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ARTICLE

Analysis of Polar Lipids in the Serum from Rats Fed Shiitake by Liquid Chromatography−Mass Spectrometry/Mass Spectrometry

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Consumption of a shiitake mushroom diet has been reported to have effects on serum phospholipids. However, much less is known about the effect on serum polar lipids including lysophospholipids and free fatty acids. In the present study, the effects of a shiitake diet were evaluated on the basis of identification and quantification of individual polar lipid components in rat serum using liquid chromatography−mass spectrometry/mass spectrometry. By comparison with standards and published data, 50 lysophospholipids and 32 free fatty acids were identified, and the concentrations of 27 polar lipids in rat serum were determined. Shiitake diets decreased the levels of all individual polar lipid components in the serum of male rat. The total level of serum polar lipids in males fed 4% shiitake diets (1365.71 mol/L) was significantly lower than that of the control (2270.26 mol/L). However, shiitake diets did not significantly affect the levels of serum polar lipids in female rats.

KEYWORDS: Shiitake mushrooms; rat serum; polar lipids; LC−MS/MS; gender differences

INTRODUCTION

Shiitake mushrooms (*Lentinus edodes*) have been popular as a health food for thousands of years in the East and more recently in the West. Shiitake is the second most highly consumed mushroom in the world (*1*−*6*). The majority of the studies of its nutritional and therapeutic effects have focused on the anticancer activity (*6*−*10*). Additionally, a few studies have centered on hypocholesterolemic effects (*11*, *12*) and mycochemical eritadenine, [2*R*,3*R*-4-(9-adenyl)butyric acid], has been documented to be a major hypocholesterolemic agent in shiitake (*13*, *14*). Furthermore, eritademine has been reported to affect several variables concerning lipid metabolism and induce the alterations of lipid molecular species profile in rats (*15*). For example, dietary eritadenine decreased the ratio of phosphatidylcholine/phosphatidylethanolamine in liver microsomes, and increased the proportion of 16:0−18:2 molecular species with a decrease in 18:0−20:4 of phosphatidylcholine in plasma in rats (*15*).

In our previous study, when compared with rats fed control diets, consumption of shiitake diets did not result in significant differences in the incidence, multiplicity, or final weights of colon tumors of rats (*16*). However, 4% shiitake diet elevated the active energy expenditure of male rats, thereby resulting in leaner animals than the control. These findings prompted us to study effects of shiitake consumption on serum levels of lipids. Lysophospholipids are major lipid components in the blood of humans and rats (*17*) and relatively polar in comparison with phospholipids. Varied polar headgroups in lysophospholipid components result in different subclasses such as lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidic acid, lysophosphatidylglycerol, lysophosphatidylinositol, and lysophosphatidylserine. Lysophospholipids have been extensively studied, and different biological activities of its subclasses were detected (*18*). Free fatty acid is also a major polar lipid class in serum and possesses biological activity (*19*, *20*).

In the present study, polar lipid components in serum from rats were identified and the concentration of individual component in the serum was determined using liquid chromatography−mass spectrometry/mass spectrometry (LC−MS/MS). The effect of shiitake was evaluated based on alteration of concentrations of individual polar lipid components in the serum from rats.

MATERIALS AND METHODS

Material. Shiitake mushrooms ("Snowcap" variety) were obtained from Shirley Community Development Corporation (Shirley, AR). The shiitake was grown in oak logs, and fruiting bodies were harvested and immediately frozen in dry ice and stored at −20 °C. Frozen shiitake were lyophilized, powdered, bagged under vacuum and stored at −20 °C.

For LC−MS/MS analysis, eighteen standards, including three lysophospholipid mixture standards, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The three lysophospholipid mixture standards were L-α-lysophosphatidylcholine mixture, L-α-lysophosphatidylethanolamine mixture and L-α-lysophosphatidylinositol mixture. The lysophosphatidylcholine mixture standard was prepared by the action of phospholipase A on soybean L-α-phosphatidylcholine and contains primarily C-18 unsaturated

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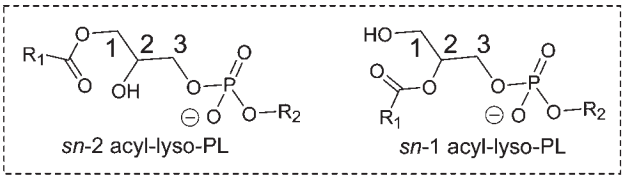

Lysophosphatidylcholine (lyso-PC): R_2=

No.	name	Skeleton	R_1
1	14:0a/lyso-PC	*sn*-2	$CH_3(CH_2)_{11}CH_2$-
2	lyso/18:2a-PC	*sn*-1	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_6CH_2$-
3	lyso/20:4a-PC	*sn*-1	$CH_3(CH_2)_4(CH{=}CHCH_2)_4CH_2CH_2$-
4	18:2a/lyso-PC	*sn*-2	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_6CH_2$-
5	20:4a/lyso-PC	*sn*-2	$CH_3(CH_2)_4(CH{=}CHCH_2)_4CH_2CH_2$-
6	lyso/16:0a-PC	*sn*-1	$CH_3(CH_2)_{13}CH_2$-
8	16:0a/lyso-PC	*sn*-2	$CH_3(CH_2)_{13}CH_2$-
9,10*	lyso/18:1a-PCs	*sn*-1	$CH_3(CH_2)_7CH{=}CH(CH_2)_6CH_2$-
11	15:0a/lyso-PC	*sn*-2	$CH_3(CH_2)_{12}CH_2$-
12	22:5a/lyso-PC	*sn*-2	$CH_3(CH_2)_4(CH{=}CHCH_2)_5CH_2$-
13,14*	18:1a/lyso-PCs	*sn*-2	$CH_3(CH_2)_7CH{=}CH(CH_2)_6CH_2$-
15	17:0a/lyso-PC	*sn*-2	$CH_3(CH_2)_{14}CH_2$-
16	lyso/18:0a-PC	*sn*-1	$CH_3(CH_2)_{15}CH_2$-
18	18:0a/lyso-PC	*sn*-2	$CH_3(CH_2)_{15}CH_2$-
19	20:1a/lyso-PC	*sn*-2	$CH_3(CH_2)_9CH{=}CH(CH_2)_6CH_2$-

Lysophosphatidylethanolamines (lyso-PE): R_2=

No.	name	Skeleton	R_1
7	16:0a/lyso-PE	*sn*-2	$CH_3(CH_2)_{13}CH_2$-
17	18:0a/lyso-PE	*sn*-2	$CH_3(CH_2)_{15}CH_2$-

Free fatty acids:

No.	name	Structure formula
20	16:1 (Palmitoleic acid)	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$
21	22:6(*Cis*-4,7,10,13,16,19 -docosahexaenoic acid)	$CH_3CH_2(CH{=}CHCH_2)_6CH_2COOH$
22	20:4 (arachidonic acid)	$CH_3(CH_2)_4(CH{=}CHCH_2)_4CH_2CH_2COOH$
23	18:2 (Linoleic acid)	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$
24	22:5	$CH_3(CH_2)_4(CH{=}CHCH_2)_5CH_2COOH$
25	16:0 (Palmitic acid)	$CH_3(CH_2)_{14}COOH$
26	18:1 (Oleic acid)	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$
27	18:0 (Stearic acid)	$CH_3(CH_2)_{16}COOH$

9,10* and 13,14* are isomers.

Figure 1. Structures of serum polar lipids.

acyloxy groups. The lysophosphatidylethanolamine mixture standard was prepared from egg yolk and contains primarily stearoyl and palmitoyl groups. The lysophosphatidylinositol mixture standard was prepared by the action of phospholipase A on soybean L-α-lysophosphatidylinositol and contains primarily palmitoyl and stearoyl groups. The three pure lysophospholipid standards were 1-palmitoyl-*sn*-glycero-3-phosphocholine (16:0a/lysophosphatidylcholine), 1-oleoyl-*sn*-glycero-3-phosphocholine (18:1a/lysophosphatidylcholine) and 1-stearoyl-*sn*-glycero-3-phosphocholine (18:0a/lysophosphatidylcholine). The free fatty acid standards included [5*S*,12*R*]-dihydroxy-[6*Z*,8*E*,10*E*,14*Z*]-eicosatetraenoic acid (leukotriene B_4), 13(*S*)-hydroxyoctadeca-9*Z*,11*E*-dienoic acid, 15(*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid, 12(*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid, *cis*-4,7,10,13,16,19-docosahexaenoic acid, eicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic acid (arachidonic acid), *cis*-9,*cis*-12-octadecadienoic acid (linoleic acid), hexadecanoic acid (palmitic acid), *cis*-9-octadecenoic acid (oleic acid), and octadecanoic acid (stearic acid). The two flavonoid standards were genistein and biochanin A.

Experimental Diets and Animals. Animal use protocols were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. The housing for rats was described previously (*16*). Rats were fed AIN-93G diets (*21*) containing 20% (w/w) casein (New Zealand Milk Products, Santa Rosa, CA), or casein supplemented with shiitake (1% or 4% wt/wt). The final total crude fiber (α-cellulose plus shiitake), protein, and energy contents in shiitake and control diets were the same, and the final composition of experimental diets is summarized in a previous publication (*16*). Thirty-six timed-pregnant Sprague−Dawley rats of similar average body weight on gestation day 4 were purchased from Charles River Laboratories (Wilmington, MA) and were divided into 3 dietary groups (casein, 1% shiitake, and 4% shiitake). A total of 450 pups were born. The pups on postnatal day 2 were weighed and culled to 5 males and 5 females per litter, and continued on their diet throughout the experiment. At postnatal day 50, rats were anesthetized with phenobarbital and euthanized, and blood was collected without anticoagulant. After the blood was kept at room temperature for 2 h, serum was prepared by centrifugation at 1500*g* for 20 min.

LC−MS Sample Preparation. For the sample preparation, a pool was made by combining sera from 5 rats per group (0.5 mL/rat). The exhaustive extraction of polar lipids from serum was described previously (*18*). Internal standard (IS) (5 ng of genistein + 10 ng of biochanin A per μL serum) was added to serum before preparation of LC−MS samples. Next, the pooled serum (40 μL) was combined with 80 μL of 100% methanol (final methanol concentration is 67%) and vortexed vigorously for 10 min followed by sonication in ice water for 10 min. The mixture was centrifuged at relative centrifuge force 153393*g* for 20 min, and the supernate as an extract was removed carefully to a sample vial. The extraction process was repeated twice with 80 μL of 100% methanol and 80 μL of 90% aqueous methanol, respectively. The volume of extracts from three extractions in the sample vial was brought to 300 μL with 100% methanol and mixed well for LC−MS/MS.

In order to examine the efficiency of the extractions, the pellet from the third extraction was extracted with 100% methanol (80 μL) and methanol: methylene chloride (1:2) (80 μL). The combined extract from these two

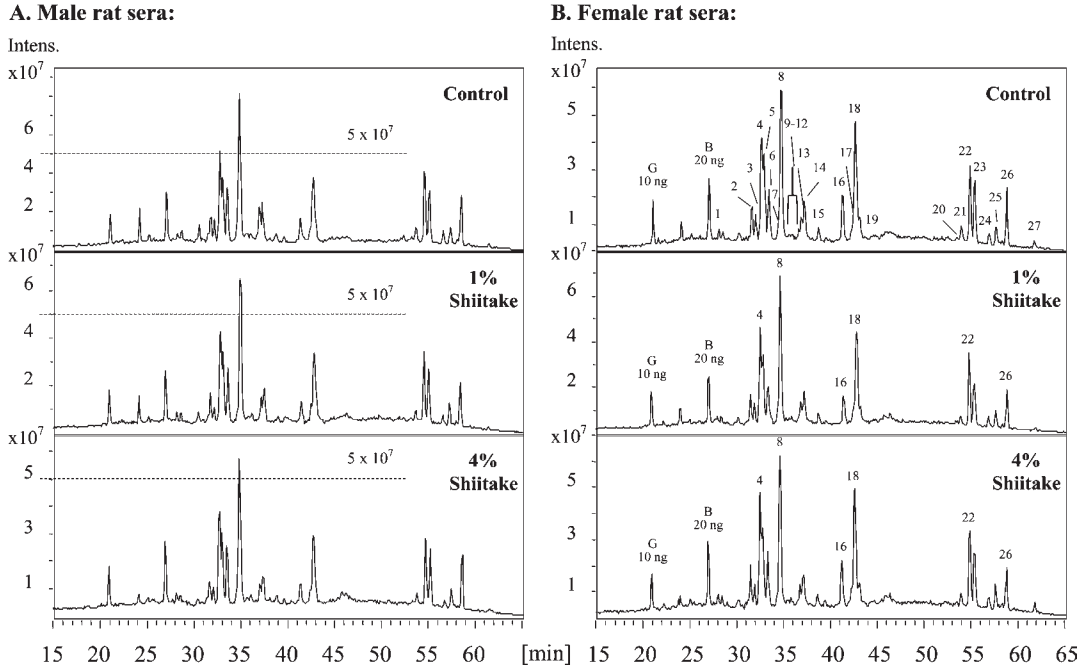

Figure 2. LC−MS total ion chromatograms (TIC) of the relative polar components of rat sera. Control = sera pooled from rats fed casein diet; 1% shiitake = sera pooled from rats fed 1% shiitake mushroom diet; 4% shiitake = sera pooled from rats fed 4% shiitake mushroom diet. G = IS genistein, B = IS biochanin A.

extractions was evaporated to dryness under N_2 followed by drying in a freeze-dryer. The dried extract was reconstituted with 150 μL of 95% methanol and analyzed by LC−MS/MS in order to examine the efficiency of the three previous extractions.

LC−MS Analysis. Fifteen microliters of the serum extract was directly analyzed without any purification. Fifteen microliters of sample was equivalent to 2.0 μL of serum containing 10 ng of genistein and 20 ng of biochanin A as IS. Lysophospholipid and free fatty acid standards were analyzed in the same LC−MS/MS conditions. LC−MS/MS analysis was performed using a Bruker Esquire-LC multiple ion trap mass spectrometer equipped with an Agilent 1100 series liquid chromatograph. An HP ChemStation was used for data collection and manipulation. The column used was a 150 mm × 4.6 mm i.d., 5 μm, Eclipse XDB-C8, with a 4 mm × 4 mm i.d. guard column of the same material (Agilent Technologies, Wilmington, DE). The LC gradient was solvent B (0.1% formic acid/acetonitrile) in solvent A (0.1% formic acid/H_2O) at a flow rate of 0.5 mL/min: 20−58% in 20 min; 58−64% from 20−33 min; 64−100% from 33−60 min; held at 100% from 60−65 min and finally back to 20% in 70 min. The constituents in the eluant were monitored with a diode-array detector (DAD) followed by electrospray ionization mass spectrometry (ESI-MS) with automatic MS/MS in both negative and positive ion modes. The DAD was set at the following five wavelengths: 200 ± 10, 240 ± 10, 290 ± 10, 320 ± 10 and 355 ± 10 nm. For optimum MS analysis, 10 mM ammonium acetate (for negative ion mode) or 2% formic acid (for positive ion mode) in methanol were used as ionization reagents and added at a flow rate of 0.1 mL/min via a tee in the eluant stream of the HPLC just prior to the mass spectrometer. Conditions for ESI-MS analysis included a capillary voltage of 3200 V, a nebulizing pressure of 33.4 psi, a drying gas flow of 10 mL/min, and a temperature of 340 °C. Parameters that control the API interface and the mass spectrometer were set via the Smart Tune with compound stability of 50% and trap drive level of 50%. Ion charge control (ICC) was set to "on", and the other settings include the following: target, 5000; maximum accumulation time, 50.00 ms; scan, m/z 80.00 to 850.00; averages, 10; and rolling averaging, off. Conditions for automatic MS/MS were as follows: width of the isolation, 4.0; fragmentation amplitude, 1.00 V; and number of parents, 1.

Quantification was performed by LC−MS using peak areas of anions generated from the extracted ion chromatogram (EIC). The areas of deprotonated molecular ions $[M - H]^-$ were used for quantification of free fatty acids, lysophosphatidylethanolamines, lysophosphatidylinositols, lysophosphatidic acids and lysophosphatidylglycerols. The areas of adduct ions $[M + COO]^-$ were used for quantification of lysophosphatidylcholines. Thirteen standards were used to create calibration curves including 16:0a/lysophosphatidylcholine (Std 1), 18:1a/lysophosphatidylcholine (Std 2), 18:0a/lysophosphatidylcholine (Std 3), *cis*-4,7,10,13,16,19-docosahexaenoic acid (Std 4), arachidonic acid (Std 5), linoleic acid (Std 6), palmitic acid (Std 7), oleic acid (Std 8), stearic acid (Std 9), 13(*S*)-hydroxyoctadeca-9*Z*,11*E*-dienoic acid (Std 10), 15(*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid (Std 11), 12(*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (Std 12) and leukotriene B4 (Std 13). Calibration curves of each standard were created with peak areas from nine concentrations (800, 400, 200, 100, 50, 25, 12.5, 6.25, and 3.125 ng) using Microsoft Excel software. The concentrations of individual components were determined from calibration curves of the corresponding standard. Compounds were quantified by calibration curves of their structure related standards when their corresponding standards were not available. Calibration curves of Std 1 were used for quantification of compounds **6** and **8**; Std 2 for **1**−**5**, **7** and **9**−**15**; Std 3 for **16**−**19**; Std 4 for **21**; Std 5 for **22**; Std 6 for **20**, **23**, **24**; Std 7 for **25**; Std 8 for **26**; Std 9 for **27**; (Figure **2**).

Statistical Analyses. Results are expressed as means ± SD of at least three replicate determinations for each assay. Using SigmaStat (Systat Software, Inc., point Richmond, CA), the data were analyzed by one-way analysis of variance, followed by Dunnett's post hoc test to compare treatments vs the control. P values below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Experimental Diets. The animal experiment was designed to evaluate effects of shiitake on serum polar lipids in male and female rats. However, the insoluble dietary fiber, α-cellulose, can reduce intestinal transit time and possesses some bioactivity (*22*, *23*). In order to eliminate any "cellulose effect" on dietary intake, the contents of final total crude fiber (α-cellulose plus shiitake fiber) were equalized for all 3 diets (control, 1% shiitake diet and 4% shiitake diet) (*16*). Thus, the changes described in this study are specific to shiitake and could not be attributed to "cellulose effect".

Extraction and Identification of Serum Components. The methanol extraction process (three times of extractions) was considered nearly complete, because there were no detectable IS and polar lipid components in the combined extract from fourth and fifth extractions. To avoid loss and contamination of analytes in

Table 1. Effects of Shiitake Diet on the Levels of Serum Lysophospholipids in Male Rats[a]

no. in **Figure 1**	casein diet (μmol)	1% shiitake diet (μmol)	effect (1% shiitake/control)	4% shiitake diet (μmol)	effect (4% shiitake/control)
			Lysophosphatidylcholines		
1	3.79 ± 0.97	1.76 ± 0.41 a	0.46 ± 0.11	1.37 ± 0.24 ab	0.36 ± 0.06
2	17.25 ± 1.28	13.06 ± 0.21 a	0.76 ± 0.01	9.02 ± 0.49 ab	0.52 ± 0.03
3	9.42 ± 0.26	5.45 ± 0.07 a	0.58 ± 0.01	4.71 ± 0.09 ab	0.50 ± 0.01
4	84.21 ± 5.32	65.23 ± 4.52 a	0.77 ± 0.05	46.90 ± 1.16 ab	0.56 ± 0.01
5	57.12 ± 4.29	35.86 ± 0.84 a	0.63 ± 0.01	30.12 ± 2.16 ab	0.53 ± 0.04
6	52.88 ± 2.99	39.56 ± 1.71 a	0.75 ± 0.03	32.46 ± 0.93 ab	0.61 ± 0.02
8	344.35 ± 16.81	256.94 ± 9.08 a	0.75 ± 0.03	220.84 ± 27.72 ab	0.64 ± 0.08
9, **10**	7.99 ± 0.73	4.61 ± 0.20 a	0.58 ± 0.02	2.79 ± 0.34 ab	0.35 ± 0.04
11	1.12 ± 0.19	0.53 ± 0.09 a	0.47 ± 0.07	0.31 ± 0.13 ab	0.27 ± 0.14
12	1.76 ± 0.11	1.00 ± 0.01 a	0.57 ± 0.01	1.42 ± 0.07 ac	0.81 ± 0.04
13, **14**	56.44 ± 2.33	32.91 ± 1.33 a	0.58 ± 0.02	21.47 ± 0.78 ab	0.38 ± 0.01
15	2.37 ± 0.33	1.60 ± 0.22 a	0.67 ± 0.11	1.46 ± 0.05 ab	0.62 ± 0.03
16	27.08 ± 1.33	16.51 ± 1.31 a	0.61 ± 0.05	13.67 ± 1.12 ab	0.50 ± 0.04
18	169.84 ± 2.38	105.12 ± 3.32 a	0.62 ± 0.02	76.92 ± 3.09 ab	0.45 ± 0.02
19	1.88 ± 0.23	1.19 ± 0.20 a	0.64 ± 0.11	0.84 ± 0.28 ab	0.45 ± 0.15
subtotal	837.51 ± 0.49	581.34 ± 7.56 a	0.69 ± 0.01	464.29 ± 29.75 ab	0.55 ± 0.03
			Lysophosphatidylethanolamines		
7	7.77 ± 0.60	5.82 ± 0.49 a	0.75 ± 0.06	4.82 ± 0.47 ab	0.62 ± 0.06
17	16.40 ± 1.36	15.47 ± 0.29	0.94 ± 0.02	12.42 ± 1.23 ab	0.76 ± 0.08
subtotal	24.17 ± 1.28	21.29 ± 0.32 a	0.88 ± 0.01	17.24 ± 0.86 ab	0.71 ± 0.04
total lysophospholipid in serum	861.69 ± 11.06	602.63 ± 7.80 a	0.70 ± 0.01	481.53 ± 30.58 ab	0.56 ± 0.03

[a] Values are expressed as means ± SD. Letters "a" and "b" indicate significant decrease ($P < 0.05$; a, 1% or 4% shiitake diet vs casein diet; b, 4% shiitake diet vs 1% shiitake diet). Letter "c" indicates significant increase ($P < 0.05$; 4% shiitake diet vs 1% shiitake diet).

extract, the extraction process did not include filtration and concentration. The crude extract was directly analyzed without further purification. Using LC−MS/MS, samples (crude extract) were separated by RP-HPLC and analyzed by DAD and ESI-MS with automatic MS/MS in both negative and positive ion modes. Polar lipid compounds could yield unique fragments and neutral losses in ESI-MS, which are effective for structural elucidation of the polar lipid compounds. The major unique fragments and neutral losses from collision-induced dissociation (CID) in positive mode are $[M + H - 77]^+$ and cation at m/z 184 u for lysophosphatidylcholine; $[M + H - 61]^+$ and 141 u neutral loss for lysophosphatidylethanolamine; $[M + H - 180]^+$ and 260 u neutral loss for lysophosphatidylinositol; cations at m/z 155 and 154 u neutral loss for lysophosphatidic acid; cation at m/z 155 and 172 u neutral loss for lysophosphatidylglycerol. In negative mode, they are $[M - CH_3]^-$ and 225 u neutral loss for lysophosphatidylcholine; anion at m/z 214 u and 197 u neutral loss for lysophosphatidylethanolamine; $[M - H - 180]^-$ and 316 u neutral loss for lysophosphatidylinositol; anion at m/z 153 u and m/z 171 u for lysophosphatidic acid, anion at m/z 153 u and 228 u neutral loss for lysophosphatidylglycerol (*24*). Since samples and standards were analyzed by the same LC−MS/MS condition, serum polar lipid components were identified by comparison of their HPLC retention time and ESI-MS/MS data with those of their corresponding standards or data from our previous study (*18*, *24*, *25*). Fifty lysophospholipids and 32 free fatty acids were identified or characterized in serum of rat.

Quantification of Serum Components. Fifteen microliters of sample was analyzed by LC−MS. The analyzed sample was equivalent to 2.0 μL of serum containing 10.0 ng of genistein and 20.0 ng of biochanin A as IS. Since genistein and biochanin A generated both DAD and ESI peaks in LC−MS/MS analysis, these two standards were used as IS for monitoring the reproducibility of extractions, and stability and sensitivity of LC−MS/MS analysis. Extraction and analysis were considered very reproducible based on the recovery rates of standards. For example, the means of peak areas of anions of IS genistein and biochanin A were $1.12 \times 10^8 \pm 3.38 \times 10^6$ and $1.81\ X10^8 \pm 5.02 \times 10^6$ (mean ± SD, $n = 32$), respectively. LC−MS/MS profiles of serum polar lipids are shown in **Figure 2**. The concentrations of 27 polar lipid components in serum were determined using LC−MS EIC peak areas and calibration curves of standards. The individual EIC peak for the quantification did not have any interference with the EIC peaks of other components in the serum extract. The calculated values are presented in **Tables 1**−**4**. Since LC−MS had different sensitivity to different compounds, the calculated value, which was not obtained from the calibration curves of its corresponding standard, did not exactly represent its real concentration in serum.

Shiitake Effects on Serum Polar Lipids. Lysophospholipids and free fatty acids are major lipid components in serum. Lysophosphatidylcholine is recognized as an important factor underlying signal transduction and plays a functional role in various diseases, including atherosclerosis, diabetes, and cancer mediated by lysophosphatidylcholine specific G-protein-coupled receptors (*26*, *27*). In the plasma of ovarian cancer patients, the percentages of palmitoyl- and stearoyl-lysophosphatidylcholine species are significantly higher than in control subjects, whereas oleoyl and particularly linoleoyl lysophosphatidylcholine are significantly lower than in control subjects (*28*). Furthermore, the relative concentration between regioisomers of lysophospholipid (*sn*-1or *sn*-2 lyso subspecies) is also important because generation of these isomers would influence their removal from blood, their uptake and acylation, and/or their catabolism in tissues (*29*, *30*). These findings emphasize a need to analyze individual polar lipid components in serum for evaluating the effect of shiitake consumption. Shiitake has been reported to exhibit a hypocholesterolemic effect in rats (*11*, *12*). Also, consumption of a 4% shiitake diet elicited an increase in active energy expenditure in rats compared to those fed a control diet in our previous study, thereby resulting in leaner and lighter male rats (*16*). In the present study, the effect of consumption of shiitake on individual serum lysophospholipid and free fatty acid

Table 2. Effects of Shiitake Diet on the Levels of Free Fatty Acids in Serum of Male Rats[a]

no. in **Figure 1**	casein diet (μmol)	1% shiitake diet (μmol)	effect (1% shiitake/control)	4% shiitake diet (μmol)	effect (4% shiitake/control)
20	13.61 ± 0.44	9.04 ± 0.45 a	0.66 ± 0.03	8.86 ± 0.36 a	0.65 ± 0.03
21	69.79 ± 7.62	23.10 ± 2.28 a	0.33 ± 0.03	17.62 ± 2.48 ab	0.25 ± 0.04
22	133.73 ± 14.34	65.61 ± 2.06 a	0.49 ± 0.02	59.94 ± 3.32 ac	0.45 ± 0.02
23	307.00 ± 13.62	201.69 ± 11.59 a	0.66 ± 0.04	165.83 ± 10.45 ab	0.54 ± 0.03
24	15.35 ± 0.96	7.61 ± 0.55 a	0.50 ± 0.04	5.54 ± 0.46 ab	0.36 ± 0.03
25	244.79 ± 11.65	175.12 ± 7.49 a	0.72 ± 0.03	163.58 ± 12.55 ab	0.67 ± 0.05
26	534.68 ± 38.34	332.86 ± 14.02 a	0.62 ± 0.03	381.21 ± 26.36 ac	0.71 ± 0.05
27	89.61 ± 13.71	84.49 ± 5.08	0.94 ± 0.06	81.60 ± 6.32	0.91 ± 0.07
total free fatty acid in serum	1408.57 ± 44.5	899.52 ± 5.16 a	0.63 ± 0.01	884.18 ± 9.94 a	0.62 ± 0.01

[a] Values are expressed as means ± SD. Letters "a" and "b" indicate significant decrease ($P < 0.05$; a, 1% or 4% shiitake diet vs casein diet; b, 4% shiitake diet vs 1% shiitake diet). Letter "c" indicates significant increase ($P < 0.05$; 4% shiitake diet vs 1% shiitake diet).

Table 3. Effects of Shiitake Diet on the Levels of Serum Lysophospholipids in Female Rats[a]

no. in **Figure 1**	casein diet (μmol)	1% shiitake diet (μmol)	effect (1% shiitake/control)	4% shiitake diet (μmol)	effect (4% shiitake/control)
			Lysophosphatidylcholines		
1	0.99 ± 0.07	1.70 ± 0.30 a	1.71 ± 0.30	1.44 ± 0.22 a	1.45 ± 0.22
2	11.86 ± 0.69	15.55 ± 2.13 a	1.31 ± 0.18	12.82 ± 0.59 b	1.08 ± 0.05
3	7.04 ± 0.58	7.62 ± 0.52	1.08 ± 0.07	6.02 ± 0.36 b	0.86 ± 0.05
4	61.50 ± 10.65	74.38 ± 7.58 a	1.21 ± 0.12	70.06 ± 6.42	1.14 ± 0.10
5	44.29 ± 2.26	49.29 ± 2.39	1.11 ± 0.05	37.81 ± 0.51 b	0.85 ± 0.01
6	30.33 ± 2.69	31.91 ± 2.79	1.05 ± 0.09	32.05 ± 3.11	1.06 ± 0.10
8	207.68 ± 28.28	239.20 ± 7.69 a	1.15 ± 0.04	208.93 ± 14.87	1.01 ± 0.07
9, **10**	3.13 ± 0.23	4.29 ± 0.24 a	1.37 ± 0.08	3.44 ± 0.27 b	1.10 ± 0.09
11	0.92 ± 0.20	0.75 ± 0.12 a	0.82 ± 0.16	0.80 ± 0.10	0.87 ± 0.12
12	2.30 ± 0.11	2.76 ± 0.29 a	1.20 ± 0.15	1.26 ± 0.28 bc	0.55 ± 0.11
13, **14**	22.95 ± 1.12	29.10 ± 1.97 a	1.27 ± 0.09	23.26 ± 0.78 b	1.01 ± 0.03
15	1.63 ± 0.16	2.43 ± 0.34 a	1.49 ± 0.22	2.19 ± 0.05 a	1.35 ± 0.02
16	36.23 ± 2.19	39.73 ± 5.88	1.10 ± 0.16	34.16 ± 3.32	0.94 ± 0.09
18	238.76 ± 22.98	242.56 ± 31.75	1.02 ± 0.13	216.93 ± 22.12	0.91 ± 0.09
19	0.58 ± 0.27	0.89 ± 0.11 a	1.53 ± 0.20	0.57 ± 0.09	0.98 ± 0.16
subtotal	670.19 ± 69.7	742.14 ± 55.83	1.11 ± 0.08	651.75 ± 46.19	0.97 ± 0.07
			Lysophosphatidylethanolamines		
7	4.20 ± 0.65	5.49 ± 0.58 a	1.31 ± 0.14	4.51 ± 0.44	1.07 ± 0.11
17	11.65 ± 0.53	15.80 ± 1.60 a	1.36 ± 0.14	13.22 ± 0.28	1.14 ± 0.02
subtotal	15.84 ± 0.16	21.29 ± 1.75 a	1.34 ± 0.11	17.73 ± 0.49	1.12 ± 0.03
total lysophospholipid in serum	686.03 ± 69.58	763.43 ± 57.54	1.11 ± 0.08	669.48 ± 46.63	0.97 ± 0.07

[a] Values are expressed as means ± SD. Letter "a" indicates significant increase ($P < 0.05$; 1% or 4% shiitake diet vs casein diet). Letters "b" and "c" indicate significant decrease ($P < 0.05$; b, 4% shiitake diet vs 1% shiitake diet; c, 4% shiitake diet vs casein diet).

Table 4. Effects of Shiitake Diet on the Levels of Free Fatty Acids in Serum of Female Rats[a]

no. in **Figure 1**	casein diet (μmol)	1% shiitake diet (μmol)	effect (1% shiitake/control)	4% shiitake diet (μmol)	effect (4% shiitake/control)
20	7.65 ± 0.43	9.05 ± 0.36 a	1.18 ± 0.05	8.15 ± 0.33 b	1.07 ± 0.04
21	43.56 ± 2.60	32.06 ± 5.39 c	0.74 ± 0.12	32.41 ± 3.21 c	0.74 ± 0.07
22	67.86 ± 7.17	83.14 ± 0.40 a	1.23 ± 0.01	77.87 ± 9.20	1.15 ± 0.14
23	166.64 ± 24.69	199.16 ± 7.49 a	1.20 ± 0.04	191.55 ± 4.06 a	1.15 ± 0.02
24	10.27 ± 1.40	10.98 ± 0.47	1.07 ± 0.05	9.89 ± 0.75	0.96 ± 0.07
25	156.20 ± 5.19	197.31 ± 26.86 a	1.26 ± 0.17	196.69 ± 7.39 a	1.26 ± 0.05
26	403.58 ± 42.23	288.39 ± 29.21 c	0.71 ± 0.07	173.49 ± 15.17 bc	0.43 ± 0.04
27	135.03 ± 16.46	131.39 ± 24.01	0.97 ± 0.18	142.68 ± 32.23	1.06 ± 0.24
total free fatty acid in serum	989.66 ± 75.20	949.51 ± 81.19	0.95 ± 0.08	831.63 ± 56.28 c	0.84 ± 0.06

[a] Values are expressed as means ± SD. Letter "a" indicates significant increase ($P < 0.05$; a, 1% or 4% shiitake diet vs casein diet). Letters "b" and "c" indicate significant decrease ($P < 0.05$; b, 4% shiitake diet vs 1% shiitake diet; c, 1% or 4% shiitake diet vs casein diet).

were evaluated. The male rats fed the shiitake diets had lower levels of almost all individual serum lysophospholipid and free fatty acid components than the male rats in the control group (**Figure 2**) (**Table 1** and **2**). Therefore, the total serum levels of polar lipids in shiitake diet male rats were significantly lower than that of the male control group. The levels of lysophospholipid and free fatty acids in 4% shiitake diet groups were 56% and 62% of those in control, respectively (**Tables 1** and **2**). The finding is in agreement with reports that 4% shiitake diet resulted in leaner animals than the control (*16*). The serum levels of lysophospholipid and free fatty acid in the 1% shiitake diet group were, respectively, 70% and 63% relative to the control group (**Table 1** and **2**), but consumption of the 1% shiitake diet did not result in leaner rats (*16*). In contrast, consumption of shiitake diets did not

significantly affect the concentrations of serum polar lipids (**Tables 3** and **4**) or the body weight of female rats (*16*).

Mycochemical eritadenine in the fruiting body of shiitake have been documented to be a major hypocholesterolemic agent in shiitake (*15*). It is reasonable to assume that eritadenine is involved in the lowering effects on the serum polar lipid concentrations detected in the present study. However, eritadenine has been reported to have different effects on different lipid molecular species. Dietary eritadenine increased the proportion of 16:0−18:2 molecular species with a decrease in 18:0−20:4 in plasma lipoprotein phosphatidylcholine in both male rats fed cholesterol-free and cholesterol-enriched diets (*15*). The different patterns of effects between consumption of eritadenine diet and shiitake diet may suggest that, in addition to eritadenine, there are other active components in shiitake that exert hypocholesterolemic effects.

In summary, consumption of 4% shiitake diet elevated the active energy expenditure of male rats, thereby resulting in leaner animals than their control (*16*). This study further demonstrated the hypocholesterolemic effect of dietary shiitake in rats. The effect of consumption of shiitake on the concentration of serum polar lipids was determined by LC−MS. The levels of serum polar lipids in males fed shiitake diets were significantly lower than those of the control group. The effects did not significantly differ among different lysophospholipid subclasses or free fatty acid subclasses. Although eritadenine was considered a major bioactive component in shiitake for its hypocholesterolemic effect, this study suggests additional bioactive mycochemicals for the hypocholesterolemic effect in shiitake. Further work is clearly needed to isolate and structurally elucidate these bioactive mycochemicals in shiitake, and to establish their hypocholesterolemic effects.

ABBREVIATION USED

ESI-MS, electrospray ionization mass spectrometry; API, atmospheric pressure interface; API-ES, atmospheric pressure interface-electrospray; ICC, ion charge control; TIC, total ion chromatogram; EIC, extracted ion chromatogram; CID, collision-induced dissociation.

Supporting Information Available: Table 1, quantification (negative mode) of lysophosphatidyllipid with standard, Table 2, quantification (negative mode) of free fatty acid with standard, and Table 3, means and SD of IS. This material is available free of charge via the Internet at http://pubs.acs.org.

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