

NOTE

Effects of Sphingolipid Fractions from Golden Oyster Mushroom (*Pleurotus citrinopileatus*) on Apoptosis Induced by Inflammatory Stress in an Intestinal Tract *in vitro* Model

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Abstract: Previously, we reported that the polar lipid fraction from the golden oyster mushroom, *Pleurotus citrinopileatus*, suppresses colon injuries which result from apoptosis induced by inflammatory stresses *in vivo* and *in vitro* (Yamashita *et al.*, *J. Oleo Sci.*, 69, 751-757 (2020)). Here, we investigated the use of lipid classes in mushroom polar lipid fraction in alleviating colon injury using differentiated Caco-2 cells as an intestinal tract model. The mushroom polar lipid fraction was separated into four fractions using silica thin layer chromatography. Each mushroom polar lipid fraction suppressed lipopolysaccharide (LPS)-induced decreases in the viability of intestinal cells, and the effects of sphingolipid fractions were significantly stronger than those of fraction that did not contain sphingolipids. Addition of sphingolipid fractions suppressed the expression of apoptosis-related proteins (e.g., death receptors and caspases) in the LPS-treated cells. Mushroom polar lipids, especially sphingolipids suppress intestinal apoptosis induced by inflammatory stress, and highly polar sphingolipids may exert stronger suppressive effects.

Key words: apoptosis, inflammation, mushroom, sphingoid base, sphingolipid

1 Introduction

The golden oyster mushroom (*Pleurotus citrinopileatus*) has recently become a popular delicacy in East Asia due to its flavor and health benefits. Previously, we reported that ethanol extract from golden oyster mushroom suppresses intestinal injury in a mouse model of inflammatory bowel disease¹, and using *in vivo* and *in vitro* experiments, the core substances responsible for this suppression were identified within polar lipid fraction².

Polar lipids found in mushrooms are different from those found in plants and mammals³. The main difference is in the fungi-specific 9-methyl-*trans*-4,*trans*-8-sphingadienine (9-Me d18:2^{4t,8t}), which is the major sphingoid base in the structures of glucosylceramide (GlcCer) found in mushrooms⁴. The main sterol derivatives found in mushrooms are comprised of ergosterol, which is absent in plants and

mammals⁵.

Dietary GlcCer from plant sources has been reported to alleviate colon inflammation and the formation of aberrant crypt foci in colon impairment *in vitro* and *in vivo*⁶⁻⁸. The mushroom lipids contain GlcCer with a sphingoid base that differs from those of plants and other sphingolipid classes like ceramide, ceramide dihexoside (CDH), inositol phosphoryl ceramide (IPC), and glycosyl IPC (GIPC). Whether mushroom GlcCer has anti-inflammatory activity in the intestinal cells is unknown. IPC and GIPC are major sphingolipids in plants and fungi, and are markedly more abundant (2-9 fold) than GlcCer⁹⁻¹¹. However, their functions have not been determined because their purification is complex and difficult. In this study, we investigated whether lipid classes in mushroom polar lipid fraction, especially sphingolipids, can alleviate colon injury using dif-

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ferentiated Caco-2 cells treated with lipopolysaccharide (LPS) as an intestinal inflammation model. We also analyzed the suppressive mechanism of mushroom-derived lipids on LPS-induced apoptosis using an apoptosis-related protein array.

2 Experimental Procedures

2.1 Fractionation of mushroom polar lipids

Ethanol extract from the golden oyster mushroom was purchased from Three-B Corporation (Sapporo, Japan). The polar lipid fraction (Fr.) was prepared by precipitation method with cold acetone from the organic layer of the Folch procedure as previously described²⁰. The polar lipids (Fr. A to D) were separated using silica thin-layer-chromatography (TLC) with 95:12 (v/v) chloroform/methanol solvent (Fig. 1).

2.2 GC-MS analysis of sphingoid bases

The sphingoid bases in Fr. A to D were analyzed as described previously¹². Briefly, each fraction was hydrolyzed using aq. methanolic 1 N HCl at 70°C. The reaction mixture was washed with hexane and adjusted to a pH of greater than 9 with 6 N KOH. The liberated sphingoid base component was extracted with diethyl ether and converted to fatty aldehydes using NaIO₄ oxidation. The resultant fatty aldehydes were analyzed with gas chromatography (GC)-mass spectrometry (MS). The column was CP-SIL-88 (50 m × 0.25 mm × 0.2 μm) (Agilent, CA, USA); the GC-MS system was equipped with GC-2030 and GCMS-QP2020NX instruments (Shimadzu, Kyoto, Japan).

2.3 Cell culture and count

Colon cancer Caco-2 cells (Riken Gene Bank, Tsukuba, Japan) were maintained and differentiated as reported previously^{2,8}. DMEM containing 0.1% BSA was the test basal medium. Cells were incubated for 48-hour after addition of an inflammatory stimulator (LPS, 50 μg/mL) and a polar lipid fraction (Fr. A, B, C, or D, 50 μM, as GlcCer equivalent). Subsequently, the cells were rinsed with PBS (Nissui, Tokyo, Japan), trypsinized, and counted using a counting chamber (improved Neubauer chamber, EM-Techcolor; Hirshmann, Eberstadt, Germany).

2.4 Apoptosis array assay

Levels of apoptosis-related protein secretion after stimulation for 24-hour were examined using a commercial Proteome Profiler Human Apoptosis Array Kit per the manufacturer's protocol (R&D Systems, Minneapolis, MN). The detected apoptosis-related proteins included: B-cell lymphoma 2 (Bcl-2); Bcl/leukemia x (Bcl-x); cellular inhibitor of apoptosis protein (cIAP)-1; cIAP-2; claspin; p21 cyclin dependent kinase 4 inhibitor 1A (p21/CIP1); livin; survivin;

X-linked inhibitor of apoptosis (XIAP); p27 cyclin dependent kinase 4 inhibitor 1B (p27/Kip1); catalase; phospho-p53 (S15); phospho-p53 (S46); phospho-p53 (S392); phospho-Rad17 (S635); Bcl-xL/Bcl-2 associated death promoter (Bad); Bcl associated X protein (Bax); cytochrome c; high temperature requirement protein-2 (HTRA2); second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC/Diablo); TNF-related apoptosis-inducing ligand receptor 1 (TRAIL R1); TRAIL R2; fibroblast-associated (Fas); TNF receptor 1 (TNF R1); pro-caspase-3; cleaved caspase-3; clusterin; Fas-associated death domain protein (FADD); hypoxia-inducible transcription factor (HIF)-1α; heme oxygenase (HO)-1; HO-2; paraoxonase 2 (PON2); heat shock protein (HSP) 27; HSP60; and HSP70.

2.5 Statistical analysis

Corresponding groups with and without LPS treatment were evaluated using the Student's *t*-test. LPS-treated groups or non-treated groups were analyzed using variance analysis and Scheffé's test. In all analyses, differences were considered statistically significant when $p < 0.05$.

3 Results

3.1 Analyses of mushroom polar lipid fractions

As shown in Fig. 1, mushroom polar lipids were isolated into 4 fractions (from upper position of TLC, Fr. A to D). These fractions were identified by R_f values and sterol-specific reaction: Fr. A, ceramides; Fr. B, steryl glycoside (SG) derivatives; Fr. C, GlcCer; and Fr. D, CDH, IPC, GIPC, and others. The sphingoid base composition for each fraction is indicated in Fig. 2 and Table 1. Aldehydes from sphingoid bases were not detected in Fr. B. The fungi-specific 9-Me d18:2^{4t,8t} was a predominant sphingoid base in Fr. C and D, whereas 4-hydroxysphinganine (phytosphingosine, t18:0) was the major base in Fr. A. In addition, Fr. A possessed various bases as sphinganine (d18:0), *trans*-4-sphingenine (sphingosine, d18:1^{4t}), 4-hydroxyicosasphinganine (t20:0), and *trans*-4,*trans*-8-sphingadienine (d18:2^{4t,8t}); d18:0 contains 4-hydroxynonadecasphinganine (t19:0) because aldehyde from d18:0 is the same as that derived from t19:0.

3.2 Effect of mushroom polar lipid fractions on cell viability in the intestinal inflammation model

As differentiated Caco-2 cells are adherent by nature, cell viability was assessed by counting the number of adherent cells. The addition of Fr. A, B, C, and D did not induce significant decreases in the cell viability; comparatively, Fr. A and D induced significant increases in viability (Fig. 3A).

The 48-hour treatment with LPS as an inflammatory

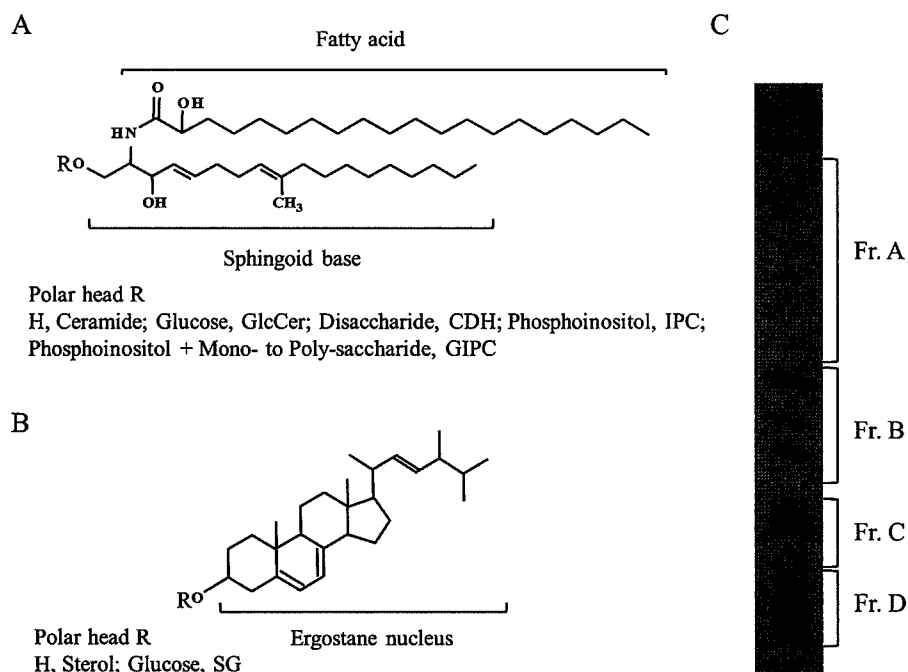


Fig. 1 Mushroom lipid structures and fractionation of polar lipids from the golden oyster mushroom using normal phase TLC.

A, Mushroom sphingolipid structures. B, Mushroom sterol structures. C, TLC profile of mushroom polar lipids. This figure is partially modified from our previous report²⁾. Mushroom polar lipids were fractionated similar to this figure. Chloroform/Methanol (95/12, v/v); Detection, 50% H₂SO₄. GlcCer, glucosylceramide; CDH, ceramide dihexoside; IPC, inositol phosphoryl ceramide; GIPC, glycosyl IPC; SG, sterylglucoside.

stress resulted in a decrease in the number of adherent cells, and the addition of the mushroom polar lipid fractions resulted in significantly higher cell viability when compared to LPS only treatment (Fig. 3B). The effects of sphingolipid fractions were significantly stronger than those of non-sphingolipid fraction (Fr. D > Fr. C ≥ Fr. A > Fr. B). When fractions with and without LPS were compared, Fr. C and D elicited similar levels of cell viability, but Fr. A and B elicited lower levels induced by LPS.

3.3 Effect of polar lipid on apoptosis-related protein expression in the intestinal inflammation model

The protein array kit used was limited to the analysis of 4 samples at a time, which necessitated the selection of LPS groups: 1) LPS + Fr. A 2) LPS + Fr. C 3) LPS + Fr. D. The values were adjusted to the LPS group average = 1.0 after being corrected using the positive control of each membrane (Fig. 4). When compared to that for a 24-hour incubation with LPS, the addition of Fr. A, C, and D resulted in lower levels of most proteins except for catalase. The significantly lower level of proteins varied between groups and was specific to the fraction: Fr. A induced 5 of 11 anti-apoptotic proteins whereas Fr. C and D induced 7 and 8 proteins, respectively; All fractions induced 2 of 4 antibody proteins; Only Fr. D induced 2 of 5 pro-apoptotic mole-

cules; Fr. A and D induced all 4 death receptors whereas Fr. C induced 3; Fr. C and D induced 2 of 2 cysteine proteases; Fr. A induced 5 of 9 other group molecules whereas Fr. C and D induced 4 and 6 proteins, respectively.

4 Discussion

In previous studies, we reported that the polar lipid fraction from the golden oyster mushroom contains components that suppressive colon injuries that result from apoptosis induced by inflammatory stresses^{1,2)}. In this study, we investigated the effects of mushroom polar lipid components and their mechanism of apoptosis suppression *in vitro*. All fractions from mushroom polar lipids maintained high levels of the cell viability, which was decreased by LPS treatment (Fig. 3B). The viabilities were higher for sphingolipid fractions of Fr. A, C, and D. The addition of Fr. A, C, or D decreased the expression of apoptosis-related proteins in intestinal cells treated with LPS (Fig. 4).

The addition of Fr. C and D resulted in identical levels of cell viability between LPS- and untreated groups, respectively (Fig. 3); Fr. D, which contained CDH, IPC, and GIPC, and produced higher viability than Fr. C, which contained GlcCer. Comparatively, the addition of LPS along with Fr. A,

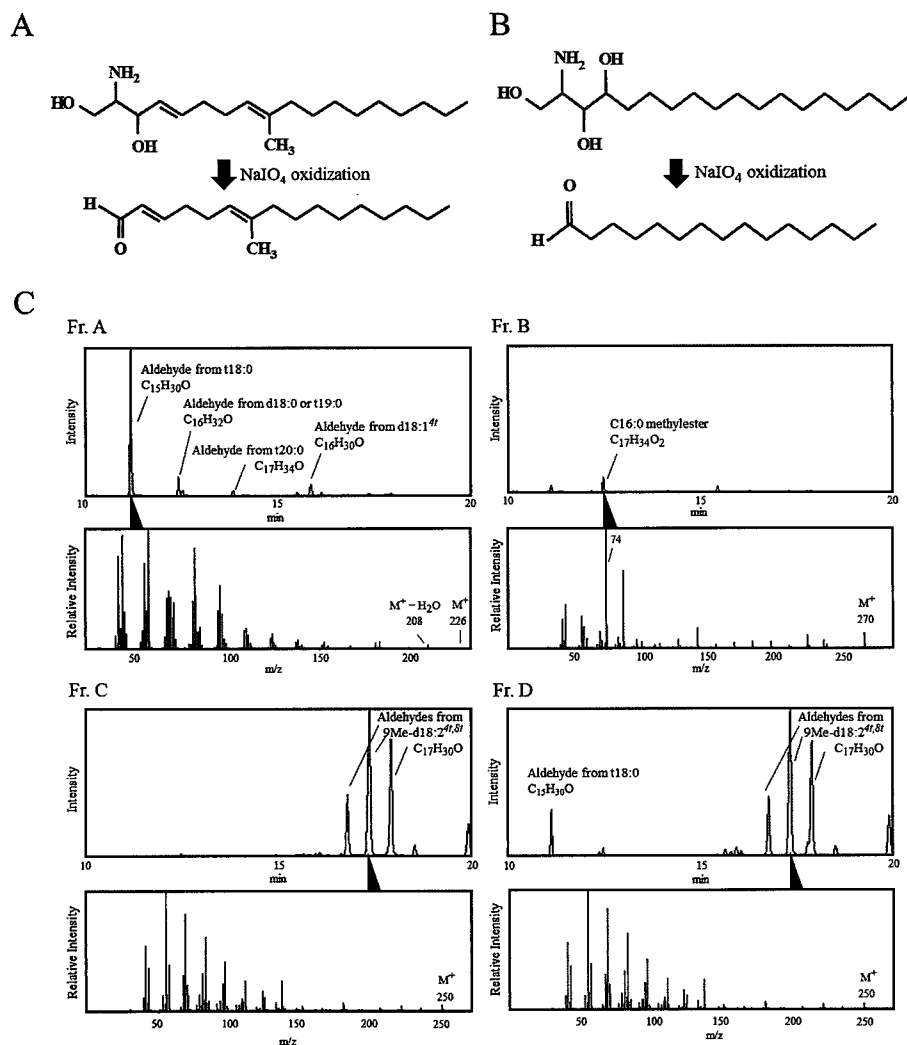


Fig. 2 Sphingoid bases in mushroom polar lipid fractions. A, NaIO₄ oxidization of dihydroxy sphingoid base. B, NaIO₄ oxidization of trihydroxy sphingoid base. C, MS analysis of aldehydes derived from each fraction. After acid hydrolysis, sphingoid bases in each fraction (Fr. A-D) were derivatized to the aldehydes by NaIO₄ oxidization. The upper layers show the total ion current chromatogram, and lower layers show mass spectrometry in the highest peak.

Table 1 Composition of sphingoid bases in mushroom polar lipid fractions.

	t18:0	t20:0	d18:0*	d18:1 ^{4t}	d18:2 ^{4t,8t}	d18:2 ^{4t,8c}	9Me-d18:2 ^{4t,8t}
Fr. A	75.3	2.8	9.7	7.5	1.8	trace	2.9
Fr. C	n.d.	n.d.	n.d.	n.d.	0.8	n.d.	99.2
Fr. D	8.8	trace	0.8	1.0	1.1	n.d.	88.3

mol%. n.d., not detected. trace, <0.5.

*d18:0 includes t19:0.

which contained ceramides, produced higher viability than treatment with LPS alone. However, this level of viability was lower than that produced by the addition of only Fr. A. Previously, we showed that plant GlcCer suppressed the apoptosis induced by LPS or TNF- α in the intestinal tract model cells used in this study⁸⁾. These results suggest that

sphingolipid classes from mushrooms also have anti-inflammatory and anti-apoptotic effects in intestinal cells. The effects are strongest in highly polar sphingolipids. Fr. D was salvaged from near the start of TLC. Therefore, it likely contains other various components. The predominant sphingoid base composed of Fr. C and D was derived from

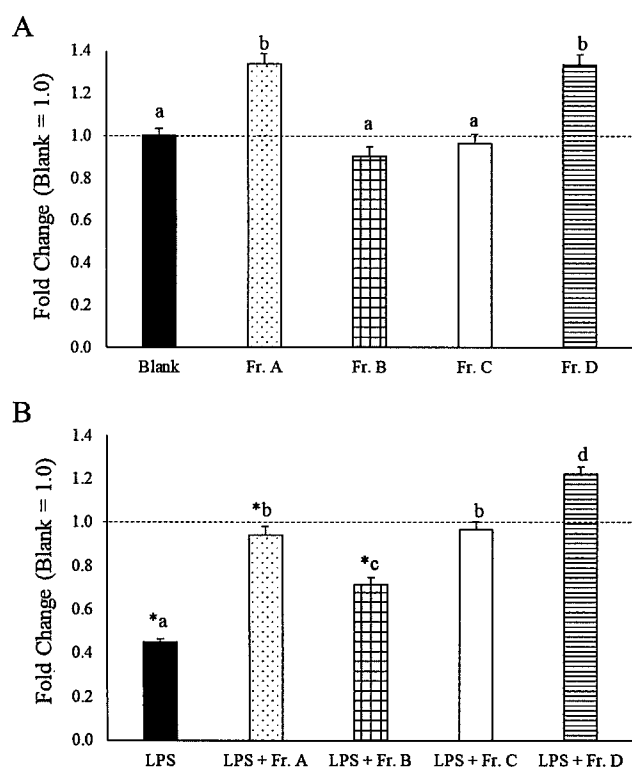


Fig. 3 Effect of mushroom polar lipid fractions on the viability of intestinal cells stimulated by inflammatory stress.

Differentiated Caco-2 cells were cultured during 48-hour treatment (LPS 50 $\mu\text{g}/\text{mL}$, each lipid fraction 50 μM as GlcCer equivalent (i.e., 36.5 $\mu\text{g}/\text{mL}$). A, Effects of mushroom polar lipid fractions on cell viability; B, Effects of mushroom polar lipid fractions on cell viability under LPS stimulation. Mean \pm SEM ($n = 4-5$). Letters a-d on the bars indicate significant differences among groups by one-way ANOVA with Scheffe's test ($p < 0.05$). Asterisks on the bars in pane B indicate significant differences from the corresponding groups in pane A by Student's t -test ($p < 0.05$).

a different base of plant-origin, 9-Me d18:2^{4t,8t}, whereas the major base of Fr. A was t18:0 (Table 1). Future studies are needed to evaluate CDH, IPC, and GIPC with higher purity to confirm this hypothesis. It would be interesting to also compare ceramides bearing 9-Me d18:2^{4t,8t}; the compounds are gotten by the hydrolysis of mushroom GlcCer.

The major sphingoid bases of Fr. A and C corresponded to fungal ceramides and GlcCer, which are predominantly composed of t18:0 and 9-Me d18:2^{4t,8t}, respectively (Table 1)^{4, 11}. In contrast, the predominant sphingoid base of Fr. D, which contains CDH, IPC, and GIPC, was 9-Me d18:2^{4t,8t}. However, it is reported that t18:0 is the predominant sphingoid base in CDH, IPC, and GIPC of fungi, and 9-Me d18:2^{4t,8t} is a small ratio (<10% of the base composition in

maitake [*Grifola frondosa*]). Generally, due to their highly polarities, IPC and GIPC are prepared from the aqueous and insoluble layers of the Folch procedure or the total extract after lipid extraction, whereas CDH is purified from the organic layer^{4, 9-11}. In this study, Fr. D was prepared from the organic layer. Therefore, Fr. D likely contained CDH, as well as IPC and GIPC bearing 9-Me d18:2^{4t,8t} and short-saccharide chains, which are fewer polar components than t18:0 and poly-saccharide chains.

The results of the apoptosis array indicated that the addition of mushroom sphingolipids resulted in reduced expression of apoptosis-related proteins as compared to LPS treatment (Fig. 4). Given that LPS treatment induced apoptosis in the same intestinal cell model^{2, 8}) and chronic colon inflammation induced expression of nearly all apoptosis-related proteins examined in the mice colon¹³), mushroom sphingolipids are thought to suppress LPS-induced expression. Mushroom sphingolipids suppress the expression of death receptors, subsequently suppress FADD as a component of death effector domain, HSPs as stress responsive factors, anti-apoptotic molecules, other downstream proteins, and finally suppress cleaved caspase-3 as an effector caspase. Due to the suppressed expression of nearly all of the anti-apoptotic molecules, mushroom sphingolipids likely inhibit the binding of LPS to Toll-like receptor 4 and/or the binding of ligands (e.g., TNF- α) to death receptors.

Under LPS stimulation, cell viability was inversely proportional to the expression of cleaved caspase-3 following the addition of mushroom sphingolipid fractions. In mushroom sphingolipids, highly polar sphingolipids are thought to have stronger anti-apoptotic effects. Dietary complex sphingolipids (e.g., sphingomyelin, GlcCer, CDH, IPC, and GIPC) are digested by intestinal enzymes and enteric bacteria to sphingoid bases via ceramides. Their bases are absorbed into lymph and the undigested lipids are excreted in the feces¹³). In addition, ceramide forms are slightly absorbed¹⁴) and the intestinal absorption rate *in vitro* of d18:1^{4t} as a major base of mammals is higher than that of 9-Me d18:2^{4t,8t} as a fungi-specific base¹⁵). Thus, in this *in vitro* study without enteric bacteria, Fr. C and D, which were composed of 9-Me d18:2^{4t,8t} and have complicated polar heads, are thought to have been absorbed significantly less than ceramides composed of d18:1^{4t} and t18:0 as major components of mammalian bases. In our previous study of plant GlcCer, following a 48-hour incubation of GlcCer with/without intestinal cells, the sum of GlcCer levels in the medium and on the cell surface was nearly identical to the level in the cell-free medium⁸). Therefore, it is quite likely that complex sphingolipids have extracellular actions. For example, they may bind to the cell surface and affect the membrane fluidity or receptor interactions, as described above.

In conclusion, mushroom lipids with sphingoid bases

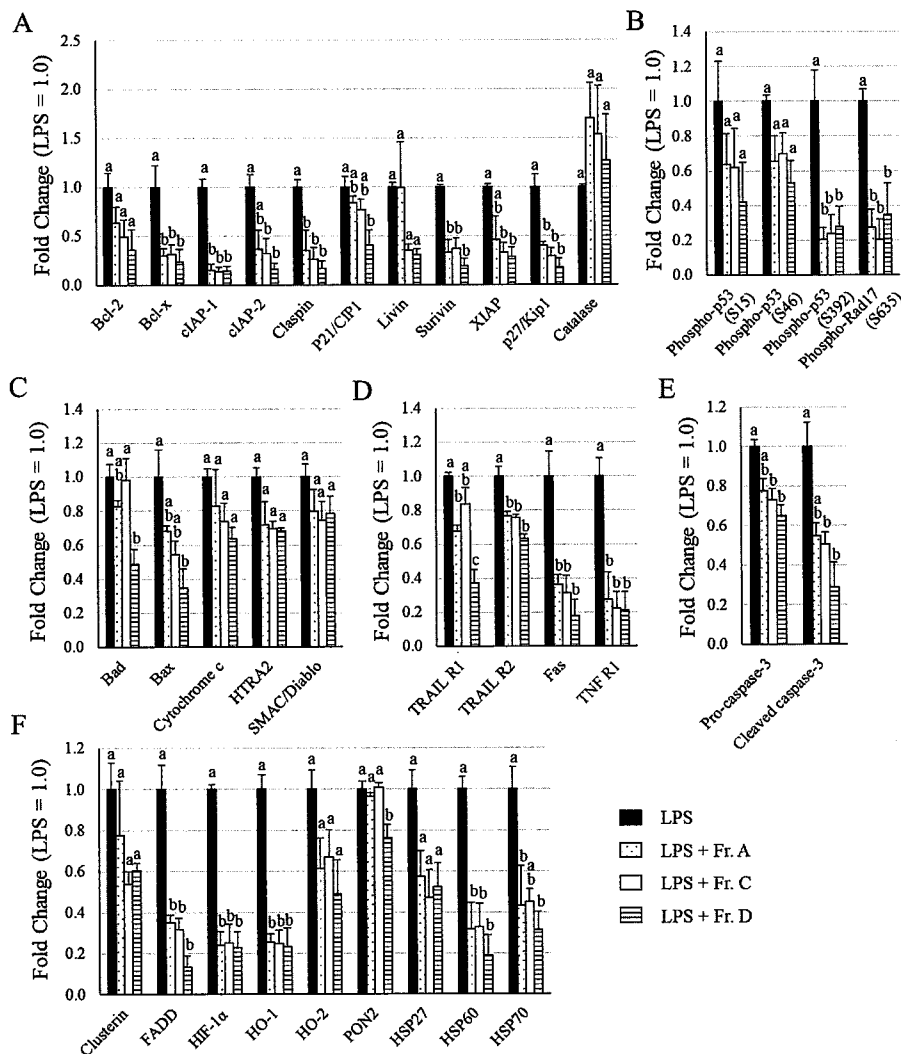


Fig. 4 Effect of mushroom polar lipid fractions on apoptosis-related proteins in intestinal cells stimulated by inflammatory stress. Differentiated Caco-2 cells were cultured during 24-hour treatment using LPS with/without 50 μM mushroom lipid fraction. A, Anti-apoptotic molecules; B, Antibody proteins; C, Pro-apoptotic molecules; D, Death receptors; E, Cysteine proteases; F, Others. Mean ± SEM (n = 4). Letters on the bars indicate significant differences among groups by one-way ANOVA with Scheffe's test (p < 0.05).

and/or saccharides protect intestinal cells from inflammatory stresses. As sphingolipids have higher polarity, they have the stronger effects on suppression of coion inflammation.

Conflict of Interest

The authors declare no conflict of interest.

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