Hypolipidemic Effect of Pleurotus eryngii Extract in Fat-Loaded Mice

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Summary Pleurotus eryngii water extract (PEE), which showed the most significant inhibitory activity against pancreatic lipase in vitro among eight edible mushrooms, was investigated to determine the mechanism of its anti-lipase activity in vitro and its hypolipidemic effect in fat-loaded mice. The inhibitory effects of mushroom extracts on pancreatic lipase activity were examined using 4-methylumbelliferyl oleate (4-MUO) or trioleoylglycerol emulsified with lecithin, gum arabic or Triton X-100 as a substrate. For in vivo experiments, blood samples were taken after oral administration of corn oil and [3H]trioleoylglycerol with or without PEE to food-deprived mice. PEE inhibited hydrolysis of 4-MUO and trioleoylglycerol emulsified with lecithin or Triton X-100, but not that of trioleoylglycerol emulsified with gum arabic. PEE suppressed the elevations of plasma and chylomicron triacylglycerol levels after oral administration of corn oil, but had no effect on lipoprotein lipase activity. [3H]Trioleoylglycerol absorption was also decreased by administration of PEE. The results of in vitro studies suggest that PEE may prevent interactions between lipid emulsions and pancreatic lipase. The hypolipidemic effect of PEE in fat-loaded mice may be due to low absorption of fat caused by the inhibition of pancreatic lipase.

Key Words lipoprotein lipase, pancreatic lipase, Pleurotus eryngii, triacylglycerol

Obesity is a one of the main worldwide health problems in modern industrialised countries, and has been implicated in the development of various diseases, such as hyperlipidemia, diabetes and atherosclerosis. The etiology of obesity is complicated and involves the interaction of numerous environmental and genetic factors. However, obesity is essentially the consequence of disequilibrium between energy intake and expenditure. In particular, excessive consumption of dietary fat plays a major contributory role in the development of obesity (1, 2). Therefore, one effective way of managing obesity is to inhibit fat absorption from the small intestine.

Pancreatic lipase is a key enzyme for the digestion of dietary triacylglycerol (TG). It is well known that dietary TG is not absorbed directly from the small intestine unless it has been hydrolysed to fatty acids and 2-monoacylglycerol by pancreatic lipase in the lumen of the proximal small intestine. Therefore, inhibition of pancreatic lipase would be an effective means of reducing fat absorption. For example, orlistat, a hydrogenated derivative of lipstatin derived from Streptomyces toxytricini, is a potent pancreatic lipase inhibitor and is used clinically to prevent obesity and hyperlipidemia (3–6).

Edible mushrooms have been used as a folk medicine for centuries in many countries. In fact, edible mushrooms are a rich source of rare minerals, amino acids and trace elements, which are involved in many biochemical processes supporting life. It has also been reported that some edible mushrooms have a wide range of pharmaceutical properties, including anti-tumour (7), hypotensive (8), hypoglycemic (9) and hypcholesterolemic (10) activities. However, little is known about their hypolipidemic activities. In this study, we screened the inhibitory activities of eight edible mushroom extracts on pancreatic lipase in vitro and examined the in vivo effects of Pleurotus eryngii water extract, which showed significant inhibitory activity.

MATERIALS AND METHODS

Reagents. Porcine pancreatic lipase, 4-methylumbelliferyl oleate (4-MUO) and lipoprotein lipase from bovine milk were from Sigma (St. Louis, MO). Trioleoylglycerol and Triglyceride E TestTM were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chol/Trig COMBO TGTM was from Helena Laboratories Japan Co. (Saitama, Japan). Glycerol tri[9, 10-3H(n)] oleate was from PerkinElmer Japan Co. (Yokohama, Japan).

Animals. Seven-week-old male ICR mice (mean body weight: 30 g) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The mice were kept for 1 wk before the experiments under a 12-h/12-h light/dark cycle in a temperature- and humidity-controlled room. The mice were given free access to food and water. The care and treatment of the mice conformed to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions in Japan.

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Preparation of mushroom extracts. The fruiting bodies of *Agaricus blazei*, *Grifola frondosa*, *Hericium erinaceus*, *Hypsizygus marmoreus*, *Lyophyllum shimeji*, *Pleurotus eryngii* and *Sparrasus crispa* were obtained from Hokuto Co. (Nagano, Japan). The fruiting bodies were powdered after drying overnight at 60°C and extracted in water or methanol for 4 h, and the solutions were freeze-dried. The extracts were recovered in water and used for experiments in vitro and in vivo. The 30% weight of dried *Pleurotus eryngii* was solubilized in water. *Pleurotus eryngii* water extract (PEE) solution was fractionated into different molecular weight (MW) ranges. >300 kDa, 30–300 kDa, 3–30 kDa and <3 kDa, using Nanosep Centrifugal Devices™ (Pall Life Sciences, East Hills, NY).

Pancreatic lipase activity assay. Pancreatic lipase activity was measured by a fluorometric assay using 4-MUO as a substrate. Aliquots of 250 μL of extract solution and 500 μL of 0.1 mM 4-MUO solution in 13 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and 1.3 mM CaCl₂ were mixed, and an aliquot of 250 μL of the lipase solution (150 μg/mL of pancreatic lipase) in the above buffer was then added to start the reaction. After incubation for 30 min at 25°C, 1 mL of 0.1 M citrate buffer (pH 4.2) was added to stop the reaction. The amount of 4-methylumbelliferone released by the action of the lipase was measured with a fluorescence spectrophotometer (Hitachi 850, Hitachi Co., Tokyo, Japan) at excitation and emission wavelengths of 355 and 460 nm, respectively. Pancreatic lipase activity was also determined by measuring the oleic acid released from trioleylglycerol. Briefly, a suspension of trioleylglycerol (80 mg), lecithin (10 mg) and taurocholic acid (0.4 mg/mL of trioleoylglycerol, 20 mg/mL fatty acid-free albumin from bovine serum, 0.1% Triton X-100 and 20% foetal bovine serum in 0.1 M Tris-HCl (pH 8.6) was prepared by sonication. Post-heparin plasma was incubated with an equal volume of 25 mM sodium dodecyl sulphate in 0.2 M Tris-HCl (pH 8.2) at 26°C for 60 min to inhibit hepatic lipase activity (14). Pre-treated post-heparin plasma was incubated with 0.2 mL of the substrate solution for 30 min at 37°C. The reaction was stopped by addition of 3.25 mL of chloroform/methanol/heptane (1 : 1.28 : 1.37, v/v/v) and 1 mL of 0.1 M K₂CO₃/H₂BO₃ (pH 10.5). The mixture was vortexed vigorously for 15 s and centrifuged at 3,000 rpm for 20 min. The radioactivity in 1 mL of the supernatant was counted in 9 mL of ACS II. LPL activity was calculated from standard curve of LPL from bovine milk.

[^3H]triolein absorption assay. Corn oil (2.5 mL/kg) containing 40 μCi of[^3H]trioleoylglycerol was administered to mice and plasma was obtained in the same way as described above. Plasma lipids were extracted by the method of Folch et al. (15). Extracted lipids were separated by thin-layer chromatography using silica gel layers (Merck, Darmstadt, Germany) with hexane/diethyl ether/acetic acid (80 : 20 : 1, v/v/v). To identify the chromatographic mobility of triglyceride and free fatty acid, standard triglyceride and free fatty acid were chromatographed simultaneously. The radioactivity was counted in 5 mL of ACS II.

Statistical analysis. The area under the curve (ΔAUIC) was calculated by the trapezoidal method. Data are expressed as means±SD. Statistical analyses were performed with Student’s t-test or factorial two way analysis of variance. Thereafter, we conducted Scheffe’s multiple comparison. Differences between means were considered significant at probability values less than 0.05.

RESULTS

Screening for the inhibitory activity on pancreatic lipase

Seven species and one variant of mushroom were screened for inhibitory activities of the extracts (100 μg/mL) on pancreatic lipase activity; PEE showed the greatest inhibitory activity (Table 1). Then, we examined the dose dependency of the effects of PEE. The result indicated that PEE inhibited pancreatic lipase at concentrations of 50–300 μg/mL in a dose-dependent manner (Fig. 1A). We further found that the high molecular weight fraction of PEE had the activity, as only the highest molecular weight fraction of PEE (MW >300 kDa) had an inhibitory activity (Fig. 1B).
Effects of heating on the inhibitory activity

To examine the thermal stability of the effective component of PEE since edible mushrooms are usually cooked with heat, the PEE solution was boiled for 5, 15, 30, 60, 90 or 120 min before measuring the pancreatic lipase activity. As shown in Fig. 2, heat treatment did not decrease the inhibitory activity of PEE until 60 min. However, further heat treatment resulted in a slight decrease in the inhibitory activity.

Effects of emulsion type on the inhibitory activity

Typical substrates for pancreatic lipase in the human body are long-chain triacylglycerols, which can be separated from the aqueous medium. However, pancreatic lipase is a water-soluble protein. Thus, for catalysis to occur, pancreatic lipase must bind to the lipid surface. Therefore, both the nature of the lipid surface and the structure of the lipase itself are important factors for lipase activity and PEE may affect their interaction. To elucidate the mechanism of lipase inhibition, the inhibitory activity of PEE on the lipase activity was measured using triolein as a substrate in different emulsifiers. Lecithin, Triton X-100 or gum arabic was used as an emulsifier to change the nature of the lipid emulsion. The results indicated that the inhibitory activity of PEE on hydrolysis of trioleoylglycerol was the strongest when trioleoylglycerol was emulsified with lecithin. PEE also inhibited hydrolysis of triolein when emulsified with Triton-X 100. On the other hand, when triolein was emulsified with gum arabic, PEE did not inhibit it (Fig. 3).

Table 1. Effects of extracts of 8 mushrooms on pancreatic lipase activity.

| Scientific name               | Lipase activity (%) |
|------------------------------|---------------------|
|                              | Water extract       | Methanol extract |
| Agaricus blazei              | 104.5               | 71.8             |
| Grifola frondosa             | 113.3               | 76.5             |
| Hericium erinaceus           | 95.9                | 68.4             |
| Hypsizygus marmoreus (brown type) | 105.8             | 110.8            |
| Hypsizygus marmoreus (white type) | 108.0             | 107.2            |
| Lepiophyllum shimeji         | 113.8               | 96.6             |
| Pleurotus eryngii            | 20.8                | 89.6             |
| Sparassus crispa             | 88.8                | 65.4             |

Pancreatic lipase activities were measured using 4-MUO as a substrate. Inhibitory effects are shown as the relative activity (%) against the control (0 μg/mL extract). The concentration of extracts used in the assay was 100 μg/mL.

Fig. 1. Inhibitory effect of Pleurotus eryngii water extract (PEE) on pancreatic lipase activity. (A) The pancreatic lipase activities of different concentrations of PEE were examined by fluorometric assay using 4-methylumbelliferyl oleate (4-MUO). (B) PEE (300 μg/mL) was fractionated into different molecular weight ranges by ultrafiltration and the fractions were diluted with the same volume of water to have equivalent amounts of the effective component to the crude PEE. The pancreatic lipase activities are shown as the relative activity (%) against the control (0 μg/mL PEE). The results are expressed as means ± SD of three independent experiments.

Fig. 2. Effects of heat-treated PEE on pancreatic lipase activity. The lipase activities were measured in an assay system using 4-MUO as a substrate, and the concentration of PEE used was 100 μg/mL. The results are expressed as means ± SD of three independent experiments.

Fig. 3. Effects of emulsion type of substrate on the inhibitory activity of PEE. Triolein was used as a substrate, and emulsified with lecithin (open circles), Triton X-100 (closed circles) or gum arabic (open squares). The lipase activities were determined by measuring the oleic acid released and the lipase activities are shown as the relative activity (%) against the control (0 μg/mL PEE). The results are expressed as means ± SD of three independent experiments.
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To clarify the effects of PEE in vivo, corn oil with or without PEE was administered orally to mice, and the serial changes in plasma TG levels were measured. Plasma TG levels were elevated after administration of corn oil, reached the maximum, and recovered to the original level at 6 h. When corn oil was added with PEE, plasma TG levels increased gradually and the maximum was much lower than that without PEE. There was a significant difference between experimental and control groups at 1.5 h by Student’s t test (*p* < 0.05).

**Effects of PEE on plasma LPL activity**

LPL catalyses the hydrolysis of plasma triacylglycerols (16). Therefore, it was possible that the observed hypolipidemic effect of PEE was due to activation of LPL. To examine this possibility, post-heparin plasma LPL activities were measured. No significant differences were observed in the LPL activities between mice administered corn oil with PEE and control mice (*n* = 6) (Fig. 6).

**Effects of PEE on lipid absorption assessed by radiolabeled trioleoylglycerol**

To assess the direct absorption of lipids, [3H]tri oleoylglycerol was administered orally to mice with or without PEE. ΔAUC of radiolabeled TG, radiolabeled FFA, and radiolabeled FFA + TG with PEE were lower at all the time points when compared to those without PEE, and the mean amount of radiolabeled FFA + TG in...
PEE group at 6 h were 42.0% lower than that in the control group (Fig. 7). There were significant differences between ΔAUC levels of radiolabeled TG, radiolabeled FFA, and radiolabeled FFA+TG in PEE group and those in the control group (n=8).

**DISCUSSION**

In the present study, we screened extracts of 8 edible mushrooms for inhibitory effects on pancreatic lipase. Our results indicated that PEE had significant inhibitory activity against pancreatic lipase in vitro and its inhibitory activity was hardly inactivated by heat treatment with boiling water in the assay system using 4-MUO as a substrate (Fig. 1). When the lipase activity was measured using trioleoylglycerol as a substrate, PEE was also found to inhibit hydrolysis of trioleoylglycerol emulsified with lecithin or Triton X-100. However, PEE did not inhibit hydrolysis of trioleoylglycerol emulsified with gum arabic (Fig. 3). The lipase inhibition of orlistat is caused by binding to the active site in the pancreatic lipase molecule (17), and is unaffected by substrate emulsifiers (18). On the other hand, the inhibition of other lipase inhibitors, such as chitin-chitosan and ε-polysylsine, is specific to the substrate emulsifier, and ε-polysylsine binds to the emulsified substrate (18, 19). Therefore, the results of the present study suggest that the inhibitory mechanism of PEE differs from that of orlistat and PEE prevents the interaction between lipid emulsion and lipase, similar to chitin-chitosan.

To examine the active component of PEE, we fractionated the PEE according to molecular weight by ultrafiltration, and found that the high molecular weight fraction (>300 kDa) had the inhibitory activity. Chitin-chitosan and citrus pectin are both high molecular weight lipase inhibitors (19, 20). However, both of these inhibitors are poorly soluble in water. Therefore, the active component of PEE may be a novel lipase inhibitor, although the details were not determined in the present study.

As pancreatic lipase inhibitors prevent postprandial hyperlipidemia (20–23), PEE may also have prevented postprandial hyperlipidemia through low intestinal absorption of dietary fat by inhibiting pancreatic lipase. To examine this possibility, we determined the plasma TG levels after oral administration of corn oil with or without PEE to mice. PEE inhibited the elevation of plasma TG levels (Fig. 4). This hypolipidemic effect was likely due to a decrease in intestinal absorption. Intestinal absorption is potentially inhibited at the stage of post-digestion of lipids or pre-digestion. Although the presence of post-enzymatic inhibition was not examined in the present study, pre-enzymatic inhibition would be the major cause of inhibition of absorption taking into consideration the distinct inhibition of pancreatic lipase. Furthermore, other factors after intestinal absorption may affect the blood TG level. Absorbed fatty acids and monoacylglycerols from the small intestine are known to be re-esterified to triacylglycerols in intestinal epithelial cells. Triacylglycerols are conjugated with cholesteryl esters, unesterified cholesterol, phospholipids and proteins to form chylomicrons, which are then secreted and transported via the lymphatic system to the blood. Chylomicron triacylglycerols are hydrolysed by LPL in the blood vessels. It was necessary to clarify that PEE did not act on LPL activity in the blood. Therefore, we measured chylomicron TG levels (Fig. 5) and post-heparin plasma LPL activities to determine whether the suppression of plasma TG level elevation was due to inhibition of fat absorption from the small intestine or activation of LPL. PEE did not affect post-heparin plasma LPL activities (Fig. 6). Furthermore, we measured the rate of direct lipids absorption by using [3H]trioleoylglycerol, and found that PEE decreased the rate significantly (Fig. 7). These results suggest that the hypolipidemic effect of PEE is due to inhibition of fat absorption from the small intestine.

It was reported that postprandial hypertriglyceridermia causes endothelial dysfunction and may lead to the development of atherosclerosis (24, 25). Furthermore, hypertriglyceridermia is known to be a risk factor of acute pancreatitis (26, 27). Thus, PEE may protect against diet-induced hypertriglyceridermia and may prevent these diseases in addition to obesity.

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