Effect of Dietary Maitake (*Grifola frondosa*) Mushrooms on Plasma Cholesterol and Hepatic Gene Expression in Cholesterol-Fed Mice

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Abstract: To investigate the effects of dietary *Grifola frondosa* on cholesterol, normal mice were fed a diet containing 1% cholesterol (HC group) or 1% cholesterol and 10% freeze-dried *G. frondosa* powder (HC+G group) for 4 weeks and hepatic and plasma lipid levels were compared with those of a cholesterol-free diet-fed mice (N group). Hepatic total cholesterol (TC), triacylglycerol contents were considerably increased and plasma TC / phospholipid (PL) was also increased significantly in the HC group compared with the N group. However, plasma TC content decreased in the HC+G group compared with the HC group. To characterize the mechanisms responsible for lowered plasma cholesterol in *G. frondosa*-supplemented mice, hepatic gene expression was profiled using DNA microarray and gene ontology. Genome analyses revealed that *de novo* cholesterol synthesis genes were suppressed following cholesterol intake. However, expression of bile acid biosynthesis and low-density lipoprotein receptor genes showed little change. *Scarb1*, *Abcg5*, and *Abcg8*, involved in cholesterol transport and excretion, were slightly upregulated in the HC+G group compared with the HC group. These data indicate the plasma cholesterol-lowering effect of *G. frondosa*. Moreover, fatty acid (FA) β -oxidation was promoted via adipocytokine signaling pathways, and *Saa*, encodes serum amyloid A related to arteriosclerosis, was suppressed in the HC+G group.

Key words: DNA microarray, cholesterol, *Grifola frondosa*, mushroom, functional properties

1 INTRODUCTION

Ischemic heart disease and stroke and other forms of cerebrovascular disease are leading causes of death globally¹. Hypercholesterolemia, an important risk factor for these diseases, is closely related to dietary habits. Therefore, functionality of foods attracts attention for the prevention and improvement of symptoms. Mushrooms have long been considered to have beneficial physiological effects. Their cholesterol-lowering²⁻⁴ and antiatherogenic⁵ effects are known. The hypocholesterolemic compound, eritadenine is isolated from *Lentinus edodes* and to a lesser extent in *Agaricus bisporus*⁶⁻⁹.

We previously evaluated functionality of foods using comprehensive analyses of genomes¹⁰⁻¹² and demonstrat-

ed the utility of DNA microarrays for elucidating metabolic and signaling mechanisms of such dietary supplements *in vivo*. Recently, we analyzed hepatic gene expression following mushroom intake and demonstrated upregulation of various genes involved in lipid metabolism and the immune system¹⁰. *Grifola frondosa* (Maitake), which is commonly consumed in Japan, has been recognized for its cholesterol-lowering effects²⁻⁵. However, with the exception of the low density lipoprotein (LDL) receptor², few genomic mechanisms have been reported.

To evaluate the effects of dietary *G. frondosa* on cholesterol intake, hepatic gene expression profiles were analyzed in the normal mice fed a 1% cholesterol-containing diet and mice fed 1% cholesterol and 10% freeze-dried *G.*

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frondosa powder using DNA microarrays. In addition, the mechanism by which *G. frondosa* improves lipid metabolism was examined.

2 MATERIALS AND METHODS

2.1 Experimental samples

G. frondosa (strain: Gf433) were cultured according to Yoneyama *et al.*¹³⁾. Harvested fruit bodies were autoclaved at 100°C for 10 min, freeze-dried, and powdered.

2.2 Animals and diets

The experimental diet was prepared according to the AIN-76 formulation. Dietary fiber content in mushrooms was calculated according to food composition data¹⁴⁾. Cellulose powder in the control diet was substituted with mushroom powder in the experimental diet, and the sucrose content was modified (Table 1).

Four-week-old male ICR mice (CLEA Japan Inc., Tokyo) were fed AIN-76 pellets for 1 week of adaptation. The mice were then randomly divided into three groups of five each and fed the normal diet (N group; AIN-76 pellets) or experimental diets containing 1% cholesterol (HC group) or 1% cholesterol and *G. frondosa* powder (HC + G group) for 4 weeks (Table 1). The mice were kept in an air-conditioned room at 23 ± 2°C with 40 ± 10% relative humidity, a 12-h light/dark cycle was followed, and free access to food and water was allowed. The mice were fasted for 16 h before being anesthetized with diethyl ether. Blood was immediately collected from hearts. Livers were isolated and stored at -80°C in RNeasy[®] (Ambion, Austin, TX, USA) until

RNA extraction. All mice were treated according to the guidelines for experimental animal use of Obihiro University of Agriculture and Veterinary Medicine, Japan.

2.3 Analysis of plasma and liver lipids

Blood was separated into plasma by centrifugation (4°C, 1500 g, 15 min). Plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), and phospholipid (PL) concentrations were measured using commercial enzymatic test kits¹⁰⁾. LDL cholesterol (LDL-C) was estimated as the difference between TC and HDL-C concentrations. Liver lipids were extracted according to Danno *et al.*¹⁵⁾. TC, TGs, and PLs in the extracts were measured using the kits. Statistical analyses were performed using ANOVA with the Tukey–Kramer HSD test ($p < 0.05$) with JMP 5.0.1 J (SAS Institute, Cary, NC, USA).

2.4 DNA microarray analysis

Total RNA was isolated from the livers using an SV total RNA Isolation System (Promega, Madison, WI, USA). Its quality was examined by its absorbance and by electrophoresis. Equal amounts of the RNA from five mice of each experiment group were pooled to normalize individual differences for DNA microarray analysis.

Labeled cRNA was synthesized using 2 µg of total RNA as a template with GeneChip[®] One-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA). cRNA was fragmented as per the standard protocol and then hybridized at 45°C for 16 h to GeneChip[®] Mouse Genome 430A 2.0 Array (Affymetrix). The array was washed and stained with GeneChip[®] Fluidics Station 450 and GeneChip[®] Hybridization, Wash, and Stain Kit (Affymetrix) and then scanned using GeneChip[®] Scanner 3000 (Affymetrix). The collected fluorescence signals were corrected for background and converted into numeric data using GeneChip[®] Operating Software (Affymetrix). Gene Spring GX 7.3.1 (Agilent Technologies, Santa Clara, CA, USA) was used to normalize data and compare the expression levels. The probes with the data flag “presence” were extracted. In comparison with the probes for the N group, the extracted probes whose signal level changed >1.5-fold were identified. Functional clustering of differentially expressed genes was assigned according to the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (<http://david.abcc.ncifcrf.gov/>) at “medium” stringency based on the Gene Ontology (GO) biological process. These genes were categorized and mapped onto pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (<http://www.kegg.jp/ja/>).

Table 1 Composition of the diets (%).

Component	Dietary group		
	N	HC	HC+G
Sucrose	50.0	49.5	46.7
Casein	20.0	19.8	18.7
Corn starch	15.0	14.9	14.0
Corn oil	5.0	5.0	4.7
Cellulose	5.0	5.0	0.0
AIN-76 mineral mixture	3.5	3.5	3.3
AIN-76 vitamin mixture	1.0	1.0	0.9
DL-methionine	0.3	0.3	0.3
Choline bitartrate	0.2	0.2	0.2
Cholesterol	0.0	1.0	0.9
Mushroom powder	0.0	0.0	10.3
Total	100.0	100.0	100.0

N, normal diet group; HC, high cholesterol group; H+G, high cholesterol plus *G. frondosa* powder group

3 RESULTS AND DISCUSSION

3.1 Effects of cholesterol and *G. frondosa* intake on liver and plasma lipids in mice

Food consumption of the HC + G group was highest in the three groups (Table 2). However, body and epididymal fat weights did not differ significantly between treatment groups throughout the experimental period (Table 2). Hepatic and plasma lipid contents were shown in Table 2 and 3, respectively. Total lipids, TC, and TG contents in livers of the HC group increased considerably compared with those of the N group (Table 2). TC/PL and LDL-C/PL in plasma significantly increased and TG/PL slightly de-

creased in the HC group (Table 3). Hepatic TC and TG contents were lower in the HC + G group than in the HC group. In the HC + G group, TC/PL in plasma were significantly lower than those in the HC group but similar to those in the N group. LDL-C/PL in the HC + G group were also lower than those in the HC group. TG/PL in the HC + G group was significantly lower than those in the N group. It has been reported that the serum TC/PL is significantly increased in the coronary heart disease group in contrast to the normal group¹⁶⁾. Our data suggest that ingestion of *G. frondosa* reduced this ratio.

Table 2 Metabolic parameters of mice fed experimental diets for 4 weeks.

	N	HC	HC+G
Initial BW (g) ^a	33.9 ± 2.2 ^A	33.3 ± 0.6 ^A	33.6 ± 1.7 ^A
Final BW (g) ^b	39.0 ± 3.9 ^A	38.6 ± 1.4 ^A	37.9 ± 3.1 ^A
Food consumption (g/5 mice per day)	26.3 ± 4.5 ^B	23.0 ± 4.5 ^B	35.9 ± 3.1 ^A
Epididymal fat (g)	1.2 ± 0.4 ^A	0.8 ± 0.3 ^A	0.7 ± 0.2 ^A
Liver			
Liver weight (g)	2.0 ± 0.4 ^A	2.4 ± 0.6 ^A	1.8 ± 0.3 ^A
Total lipid content (%)	7.5 ± 2.3 ^B	18.9 ± 4.2 ^A	16.8 ± 6.9 ^A
Total cholesterol content (%)	0.53 ± 0.15 ^B	3.29 ± 0.50 ^A	2.53 ± 1.09 ^A
Triacylglycerol content (%)	4.2 ± 1.9 ^B	9.0 ± 1.3 ^A	7.9 ± 3.7 ^{AB}

N, normal diet group; HC, high cholesterol group; H+G, high cholesterol plus *G. frondosa* powder group

Values are expressed as the mean ± SD (n = 5).

Values not sharing the same superscript letters (A, B, C) within each family are significantly different, as indicated by the Tukey–Kramer HSD test ($p < 0.05$).

^aInitial body weight was measured before starting dietary protocols.

^bFinal body weight was measured before sacrifice.

Table 3 Plasma lipid of mice fed experimental diets for 4 weeks.

	N	HC	HC+G
Total cholesterol (mg/dL)	99.5 ± 25.0 ^A	134.1 ± 39.1 ^A	98.2 ± 27.5 ^A
Total cholesterol / PL	0.47 ± 0.04 ^B	0.63 ± 0.05 ^A	0.50 ± 0.05 ^B
HDL-cholesterol (mg/dL)	82.9 ± 16.6 ^A	96.5 ± 30.8 ^A	76.6 ± 18.1 ^A
HDL-cholesterol / PL	0.40 ± 0.06 ^A	0.46 ± 0.04 ^A	0.39 ± 0.02 ^A
LDL-cholesterol (mg/dL)	16.6 ± 14.7 ^A	37.6 ± 13.3 ^A	21.6 ± 11.0 ^A
LDL-cholesterol / PL	0.07 ± 0.06 ^B	0.17 ± 0.06 ^A	0.11 ± 0.04 ^{AB}
Triglyceride (mg/dL)	184.0 ± 77.0 ^A	132.9 ± 40.5 ^{AB}	94.5 ± 23.9 ^B
Triglyceride / PL	0.86 ± 0.27 ^A	0.64 ± 0.17 ^{AB}	0.49 ± 0.12 ^B
Phospholipids (mg/dL)	210.1 ± 45.4 ^A	210.6 ± 57.0 ^A	194.1 ± 37.1 ^A

N, normal diet group; HC, high cholesterol group; H+G, high cholesterol plus *G. frondosa* powder group

Values are expressed as the mean ± SD (n = 5).

Values not sharing the same superscript letters (A, B, C) within each family are significantly different, as indicated by the Tukey–Kramer HSD test ($p < 0.05$).

3.2 Altered expression of hepatic genes following cholesterol and *G. frondosa* supplementation

To characterize the mechanisms leading to differences in liver and plasma cholesterol and TG contents between the experimental diet groups, hepatic gene expression was analyzed using DNA microarray. Table 4 shows significantly altered ($p < 0.05$) GO terms related to lipid metabolism. In lipid metabolic processes, steroid metabolic processes, and regulation of lipid metabolic processes, no differences were observed between the number of genes with altered ex-

pression in the experimental diet groups.

3.3 Effects of 1% cholesterol intake on the expression of lipid metabolism-related genes

Table 5 shows the expression levels of genes belonging to the GO term involved in cholesterol metabolism (Table 4). The expression levels in the HC group were compared with those in the N group.

In GO:0042632, cholesterol homeostasis, *Abcg5* (ATP-binding cassette, sub-family G, member 5) expression was

Table 4 Functional annotation clustering of differentially expressed genes.

GO number	GO term (biological process)	HC		HC+G	
		No. ^a	P value	No. ^a	P value
GO:0055088	Lipid homeostasis	12	1.01E-03		
GO:0055092	...Sterol homeostasis	9	6.84E-03		
GO:0042632	...Cholesterol homeostasis	9	6.84E-03		
GO:0006629	Lipid metabolic process	104	3.54E-03	104	2.04E-02
GO:0044255	...Cellular lipid metabolic process	74	7.52E-03		
GO:0008610	...Lipid biosynthetic process	46	1.59E-02	47	2.86E-02
GO:0008202	Steroid metabolic process	30	8.87E-03	32	6.47E-03
GO:0016125	...Sterol metabolic process	18	5.56E-03	17	2.21E-02
GO:0008203	...Cholesterol metabolic process	16	1.17E-02	17	9.07E-03
GO:0006695	...Cholesterol biosynthetic process	8	1.20E-02		
GO:0006694	...Steroid biosynthetic process	16	1.33E-02		
GO:0016126	...Sterol biosynthetic process	10	5.06E-03		
GO:0032787	...Monocarboxylic acid metabolic process	46	5.39E-03		
GO:0044255	...Cellular lipid metabolic process	74	7.52E-03		
GO:0019216	Regulation of lipid metabolic process	13	2.94E-02	15	8.69E-03
GO:0046890	...Regulation of lipid biosynthetic process	8	9.21E-03	8	1.29E-02
GO:0042304	...Regulation of fatty acid biosynthetic process	5	1.35E-02	5	1.68E-02
GO:0045834	...Positive regulation of lipid metabolic process	7	4.92E-02		
GO:0019218	...Regulation of steroid metabolic process			7	4.36E-02
GO:0006643	Membrane lipid metabolic process			15	2.05E-02
GO:0006665	...Sphingolipid metabolic process	13	4.67E-02	14	3.44E-02
GO:0046519	...Sphingoid metabolic process	10	2.50E-02		
GO:0045834	Positive regulation of lipid metabolic process	7	4.92E-02		

HC, high cholesterol group; H+G, high cholesterol plus *G. frondosa* powder group

^aThe number of genes altered by cholesterol and *G. frondosa* intake belonging to each GO category ($p < 0.05$).

upregulated 1.71-fold compared with that in the N group. The *Abcg5* gene functions after forming a heterodimer with *Abcg8*, i.e., ABCG5/ABCG8. *Abcg8* expression was also slightly upregulated 1.49-fold. The heterodimer ABCG5/ABCG8 is involved in cholesterol excretion from the liver

to the bile^{17, 18)}. In an agreement with the present data, Berge *et al.* reported that *Abcg5* and *Abcg8* were expressed at highest levels in the human liver and were up-regulated in the livers of the mice fed 2% cholesterol¹⁹⁾. Dietary cholesterol increases oxysterol in the body

Table 5 Change (vs N group) in expression of hepatic genes involved in cholesterol homeostasis, cholesterol metabolic process, and regulation of lipid metabolic process.

GenBank accession	Gene symbol	Gene name	HC vs N	HC+G	
				vs N	vs HC
GO:0042632 cholesterol homeostasis					
NM_031884	<i>Abcg5</i>	ATP-binding cassette, sub-family G (WHITE), member 5	1.71	1.71	1.00
AF324495	<i>Abcg8</i>	ATP-binding cassette, sub-family G (WHITE), member 8	1.49	1.89	1.26
AI785548	<i>Apob</i>	Apolipoprotein B	0.38	0.75	1.96
AB029929	<i>Cav1</i>	Caveolin, caveolae protein 1	1.54	0.75	0.49
NM_009846	<i>Cd24a</i>	CD24a antigen	1.64	1.73	1.05
BC002148	<i>Fabp4</i>	Fatty acid binding protein 4, adipocyte	4.16	0.96	0.23
NM_008061	<i>G6pc</i>	Glucose-6-phosphatase, catalytic	0.43	0.62	1.45
BB769209	<i>Npc1</i>	Niemann Pick type C1	1.51	1.04	0.69
BB654116	<i>Pex2</i>	Peroxin 2	1.77	1.46	0.82
BG069809		Transcribed locus	0.64	0.88	1.38
GO:0008203 cholesterol metabolic process					
AF125307	<i>Cln8</i>	Ceroid-lipofuscinosis, neuronal 8	0.76	0.54	0.70
AV214220	<i>Cln8</i>	Ceroid-lipofuscinosis, neuronal 8	0.75	0.62	0.83
NM_018887	<i>Cyp39a1</i>	Cytochrome P450, family 39, subfamily a, polypeptide 1	0.28	0.38	1.35
NM_010010	<i>Cyp46a1</i>	Cytochrome P450, family 46, subfamily a, polypeptide 1	0.96	0.10	0.11
NM_020010	<i>Cyp51</i>	Cytochrome P450, family 51	0.64	0.53	0.83
BG295389	<i>Dhcr24</i>	24-Dehydrocholesterol reductase	0.39	0.10	0.27
BG065877	<i>Hdlbp</i>	High density lipoprotein (HDL) binding protein	2.20	1.46	0.66
BB705380	<i>Hmgcs1</i>	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1	0.63	0.60	0.95
AK004902	<i>Hmgcs2</i>	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2	0.55	0.40	0.73
NM_010476	<i>Hsd17b7</i>	Hydroxysteroid (17-beta) dehydrogenase 7	0.54	0.76	1.39
BC004801	<i>Idi1</i>	Isopentenyl-diphosphate delta isomerase	0.46	0.66	1.43
AV257512	<i>Insig2</i>	Insulin induced gene 2	0.56	0.97	1.72
U42467	<i>Lepr</i>	Leptin receptor	1.42	1.62	1.14
U58862	<i>Lepr</i>	Leptin receptor	1.46	1.55	1.06
NM_010719	<i>Lipe</i>	Lipase, hormone sensitive	1.49	1.70	1.14
NM_011314	<i>Saa1</i>	Serum amyloid A 1	2.09	1.40	0.67
BI412871	<i>Scap</i>	SREBP cleavage activating protein	0.75	0.59	0.79
BB224405	<i>Scarb1</i>	Scavenger receptor class B, member 1	1.16	1.53	1.32
BG064396	<i>Soat1</i>	Sterol O-acyltransferase 1	1.30	1.71	1.32
AI326423	<i>Srebf1</i>	Sterol regulatory element binding factor 1	3.15	2.74	0.87
BC014769	<i>Tm7sf2</i>	Transmembrane 7 superfamily member 2	0.59	0.55	0.94
BB628702	<i>Vldlr</i>	Very low density lipoprotein receptor	0.77	1.61	2.09

N, normal diet group; HC, high cholesterol group; H+G, high cholesterol plus *G. frondosa* powder group

Table 5 Continued.

GenBank accession	Gene symbol	Gene name	HC vs N	HC+G	
				vs N	vs HC
GO:0019216 regulation of lipid metabolic process					
BE630294	<i>Adora1</i>	Adenosine A1 receptor	1.41	1.60	1.13
AK018763	<i>Agt</i>	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	0.61	0.66	1.09
AK014564	<i>Angptl4</i>	Angiopoietin-like 4	0.50	0.60	1.19
NM_020581	<i>Angptl4</i>	Angiopoietin-like 4	0.57	0.37	0.64
AI785548	<i>Apob</i>	Apolipoprotein B	0.38	0.75	1.96
BC003248	<i>Brca1</i>	Breast cancer 1	1.61	0.59	0.36
AB029929	<i>Cav1</i>	Caveolin, caveolae protein 1	1.54	0.75	0.49
BF580781	<i>Hnf4a</i>	Hepatic nuclear factor 4, alpha	0.78	0.58	0.74
BC011437	<i>Il1b</i>	Interleukin 1 beta	1.36	1.65	1.22
AV257512	<i>Insig2</i>	Insulin induced gene 2	0.56	0.97	1.72
BB345784	<i>Irs1</i>	Insulin receptor substrate 1	0.55	0.72	1.30
NM_008173	<i>Nr3c1</i>	Nuclear receptor subfamily 3, group C, member 1	1.28	1.45	1.13
BB654116	<i>Pex2</i>	Peroxin 2	1.77	1.46	0.82
BC016892	<i>Ppara</i>	Peroxisome proliferator activated receptor alpha	0.83	0.54	0.65
BB087946	<i>Rac1</i>	RAS-related C3 botulinum substrate 1	0.54	0.69	1.26
BI412871	<i>Scap</i>	SREBP cleavage activating protein	0.75	0.59	0.79
AI326423	<i>Srebf1</i>	Sterol regulatory element binding factor 1	3.15	2.74	0.87
L36062	<i>Star</i>	Steroidogenic acute regulatory protein	20.28	15.65	0.77
U36502	<i>Stat5a</i>	Signal transducer and activator of transcription 5A	1.28	1.52	1.19
AW490135	<i>Stub1</i>	STIP1 homology and U-Box containing protein 1	1.43	1.65	1.16
BC027242	<i>Vav3</i>	Vav 3 oncogene	2.16	2.60	1.50

N, normal diet group; HC, high cholesterol group; H+G, high cholesterol plus *G. frondosa* powder group

and induces ABCG5/ABCG8 upregulation by activating the liver X receptor (LXR)²⁰. Thus, it is expected that the system for cholesterol excretion from the liver to the bile is upregulated in the cholesterol-supplemented mice. In contrast, the gene encoding Apolipoprotein B (*Apob*), the primary apolipoprotein responsible for endogenous lipoprotein metabolism in VLDLs, was unexpectedly downregulated 0.38-fold in the HC group. These results indicate that VLDL excretion from the liver was lowered in these animals. Interestingly, plasma *Fabp4* (adipocyte FA-binding protein 4), was reportedly increased in metabolic syndrome patients²¹ and upregulated 4.16-fold. TG content in the livers of the HC group was higher than that of the N group (Table 2), and *Fabp4* expression was positively correlated with liver fat content in humans²². Moreover, Fu *et al.* showed that *Fabp4* overexpression in human THP-1 monocytic cells accelerates cholesterol and TG accumulation in macrophage foam cells²³.

In GO:0008203 cholesterol metabolic process, *Hmgcs1*, *Hmgcs2*, *Idi1*, *Cyp51*, *Tm7sf2*, *Hsd17b7*, and *Dhcr24*

were downregulated (Fig. 1). Increases in dietary cholesterol are likely to suppress *de novo* cholesterol synthesis, leading to suppression of these genes. It was expected that dietary cholesterol would promote primary bile acid biosynthesis in the liver as cholesterol catabolism was increased. However, *Cyp39a1* and *Cyp46a1* of the 24-hydroxylase pathway were not upregulated. In addition, upregulation of cholesterol 7 α -hydroxylase (*Cyp7a1*), which performs the rate-limiting step of bile acid biosynthesis and is controlled by LXR α , was not observed (data not shown). These results show that increased liver cholesterol does not necessarily upregulate genes involved in bile acid biosynthesis.

Srebf1, which encodes sterol regulatory element binding factor 1 (SREBP1), was upregulated 3.15-fold in cholesterol-fed mice (Table 5). Yabe *et al.* reported that SREBP1c is mainly expressed in liver and adipose tissue and controls genes involved in FA and TG synthesis²⁴. In addition, decrease in *Insig2* expression might allow SREBP1c to be processed even when hepatic cholesterol concentrations

Fig. 1 Expression of Steroid Biosynthesis Genes Induced by Ingestion of Cholesterol and *G. frondosa*. HC, high cholesterol group; H+G, high cholesterol plus *G. frondosa* powder group.

are relatively high and FA synthesis is activated. In the present study, FA synthase (*Fasn*) was not upregulated following cholesterol intake (1.27-fold), but *Insig2* downregulation (0.56-fold) and *Srebfl* upregulation were observed. Therefore, these transcriptional changes may be responsible for the significant increase in liver TG (Table 2).

Expression of the lipoprotein metabolism gene *Ldlr* was unchanged in the cholesterol-fed mice. However, *Hdlbp*, which encodes the HDL-binding protein, was increased 2.2-fold, suggesting that the HDL-binding protein facilitates removal of excess cholesterol from cells²⁵. In contrast, expression of *Scarb1*, which encodes the HDL receptor, was not upregulated (1.16-fold). Cai *et al.* reported that SAA, which is a ligand for the scavenger receptor class B type I (SR-B1), inhibits HDL binding and selective cholesterol ester (CE) uptake and thereby influences HDL-C metabolism²⁶. In agreement, *Saa1* was increased 2.09-fold in the HC group. In the liver, SR-B1 plays a key role in the clearance of HDL CE and facilitates cholesterol transport from the periphery to the liver²⁶. Therefore, it is supposed that reverse cholesterol transport through SR-B1 was inhibited by increased SAA production.

Angiopoietin-like 4 (*Angptl4*) is a known inhibitor of lipoprotein lipase (LPL) activity. In this study, *Angptl4* expression was decreased (0.50- and 0.57-fold). When LPL-dependent VLDL lipolysis is inhibited, plasma TG concentrations rise. Therefore, *Angptl4* downregulation may have contributed to decreased plasma TG concentrations in the HC group. Lichtenstein *et al.* also suggested that *Angptl4* induced cholesterol synthesis in the liver is secondary to inhibition of LPL- and hepatic lipase-dependent hepatic

cholesterol uptake²⁷. Although hepatic and plasma cholesterol concentrations were increased by dietary cholesterol (Table 2 and 3), suppression of cholesterol synthesis in the liver may be related to *Angptl4* downregulation. Tomita reported that *Angptl4* suppression is involved in the pathology of non-alcoholic steatohepatitis (NASH)²⁸. Thus, this observation may indicate NASH development in the HC group.

Caveolin, caveolae protein 1 (*Cav1*), which encodes a cholesterol-binding protein and plays an important role in intracellular cholesterol transport, was upregulated 1.54-fold. Removal of this protein is essential for activation of endothelial nitric oxide synthase (eNOS), which is an important antiarteriosclerotic mechanism. Feron *et al.* demonstrated that high caveolin-1 expression decreased eNOS activation²⁹. In addition, increased LDL-C significantly upregulates caveolin expression. Consistent with this, LDL-C was increased in the HC group (Table 3) and may have led to slight *Cav1* upregulation.

3.4 Effect of *G. frondosa* intake on the expression of lipid metabolism genes in cholesterol-loaded mice livers

There were differences in the expression of lipid metabolic processes (GO:0006629) (Table 5). In the HC + G group, *Abcg8* expression (1.89-fold) was higher than that in the HC group. ABCG5/ABCG8 expression increases in the presence of nuclear receptor LXR agonists, leading to increased cholesterol excretion into bile³⁰. Our data therefore indicate that *G. frondosa* contains LXR agonists that promote cholesterol excretion.

With regard to primary bile acid synthesis, *Cyp39a1* and

Cyp46a1 were not upregulated in the HC + G group as well as in the HC group. Substantial differences in the expression of the genes of *de novo* cholesterol synthesis and LDL receptors were not found between the HC + G and HC groups. Although there were no significant differences in liver cholesterol, enhanced cholesterol excretion led to significant decrease in plasma cholesterol in the HC + G group compared with the HC group. Moreover, *Apob* expression in the HC + G group was similar to that in the N group.

Srebf1 expression is associated with fatty liver and hyperlipidemia. In these experiments, *Srebf1* expression was elevated in both cholesterol-fed mice groups, but it was lower in the HC + G group (2.74-fold) than in the HC group (3.15-fold). These results suggest decreasing hepatic TG content in the HC + G group. Moreover, *Fabp4* expression was decreased to the level in the N group in the *G. frondosa*-supplemented mice, and *Cav1* was upregulated in the HC group but not in the HC + G group. Hence, caveolin-mediated eNOS inhibition may have been limited in the HC + G group.

G. frondosa intake promoted cholesterol metabolism in a previous study⁴⁾. However, similar to a study using rats by Fukushima *et al.*²⁾, upregulation of bile acid metabolism was not observed in this study. The cholesterol-lowering effects of these mushrooms are thought to be caused by increased excretion of cholesterol and bile acid in feces^{2, 4)}, which may involve mushroom dietary fiber as discussed. *G. frondosa* contains soluble (0.3 g/100 g boiled mushroom) and insoluble (2.4 g/100 g boiled mushroom) fibers¹⁴⁾. These dietary fibers were corrected using insoluble cellulose powder so that the fiber content was the same in all groups. The dietary actions of soluble and insoluble fibers differ. Fukushima *et al.* reported plasma cholesterol-lowering effects of soluble dietary fiber²⁾. β -glucan is a soluble fiber found in *G. frondosa* and is a candidate compound for cholesterol lowering. However, the main objective of

this study was to examine hepatic gene expression following dietary supplementation with *G. frondosa*. Hence, further investigation is needed to determine the active components. Fukushima *et al.* found that fecal cholesterol excretion was promoted by dietary *G. frondosa*²⁾, but the mechanism of this effect was unclear. Kubo and Nanba also showed that cholesterol and bile acid in feces of rats with 1% cholesterol (10% lard and 2% corn oil) were increased by *G. frondosa* intake⁴⁾. As mentioned above, although a significant increase in *Cyp7a1* expression was not observed, genes related to cholesterol transport and excretion into bile were slightly upregulated in the HC + G group. Hence, these minor transcriptional changes may have cumulatively influenced plasma cholesterol content.

3.5 Effects of cholesterol and *G. frondosa* intake on the expression of other genes

As shown in Table 5, the leptin receptor gene *Lepr* was upregulated in the HC + G group (1.62-fold). Therefore, we focused on the gene expression of adipocytokine signaling components (Fig. 2). As for 5'-AMP-activated protein kinase, which controls sugar and lipid metabolism and plays an important role in energy metabolism, Ca^{2+} -calmodulin-dependent protein kinase kinase 1 (*Camkk1*) was upregulated 2.27-fold in the HC + G group. Downstream, carnitine palmitoyltransferase (*Cpt1a*), which is a key enzyme for β -oxidation of long-chain FAs in liver mitochondria³¹⁾, was up-regulated 3.02-fold. This may have promoted FA oxidation, which led to decrease in liver and plasma TG. *Cpt1a* levels in the experimental diet groups were significantly higher than those in the N group, and the level in the HC + G group was highest among the three groups. Hence, *Cpt1a* upregulation may moderate hepatic TG accumulation by excess cholesterol intake. This result indicates the presence of compounds in *G. frondosa* that induce *Cpt1a* upregulation. Because the mRNA level does

Fig. 2 Expression of Adipocytokine Signaling Genes Induced by Ingestion of Cholesterol and *G. frondosa*. HC, high cholesterol group; H+G, high cholesterol plus *G. frondosa* powder group.

not necessarily accord with enzyme activity, metabolic changes are merely inferred from analyses of mRNA expression. Nonetheless, these data offer an explanation for the decline in hepatic and plasma TG concentrations in the HC + G group compared with the HC group. Thus, β -oxidation was promoted via adipocytokine signaling following supplementation with *G. frondosa*, and the related genes (*Lepr*, *Camkk1*, *Prkg2*, *Cpt1a*) were elevated in the HC + G group compared with the HC group. Hence, dietary intake of *G. frondosa* during high cholesterol feeding is expected to promote fat catabolism.

As suggested above, SAA inhibits cellular hepatic cholesterol reverse transport. Accordingly, *Saa1* was downregulated 1.40-fold and *Scarb1* was upregulated 1.53-fold in the HC + G group compared with the HC group (Table 5), suggesting that cholesterol reverse transport was promoted by dietary *G. frondosa*. A previous study indicates that elevated SAA expression is associated with cardiovascular risks, including obesity, insulin resistance, metabolic syndrome, and diabetes³². In this study, expression of SAAs (*Saa1*, *Saa2*, *Saa3*, and *Saa4*) was significantly upregulated in the HC group compared with the N group (1.54- to 3.56-fold), but it was suppressed in the HC + G group (1.19- to 1.54-fold). These data suggest that dietary intake of *G. frondosa* suppresses the expression of SAAs that induce arteriosclerosis.

These data indicate that dietary *G. frondosa* during cholesterol feeding promotes fat catabolism by inducing adipocytokine signaling and β -oxidation, and subsequently leads to antiatherogenic effects. Hence, dietary *G. frondosa* can prevent and improve the symptoms of obesity, diabetes, and arteriosclerosis induced by hypercholesterolemia.

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