

In vitro and in vivo antidiabetic effects of the ethanol extract from *Lentinula edodes* (Shiitake)

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Abstract

We examined the expression patterns of glucose transporters (GLUTs) in Caco-2 human colon cancer cells that spontaneously or chemically differentiated into intestinal epithelial cells. RT-PCR analysis demonstrated that the expression of intestinal glucose transporters such as GLUT-1 and -2 was increased by both types of differentiation. We evaluated the inhibitory effects of extracts from *Lentinula edodes* on glucose transport in intestinal Caco-2 cells induced to differentiate by 5 mM sodium butyrate. The ethanol extract of *L. edodes* (LEE) at 0.1 mg/ml markedly inhibited glucose intake through the monolayer of differentiated Caco-2 cells; however, neither water nor the 50% (v/v) ethanol extract affected glucose transport through these cells at the same concentration. We also investigated the effects of LEE on plasma glucose levels in mice with hyperglycaemia induced by feeding with a high fat diet (HFD). The results obtained showed that LEE reduced plasma glucose levels by 64.0% in HFD-fed mice.

Introduction

Hyperglycaemia is a sign of diabetes and caused by a number of factors including abnormal sugar intake, decreases in plasma insulin levels, and insulin resistance, and, thus, antidiabetic agents have been classified into different types [1]. One category of ameliorating agents for hyperglycaemia consists of inhibitors of glucose transporters (GLUTs) in intestinal and proximal tubule cells [2–4]. Caco-2 human colon cancer cells were previously shown to spontaneously or chemically differentiate into mature intestine-like cells [5, 6]. Differentiated Caco-2 cells have been used as models for the intestinal transport of sugars, amino acids and other nutrients [7–9], and screening for GLUT inhibitory activity in food components, particularly some flavonoids, has been conducted using these cells [10–12].

We previously determined the lipoprotein profiles of chemically differentiated Caco-2 cells, and developed a system to evaluate antidyslipidaemic activity [13]. However, it currently remains unclear whether other differentiation indexes in Caco-2 cells, such as sugar transport, are similar in spontaneously and chemically differentiated cells. Therefore, we briefly investigated the gene expression levels of major intestinal glucose transporters in chemically and spontaneously differentiated Caco-2 cells.

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In Japan, the fruiting bodies of *Lentinula edodes* are the most common edible mushroom. A number of biological activities of this mushroom including its anticancer, antioxidant and immune-enhancing effects, have already been studied [14–16]. Furthermore, some antimetabolic syndrome effects, particularly its antidiabetic activity, have been demonstrated [17]; however, inhibition of GLUT by *L. edodes* has not yet been investigated in detail. In the present study, we evaluated the inhibitory activity of *L. edodes* on glucose transport in intestinal Caco-2 cells, and found that the ethanol extract of *L. edodes* (LEE) exhibited marked activity. We also showed that LEE improved hyperglycaemia in metabolic syndrome model mice.

Materials and methods

L. edodes extracts

Commercially available fruiting bodies of *L. edodes* were used after lyophilization. Freeze-dried mushrooms (1 g) were extracted in 50 ml of distilled water, 50% (v/v) ethanol or ethanol, and the extracts collected by centrifugation. The water extract was dried by lyophilization, and the 50% ethanol and ethanol extracts by evaporation. The procedure yielded 213.3 mg of extract from the water, 217.3 mg from the 50% ethanol, and 46.6 mg from the ethanol.

Induction of Caco-2 cell differentiation in transwell chambers

Caco-2 cells were seeded at a density of 4.0×10^5 cells per well on a 12-well transwell chamber (12 mm diameter and 0.4 μm pore size; Greiner Bio-One, Kremsmünster, Austria) precoated with collagen, and were attached to the membrane by culturing for 12 h in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin–100 mg/ml streptomycin. In the Caco-2 cell preculture in the transwell chamber, the day when the cells reached a confluent monolayer was considered day 0. Spontaneous differentiation of Caco-2 cells on membranes was induced by consecutive incubation by day 21, while chemical induction was achieved by treatment with 5 mM sodium butyrate for the first 4 days.

Effects of extracts from *L. edodes* on glucose transport in Caco-2 cells

Caco-2 cells induced to differentiate by sodium butyrate in the transwell chamber were incubated in DMEM (1 ml, apical side) for 6 h with or without the three extracts of *L. edodes* at 0.1 mg/ml, and the secretion of glucose on the basolateral side (PBS, 0.5 ml) was determined using high-performance anion-exchange chromatography (HPAEC).

Determination of glucose concentrations on the basolateral side

HPAEC was performed using a Dionex DX-500 system (Thermo Fisher Scientific, Waltham, MA, USA) with a pulsed amperometric detector. A CarboPac PA-1 column (4 mm i.d. \times 250 mm) with a PA-1 guard column was used. D-Glucose was analyzed using the following gradient elution conditions: 0.1 M NaOH (eluent A) and 0.1 M NaOH containing 0.5 M sodium acetate (eluent B) at a flow rate of 1 ml/min; 0–1 min, 100% eluent A; 1–25 min, a linear gradient of eluent B from 0% to 5% (v/v); and 25–30 min, a linear gradient of eluent B from 5% to 100%.

RNA extraction and cDNA synthesis

Total RNA was isolated from Caco-2 cells using a QuickGene RNA cultured cell kit S (Fujifilm, Tokyo, Japan). Template cDNA synthesis was performed with 5 μg of total RNA using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan).

Real-time RT-PCR

In a fluorescent temperature cycler (Chromo4; Bio-Rad Laboratories, Hercules, CA, USA), 2.5% of each RT reaction solution was amplified in 25 μl of $1 \times$ SYBR Premix Ex Taq II (Takara Bio) containing 0.2 μM of each primer. Samples were incubated in the thermal cycler for an initial denaturation at 95°C for 10 s, followed by 40 PCR cycles. Each cycle consisted of 95°C for 5 s and 60°C for 30 s. The oligonucleotide primers used in the experiment are indicated in Table 1. In order to confirm the amplification of specific transcripts, melting curve profiles (cooling the sample to 60°C and heating slowly to 95°C with continuous measurement of fluorescence) were produced at the end of each PCR cycle. The relative expression levels

Gene name	Relative intensity/GAPDH (%)			
	Day 0	Day 4	Butyrate day 4	Day 21
IAP	100.0±2.9	75.0±0.4	800.1±53.8	421.4±26.5
GLUT-1	100.0±4.9	77.1±1.6	313.4±25.0	208.3±17.5
GLUT-2	100.0±6.3	191.2±1.5	468.2±80.7	348.8±44.7
GLUT-5	100.0±4.8	361.2±17.0	1167.5±31.7	1638.8±34.8
SGLT-1	100.0±6.9	94.2±7.9	4343.9±85.9	2342.8±98.7

Values represent means±S.E.M. (n=6)

Table 1 - Changes in glucose transporter gene expression through Caco-2 cell differentiation

of both mRNAs were normalized by the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

RNA extraction and cDNA synthesis

Total RNA was isolated from Caco-2 cells using a QuickGene RNA culture cell kit S. Template cDNA synthesis was performed with 5 µg of total RNA using the PrimeScript RT reagent kit (Takara Bio).

Animal experiments

Animal experiments were carried out at the Animal Research Laboratory, Bioscience Education and Research Center, Akita University, Japan. Male 6-week-old C57BL/6J mice were purchased from CLEA Japan. They were housed in groups of four or five in wire-topped, polycarbonate cages with wood shavings for bedding in a room at constant temperature (23±2°C) and humidity (50±10%) under a 12 h light–dark cycle (7:00–19:00). They had free access to a fresh diet and sterile drinking water during the experimental period. They were fed a normal diet (CE-7; CLEA Japan) for 7 days and a high fat diet (HFD32; CLEA Japan) for 28 days, and were then divided into two groups: a control group (n=6) and a LEE group (n=6). The control and LEE groups were administered the CE-7 diet and the CE-7 diet supplemented with 2% (w/w) of LEE, respectively, for 7 days. Food intake was recorded every 2 or 3 days, and body weights were measured every week. At the end of the experimental period, mice were sacrificed under light isoflurane anaesthe-

sia after 16 h of fasting. Blood was collected from the abdominal aorta, and the heparinized plasma obtained was stored at –80°C until analysis. Livers as well as mesenteric and epididymal adipose tissue were excised and weighed. Animal experimentation protocols were previously approved by the Animal Research Committee, Akita University (approval number: a-1-2504). The entire experiment closely followed the university's regulations for animal experimentation, which are in strict accordance with government legislation in Japan.

Plasma parameters

Plasma levels of glucose, aspartate transaminase (AST) and alanine transaminase (ALT) were measured using an automatic analyzer (FUJI DRICHEM 3500V; Fujifilm). Triglyceride and cholesterol levels in the four major lipoprotein fractions were determined by LipoSEARCH® (Skylight Biotech, Akita, Japan) [18].

Results and discussion

Briefly, we assessed the expression patterns of differentiation markers, such as sugar transporters, in spontaneously or chemically differentiated intestinal Caco-2 cells using RT-PCR (Table 1). The primer sequences are shown in Table 2.

Gene name		
GAPDH	(Foward)	5'-GCACCGTCAAGGCTGAGAAC-3'
	(Reverse)	5'-TGGTGAAGACGCCAGTGGA-3'
IAP	(Foward)	5'-GCAACCCTGCAACCCACCAAGGAG-3'
	(Reverse)	5'-CCAGCATCCAGATGTCCCGGGAG-3'
GLUT-1	(Foward)	5'-ATGGCGGGTTGTGCCATA-3'
	(Reverse)	5'-TGCTCAGATAGGACATCCAGGGTAG-3'
GLUT-2	(Foward)	5'-GCTGCGAATAAACAGGCAGGA-3'
	(Reverse)	5'-CAGCACAGCAGTGATGACAGTGA-3'
GLUT-5	(Foward)	5'-TGGAGCAACAGGATCAGAGCA-3'
	(Reverse)	5'-GATGACCCAAAGGCAGCTATCAG-3'
SGLT-1	(Foward)	5'-GCCCAACTCTGATTGCATTTA-3'
	(Reverse)	5'-CTGGTTCTACTTCACCTGAGCAC-3'

Table 2 - Primer sequences used in this study

The amount of mRNA of intestine alkaline phosphatase (IAP), an intestinal differentiation marker of Caco-2 cells, was elevated by the 21-day incubation (spontaneous differentiation). Chemical induction with 5 mM sodium butyrate up-regulated IAP in Caco-2 cells, while the IAP mRNA level was 1.9-fold higher than that in spontaneously differentiated cells. We determined whether the genes GLUT-1, GLUT-2, GLUT-5 and SGLT-1, which are major sugar transporters in intestinal Caco-2 cells [19], were up-regulated in chemically and spontaneously differentiated Caco-2 cells prior to investigating the inhibitory effects of the mushroom extracts on glucose transport. The gene expression of these transporters in Caco-2 cells was up-regulated by both chemically induced and spontaneous differentiation (Table 1). These results indicated that 5 mM sodium butyrate stimulated Caco-2 cell differentiation without lengthy incubation; therefore, we used chemically induced Caco-2 cells as an intestinal glucose transport model. Fig. 1 shows the effects of the three extracts of *L. edodes* on glucose transport in intestinal Caco-2 cells. Transport through intestinal Caco-2 cells treated with 100 μ M epicatechin gallate (Wako Pure Chemical Industry, Osaka, Japan), which was used as the positive control [10–12], was inhibited by 60% compared to that in untreated cells. The water and 50% ethanol extracts had no inhibitory effects on glucose transport in the cells, but transport in cells treated with LEE was inhibited by 37.2% compared to that in untreated cells.

We also examined the effects of LEE on lipid and sugar metabolism in mice with dyslipidaemia and hyperglycaemia (metabolic syndrome model) induced by feeding with HFD for 6 weeks. The actual diet compositions were determined by the Japan Functional Food Analysis and Research Center (Table 3). The effects of LEE on the accumulation of adipose tissue and plasma biochemical parameters are summarized in Tables 4 and 5, respectively. LEE markedly decreased the accumulation of mesenteric adipose tissue, but not body, liver or adipose tissue weights in metabolic syndrome model mice. LEE did not affect plasma cholesterol or triglyceride levels in mice with HFD-induced dyslipidaemia (Table 5); however, the extracts markedly suppressed the accumula-

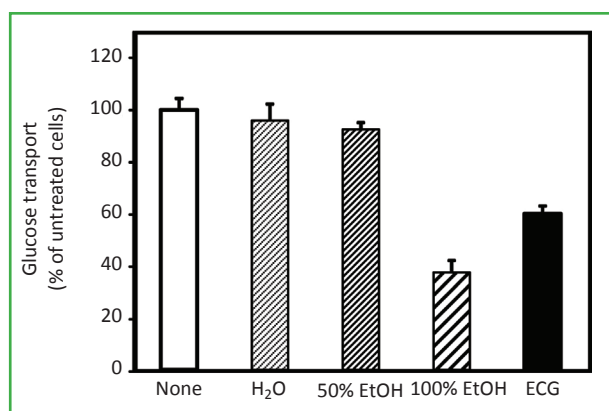


Figure 1 - Effects of extracts from *Lentinula edodes* on glucose transport in intestinal Caco-2 cells. Intestinal Caco-2 cells on a transwell chamber were incubated in DMEM with or without three extracts of *L. edodes* at 0.1 mg/ml or 100 μ M epicatechin gallate for 6 h, and the secretion of glucose on the basolateral side was determined using high-performance anion-exchange chromatography. The amount of glucose on the basolateral side is represented as a percentage of that in non-treated intestinal Caco-2 cells (values represent means \pm S.E.M., n=3). * p <0.05 and ** p <0.01 vs the control group (Kruskal-Wallis multiple comparisons with Dunn's test)

tion of mesenteric adipose tissue (Table 4), and elevated the plasma HDL-cholesterol to total cholesterol ratio (Table 5). On the other hand, LEE markedly decreased plasma glucose levels by 64.0% in HFD-fed mice.

Extracts or compounds from many mushrooms have been shown to improve diabetes and its complications in vitro and/or in vivo. Terpenoids from

	Control diet	LEE diet
Moisture (g/100 g)	9.5	2.7
Protein (g/100 g)	12.0	12.7
Lipid (g/100 g)	3.9	4.8
Ash (g/100 g)	2.3	2.5
Carbohydrate (g/100 g)	69.7	74.2
Dietary fibre (g/100 g)	2.6	2.5
Energy (kcal/100 g)	415.0	449.0
Food intake (g/day)	1.8	1.7

Table 3 - Contents of the control and ethanol extract of *Lentinula edodes* (LEE) diets

	Control group	LEE group
Body weight (g)	33.1 \pm 0.8	32.4 \pm 0.8
Liver weight (mg)	1048.7 \pm 14.3	1100.2 \pm 62.3
Mesenteric adipose tissue (mg)	447.2 \pm 48.6	312.3 \pm 23.0*
Epididymal adipose tissue (mg)	1497.2 \pm 110.0	1480.2 \pm 109.9

Values represent means \pm S.E.M. (n=6)

* p <0.05 vs control group (Mann-Whitney U test)

Table 4 - Effects of ethanol extract of *Lentinula edodes* (LEE) on visceral adipose tissue weights

	Control group	LEE group
Cholesterol (mg/dl)		
Total	63.9±7.8	67.0±6.7
CM	0.2±0.1	0.1±0.0
VLDL	5.6±1.4	4.0±0.6
LDL	10.9±2.6	7.1±1.2
HDL	47.2±5.6	55.8±5.5
HDL/total (%)	73.6±3.8	83.3±3.3*
Triglycerides (mg/dl)		
Total	26.0±5.0	32.6±7.5
CM	1.5±0.6	1.0±0.3
VLDL	14.8±4.2	18.7±6.0
LDL	8.1±0.7	10.7±1.2
HDL	1.9±0.4	2.2±0.3
Glucose (g/dl)	240.0±4.4	153.7±6.7**
AST (U/dl)	56.0±2.3	48.2±4.2
ALT (U/dl)	31.5±2.9	30.3±8.9

Values represent means±S.E.M. (n=6)

* $p < 0.05$; ** $p < 0.01$ vs control group (Mann-Whitney U test)

Table 5 - Effects of ethanol extract of *Lentinula edodes* (LEE) on plasma biochemical parameters

Inonotus obliquus, and *p*-terphenyl derivatives and some telephantins from *Hydnellum concrescens* were previously reported to inhibit α -glucosidase activity [20, 21]. An extract of *Ganoderma lucidum* was found to exert inhibitory effects on α -glucosidase and aldose reductase, which is a key enzyme associated with diabetic complications [22]. Furthermore, a polysaccharide fraction from *Pleurotus sajor-caju* prevented glucose intolerance and insulin resistance in hyperglycaemic mice [23]. However, the inhibitory effects of mushrooms on GLUT have not yet been determined. In the present study, we have shown that LEE is very effective against hyperglycaemia in metabolic syndrome model mice. These effects may be due to the inhibition of intestinal sugar transport. We intend to identify the active compounds of *L. edodes* in our future research. Furthermore, our antidiabetic data for *L. edodes* are limited to in vitro and experimental animal studies, so we hope to perform clinical tests in diabetic subjects.

Conclusions

LEE strongly inhibited glucose transport in human intestinal epithelium-like cells. This extract also improved plasma glucose levels and the accumulation of mesenteric adipose tissue in metabolic syndrome model mice fed HFD.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Animal Rights

All institutional and national guidelines for the care and use of laboratory animals were followed.

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