The following gene specific-primers were used: MCP-1 (sense: 5′-TCT GGA CCC ATT CCT TCT TG-3′, antisense: 5′-CCC AAT GAG TAG GCT GGA GA-3′), PGC-1α (sense: 5′-CGG TGT CGT TAG TGG CTT GA-3′, antisense: 5′-ATG TGT CGC CTT CTT GCT CT-3′), TNFα (sense: 5′-TGA GAG GGA GCC CAT T-3′, antisense: 5′-AGC TGG AAC TGG CAG AAG AG-3′), 36b4 (sense: 5′-TCT CCA GAG CTG GTT TGT TTC R-3′, antisense: 5′-CTT CAT TGT GGG AGC AGA CA-3′), GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (sense: 5′-GAC CAC AGT CCA TGC CAT CAC-3′, antisense: 5′-CTT CAT TGT GGG AGC AGA CA-3′), GPDH (glyceraldehyde 3-phosphate dehydrogenase) (sense: 5′-GAG TAC CAC TGA TGC CAT CAC-3′, antisense: 5′-CCA CCC TGT TGC TGT AG-3′), UCP3 (sense: 5′-CGG TGG ATG TGG TAA AGA CC-3′, antisense: 5′-AAA GGA GGG CAC AAA TTC TT-3′). After the PCR reaction, each PCR product was confirmed for its single amplification by analyzing the melting curves of the PCR products.

Statistical analysis. Data are expressed as means±SE. Data were analyzed employing the unpaired Student’s t-test. A p-value <0.05 was accepted as statistically significant.
Results

As shown in Fig. 1A, body weights in the EHM and control groups during the 12 wk of treatment did not differ significantly. While total food intake in the EHM group (88.0 ± 5.4 g) appeared to be higher than that in the control group (83.2 ± 3.3 g), the difference did not reach statistical significance (Fig. 1B). The levels of fasting plasma insulin (p=0.061, Table 1) in the EHM group tended to be lower, compared to that in the control group after 12 wk of treatment, but fasting plasma glucose, the plasma glucose level 2 h after intra-peritoneal glucose tolerance test (IPGTT) (p=0.054) after 11 wk of treatment (the EHM group: 149 ± 25 mg/dL, the control group: 239 ± 38 mg/dL), and the area under curve (AUC) of glucose of IPGTT did not differ markedly between the two groups (data not shown).

On the other hand, the mRNA expressions of the pro-inflammatory adipokines TNFα and MCP-1 in the EHM group were lower, compared to those in the control group (Fig. 3A). To evaluate the lower fat composition in the EHM group, we assessed the mRNA expressions of genes related to nutritional metabolism and energy expenditure in the liver, soleus muscle and epididymal fat. As shown in Fig. 3B, we found that the mRNA expressions of uncoupling protein 3 (UCP3) and peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) in the soleus muscle were both significantly elevated in the EHM group as compared to those in the control group.

Discussion

Only 5 mg of EHM obtained with ethanol was used on each mouse to investigate the effects on pro-inflammatory cytokines (12). Considering the results of the ethanol extraction experiment, we instead used 10 mg and 1 mg of EHM/20 g of body weight per mouse for once-daily treatment to determine the effects of EHM on...
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KK-\(\text{A}^\text{V}\) mice. The characteristics of the 1 mg treatment group were very similar to those of the PBS treatment group serving as a control (group control). Thus, we analyzed and present herein the data from the 10 mg EHM treatment group (EHM group).

In this study, we showed for the first time that EHM treatment prevents fat deposition in KK-\(\text{A}^\text{V}\) diabetic mice (Fig. 2). We speculate that increased expression of PGC-1\(\alpha\) is involved in mitochondrial biosynthesis (15) and that the subsequent increase in UCP3 expression raised energy expenditure via thermogenesis (16) in the soleus muscle. Such effects could explain the lower the fat accumulation in the EHM group. A slight increase in total food intake resulted in similar weight gains in the two groups, but the fat deposits in the EHM group were still lower. We speculate that body weight in the EHM group would be lower than that in the control group had the mice been treated for a longer time. To test these speculations, we will in future conduct further studies under paired-feeding conditions with examination of energy expenditure. However, an anti-proliferative effect of EHM on adipocytes, possible components of which include hypsiziprenol, hypsin, or polysaccharides as demonstrated by previous studies in vivo only (7–10, 17) cannot be ruled out. Moreover, dietary polyphenols, such as resveratrol, were reported to increase the expression of the UCP or other obesity-related genes, including those responsible for \(\beta\)-oxidation and lipolysis, whose expressions were unchanged in both groups (data not shown) (18), and thereby to prevent obesity. We are now investigating these components and others exerting effects similar to those observed in this experiment.

The lower levels in TNF\(\alpha\) and MCP-1 mRNA expression in the EHM group might be a consequence of diminished fat deposits. Recently, treatment of BALB/c mice with ethanol extracts of HM was shown to lower the TNF\(\alpha\) or interleukin 6 expression, much as in our present study, but there were no changes in body weight (12). Furthermore, the components of the ethanol extract of HM were also not identified in this study.

Pro-inflammatory adipokines such as TNF\(\alpha\) or and MCP-1 are increased in the insulin-resistant state (5). Moreover, the mRNA expression of adiponectin in epididymal fat did not differ between the two groups used in this study (data not shown). The amelioration of adipocyte inflammation might be explained in part by the phenotype of small adipocytes showing elevated adiponectin expression concomitantly with decreased TNF\(\alpha\) or and MCP-1 expression (19). In this experiment, adipocyte sizes were similar in the two groups, as demonstrated by hematoxylin and eosin staining (data not shown). Thus, we speculate that EHM treatment might improve insulin resistance in KK-\(\text{A}^\text{V}\) mice. The tendency for levels of fasting plasma insulin and plasma glucose 2 h after IPGTT to be lower may support our contention that EHM improves these parameters. Water soluble fibers lower the absorption of sterols in the intestine, thereby decreasing the serum cholesterol levels (20). Thus, fiber exerts its effect mainly because it comprises a large percentage of the chow. Once-daily treatment with no more than 200 \(\mu\)L of the EHM solution might not influence the sterol absorption in mice fed chow ad libitum. Thus, as yet, we cannot explain the worsening of serum cholesterol observed in the EHM group.

Taken together, our results indicate that the components of EHM may have beneficial effects on obesity and insulin resistance. Further studies are underway to elucidate the mechanisms underlying these beneficial metabolic effects.

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REFERENCES


