EFFECT OF EXOGENOUS SPHINGOLIPIDS ON GROWTH AND METABOLISM IN SURFACE CULTURES OF BASIDIAL FUNGI

SVETLANA V. SENIK[1], E.R. KOTLOVA[1], N.V. PSURTSEVA[1], A.V. NOVIKOV[2], V.A. KHANIN[1], A.L. SHAVARDA[1]

[1] Komarov Botanical Institute of the Russian Academy of Sciences
Prof.Popov st., 2, St. Petersburg, Russia

[2] Institute for Analytical Instrumentation of the Russian Academy of Sciences
Rizhsky Pr., 26, St. Petersburg, Russia
senik_sv@mail.ru

ABSTRACT

Sphingolipids (sphingoid bases, ceramides and glycosylceramides) being components of signal cascades and/or lipid rafts were shown to regulate diverse processes in fungi. Particularly as it was demonstrated, glycosylceramides (GlCer) are involved in the regulation of fungal cell growth and differentiation. In this study we analyzed a range of modifications including changes in cell growth, morphology and metabolic pathways following the supplementation of medium by sphingolipids (GlCer and sphingoid bases) extracted from different organisms. Our findings suggest that effects of exogenous sphingolipids on growth rate and metabolic profiles of *Flammulina velutipes* can vary depending on the structure of sphingoid base and acyl group. The treatment of *F. velutipes* cultures with the exogenous GlCer from animal, plant and mushroom sources reveals growth-inhibitory and fruiting-stimulatory activity of these lipids.

Keywords: Basidial Fungi; *Flammulina velutipes*; Sphingolipids; Membrane Lipids; Growth Regulation

INTRODUCTION

Fungal growth, differentiation and morphogenesis are modulated by a number of universal and specific regulatory systems including adenylate cyclases, MAP kinases, G-proteins, as well as cascades mediated by lipid molecules. One of the most intriguing group of bioactive lipids is complex sphingolipids, which consist of amino-alcohol backbones (sphingoid bases) N-acylated by fatty acids and O-glycosylated by one or more sugar residues, mainly glucose and/or galactose.

Previously considered to play only a structural role in cell membranes, sphingolipids together with their precursors and the breakdown products are now also recognized as the important part of the signaling systems that regulate cell functions. Particularly, GlCer with one sugar residue (monohexosylceramides) has attracted increasing attention, because they were found to be highly bioactive molecules mediating growth, proliferation and apoptosis. In fungi they exhibit some specific activities participating in cell recognition, yeast-mycelium phase transition, budding, spore germination and fruiting [1, 2, 3, 4]. However, the study of GlCer functions in the regulation of fungal growth is in its infancy.

Recently it has been shown that functional activity of GlCer strongly depends on their structural features: length of carbon chains, number and position of double bonds in sphingoid bases and fatty acids [2, 5].

There are some differences in GlCer structures among plants, animals and fungi. Whereas animals have GlCer derived from sphingosine (d18:1^4) and phytosphingosine (t18:0), plants predominantly accumulate GlCer with 4,8-sphingadienine (d18:2^{4,8}) as a sphingoid base. Fungal GlCer have a number of structural features, including 9-methyl group branching of the sphingoid base.

This work was carried out to investigate morphological and metabolic effects of exogenous sphingoid bases and GlCer (monohexosylceramides) from animal, plant and fungi sources on the basidiomycete *Flammulina velutipes*. Poviding data about the cell biochemical status, the metabolom analysis is an excellent approach for revealing the sphingolipid functions.

MATERIALS AND METHODS

Fungi Material and Growth Conditions. The culture of basidial fungus *Flammulina velutipes* (Curt.:Fr.)Sing. (strain 1483 from the collection of Komarov Botanical Institute RAS) was used as a model. The mycelium was spotted on the ale-wort agar medium in the center of a Petri dish and it was grown at 25ºC as a surface culture for 8 days in darkness.

Experimental Conditions. To study the effects of exogenous sphingolipids the medium was supplemented by animal GlCer (from bovine brain, Sigma, Germany), mushroom GlCer (extracted from fruit bodies of basidiomycete *Pholiota nameko*), plant GlCer (extracted from wheat roots) and synthetic sphingosine (Sigma, Germany) in the concentrations of 0.04 mg/ml of the medium. All the sphingolipids were previously dissolved in ethanol (the final concentration of ethanol was 0.4 μl/ml, including control samples).

Bioassay of Growth and Fruiting Activity. Two days after the inoculation of *F. velutipes* mycelium on a Petri dish, 4 paper discs (1 cm in diameter), each charged with 10 mkl of solvent-dissolved sample, were dried and placed at the margin of the plate together with a control disc. The concentrations of each sphingolipid applied by this technique were 0, 10, 50 and 100 μg per disc. The plates with discs were further incubated for 4 weeks under the light (2000 lux) in the growth incubator (Sanyo MLR-351H).

Micromorphology of the mycelium was observed by means of light microscope Carl Zeiss Axio Scope A1 (differential interference contrast microscopy (DIC)).

Metabolome Analysis. Soluble metabolites (sugars, sugar alcohols, amino acids, organic acids) were extracted with methanol and chemically derivatized by silylation reagent BSTFA. The samples were analyzed by GC-MS. Peaks obtained were normalized using the amount of the sample dry weight and internal standard (hydrocarbon C23). The identification of analytes was carried out by the mass spectral comparison with the custom mass spectral libraries of genuine compounds. The targeted analysis of lipids (phospholipids, GlCer) extracted with isopropanol-chlorophorm (1:1) at 70ºC by the method of Nichols [6] was carried out by HPTLC [7]. Lipid classes were quantified densitometrically.

Individual molecular species of GlCer were identified by means of the electrospray ionization mass spectrometry (ESI$^+$-MS) and the low energy tandem collision-induced dissociation mass spectrometry.

Statistical Analysis of the data was performed with the Origin 7.5 software. The statistically significant changes are only discussed in the article. Data are presented as mean±se (n=3-4).

RESULTS AND DISCUSSION

ESI-MS/MS analysis of exogenous GlCer (monohexosylceramides) used in the study revealed significant differences in their structure. Whereas mushroom GlCer contained predominantly one molecular species d18:2^{9Met}/16:0-OH, animal and plant GlCer were presented by the mixture of molecules with sphingosine (d18:1), phytosphingosine (t18:0) and sphingadienine (d18:2) as a sphingoid base acylated by different fatty acids (Table 1, Figure 2). In addition, animal GlCer contained more short and middle-chain (14–18 carbons atoms) fatty acyls, than plant GlCer which comprised 60% of long-chain fatty acyls in the composition of GlCer. The fatty acids with the chain shorter than 24 atoms and/or having a 2-hydroxy group were shown to have the stronger activity for fruiting regulation [8, 9].

Table 1. Molecular composition* of GlCer used to supplement the medium

mushroom GlCer (fungal fruit bodies)	**plant GlCer** (wheat roots)	**animal GlCer** (bovine brain)
97% d18:2^{9Met}/ 16:0-OH	10% d18:1 / 16:0(OH)	37% d18:1/ 14:0(OH) 18:0(OH) 18:0
	30% d18:2 / 16:0(OH) 18:0(OH)	39% d18:1 / 24:1(OH) 20:1(OH) 24:1
	25% d18:2 / 24:1(OH) 24:0(OH) 20:1(OH)	24% t18:0 / 14:0(OH) 18:0(OH)
	35% t18:1 / 24:0(OH) 24:1(OH)	

* Structure of sphingoid base of GlCer (before slash) and fatty acyl (after the slash) of GlCer.
Symbols: the number of carbon atoms: the number of double bonds, **d** - dihydroxy-, **t** - trihydroxy-.

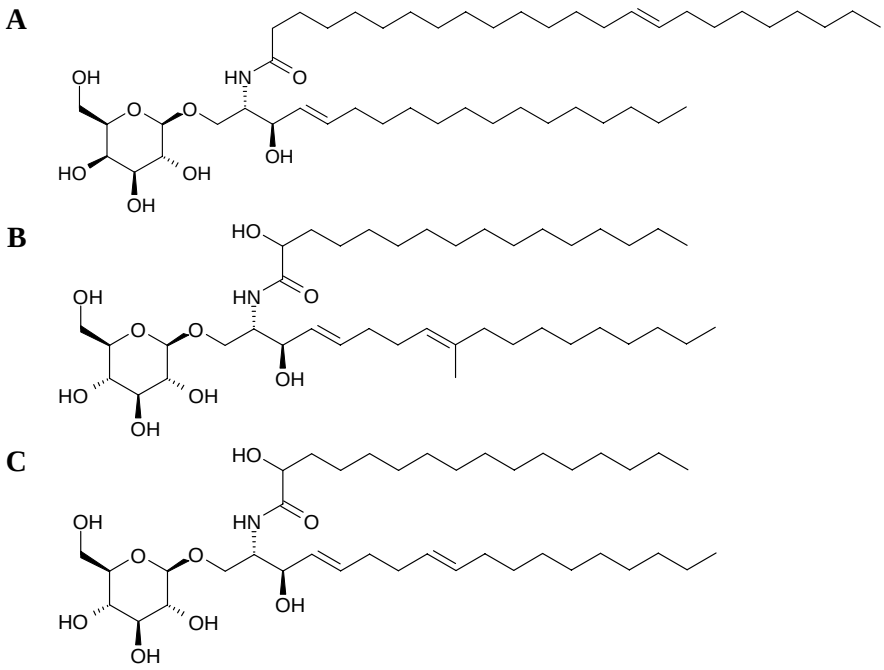

Figure 1: Structure of predominant GlCer of animal (A), mushrooms (B) and plants (C)

Growth and Fruiting Activity. Treatment of *F. velutipes* cultures with exogenous GlCer spotted on paper discs showed its growth-inhibitory effect in dose dependent manner. The growth intensity of a fungal colony was radically reduced by the highest concentrations (50 and 100µg per disc) of mushroom and plant GlCer, in a less degree by animal GlCer. These tendencies were reproduced in experiment when GlCer have been dissolved in growth medium (Figure 2).

Control Animal GlCer Mushroom GlCer Plant GlCer Sphingosine

Figure 2: Effect of GlCer on growth of *Flammulina velutipes*, the 8th day of cultivation in darkness

Morphological observations revealed that at optimal conditions the culture of *F. velutipes* was presented mainly by undifferentiated thin vegetative hyphae as well as conidiogenic ones. On the later stages of its development the part of highly differentiated cells such as thick-wall hyphae or the hyphae with granular content or curved hyphae increased. Plant GlCer induced the formation of spiral hyphae at an early stage of the cultivation (the 8th day).

Treatment of *F. velutipes* cultures with sphingosine (d18:1) also resulted in growth inhibition. Besides, this effect was accompanied by the production of vacuolated and thick-wall empty hyphae. This observation is in agreement with already described growth-inhibitory [10] and apoptosis-inducing [11] activities of sphingoid bases in fungal cells.

Bioassay with paper discs charged with GlCer was used for the study of their fruiting-inducing activity. In the case of mushroom GlCer, fruiting bodies were observed around the test discs in a dose-dependent manner. Other GlCer didn't affect fruiting. This fact is in accordance with the earlier observation that the fruiting-inducing activities of some plant GlCer were considerably lower than the ones from fungi [9]. So methyl group seems to be essential for the high fruiting-inducing activity.

According to the results obtained by means of ESI-MS/MS, GlCer of *F. velutipes* were presented by glucosylceramides containing long chain base d18:2^{9Met} and different fatty acid residues, mainly 16:0-OH (Fig. 1B). The treatment of cultures with exogenous GlCer didn't change the proportion of molecular species of GlCer.

Metabolomics. From the approximately 50 metabolites that were identified by GC-MS, the amount of more than 35 of them were significantly changed in response to supplementation by at least one of the exogenous sphingolipids. These metabolites included amino acids, sugar and sugar alcohols, organic acids, free fatty acids, phospholipids.

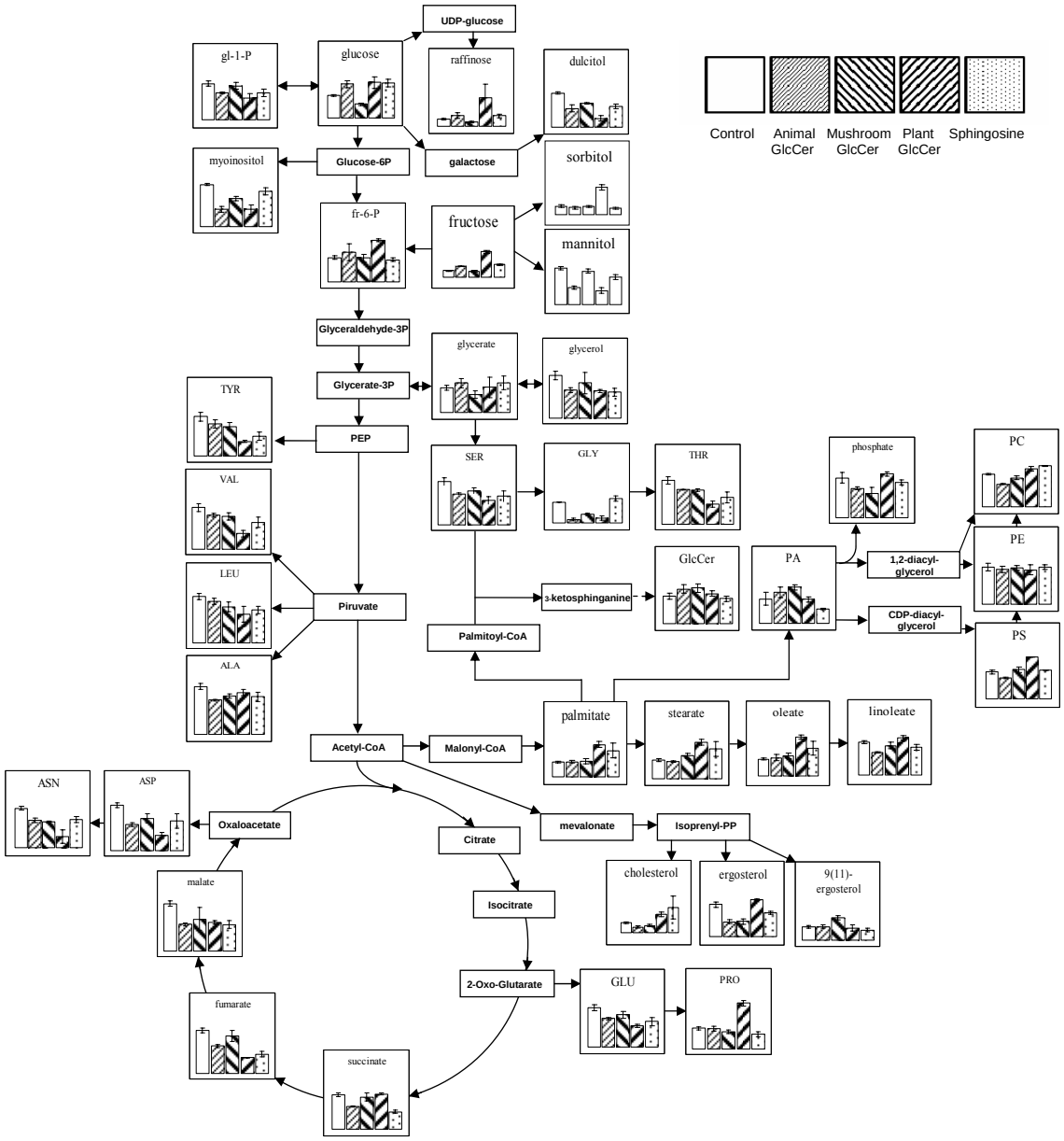

Figure 3: Shift of metabolic pathways in response to exogenous sphingolipids.
PEP, phosphoenolpyruvate; gl-1-P, glucose-1-phosphate; fr-1-P, fructose-1-phosphate; PA, phosphatidate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. Amino acids are abbreviated with their common three-letter code. Data are mean+se (n = 3–4).

Most of the metabolites demonstrated different responses to diverse molecular species of GlCer. However plant GlCer had the most significant effects on metabolom. Almost all analyzed amino acids were run down into the cultures, grown on medium supplemented by plant GlCer – TYR, VAL, LEU, ASP, ASN, GLU, SER, THR, except PRO, which amount strongly increased. Among sugars and sugar alcohols the quantity of fructose, fructose-6-phosphate, sorbitol, raffinose increased. Besides, free fatty acids (palmitate, stearate, oleate) and PS were accumulated.

General response of metabolom to exogenous GlCer independent of their source was shown by minor sugars myoinositol, dulcitol, as well as SER, GLY, Thr.

In addition, exogenous GlCer affected the PC/PE ratio that usually correlates with the stage of fungal development. Animal GlCer increased a proportion of PE that is typical for differentiated

state on the later stages of *F. velutipes* development. Plant GlCer, as well as sphingosine, by the contrary, reduced proportion of PE. Mushroom GlCer had no effect on PC/PE ratio.

Sphingosine induced some changes in sugar exchange (dulcitol, mannitol) and decreased amount of some amino acids (ASN, GLU, LEU, SER, THR, TYR) and organic acids (cuccinate, fumarate, malate). The effect of sphingosine on phospholipid metabolic pathways was unexpected. Phosphatidic acid metabolism is known to be an important target for sphingoid base action [12]. Sphingosine inhibits the activity of phosphatidic acid phosphohydrolase [13,14], the enzyme which degrades phosphatidic acid to diacylglycerol. However in present study the sphingosine treatment resulted in a depleted amount of phosphatidic acid and glycerol, but increased amount of phosphatidylcholine.

It is interesting to note that exogenous GlCer also influenced on the sterol composition. Animal and mushroom GlCer decreased by twice the amount of ergosterol and cholesterol, however under the treatment with plant GlCer the quantity of ergosterol stayed constant, but cholesterol and Δ9(11)ergosterol were accumulated. It is known that sterols and sphingolipids interact specifically in biological membranes, increasing the lipid order and creating membrane microdomains, which are shown to be signal platforms. Besides, the composition of sterols in membranes, particularly in lipid rafts, differs from kingdom to kingdom: animals contain the high amount of cholesterol; plants have sitosterol, campesterol and stigmasterol, whereas fungi have mainly ergosterol. Our data are likely to show the existence of some mechanisms that allow fungi to respond to the presence of specific sphingolipid structures in their membranes and to adjust their sterol composition accordingly. The comparable situation (changes in sphingolipid metabolism in response to specific sterol composition) has recently been shown in yeast [15].

Reduction of pool of some primary metabolite levels (fumarate, malate, SER and THR) in the combination with the growth suppression can indicate the depression of metabolic activity under the treatment by all the sphingolipids used in the work.

CONCLUSIONS

In this study, we used a combination of morphological and metabolomics approaches to determine putative functions of GlCer in basidiomycetes. We demonstrated that bioactive properties of GlCer depend on their structure.

The treatment of *F. velutipes* cultures by exogenous sphingolipids allow us to conclude the following:

1) Exogenous sphingolipids affect the growth and differentiation of fungal mycelium. 2) Regulatory effects of GlCer depend on the structure of their hydrophobic part: length and hydroxylation of fatty acyls, unsaturation and methylation of sphingoid base. 3) Exogenous sphingolipids affect phospholipid metabolism. Animal GlCer increase proportion of PE that is typical of differentiated state of fungal colony, whereas plant GlCer and sphingosine, by contrast, decrease PE. 4) Among diverse changes in metabolite profile in response to exogenous GlCer two principal tendencies can be separated: unspecific decrease in the levels of some primary intermediates (fumarate, malate, SER, GLY and THR) and specific changes in amino acid, phospholipid and sterol metabolism.

ACKNOWLEDGEMENT

This work was supported by the Federal program of Ministry of Education and Science of Russia (P 1167) and the Russian Foundation for Basic Research (09-04-0163, 11-04-01704).

REFERENCES

[1] Rhome R., Del Poeta M. (2010) Sphingolipid Signaling in Fungal Pathogens. edited by Charles Chalfant and Maurizio Del Poeta. *Adv. Exp. Med. Biol*. Landes Bioscience and Springer Science+Business Media. 688: 232 – 237.

[2] Kawai G., Ikeda Y. (1983) Chemistry and functional moiety of a fruiting-inducing cerebroside in *Schizophyllum commune*. *Biochem. Biophys. Acta*. 754: 243 – 248.

[3] Rodrigues M.L., Travassos L.R. (2000) Miranda K.R., Franzen A.J., Rozental S., De Souza W., Alviano C.S., Baretto-Bergter E. Human Antibodies against a Purified Glucosylceramide from *Cryptococcus neoformans* Inhibit Cell Budding and Fungal Growth. *Infection And Immunity*. 68: 7049 – 7060.

[4] Pinto M.R. et al. (2002) Characterization of glucosylceramids in *Pseudallescheria boydii* and their involvment in fungal differentiation. *Glicobiology.* 12: 251 – 260.

[5] Dyatlovitskaya E.V. Dependence of bioeffector role of sphingolipids on their hydrophobic fragment structure. *Biochemistry (Moskow)*. 1998. 63: 67 – 74.

[6] Nichols B.W. (1963) Separation of the lipids of photosynthetic tissues: improvements in analysis by thin-layer chromatography. *Biochem. Biophys. Acta*. 70: 417 – 425.

[7] Bening C. et al. (1993) Sulphoquinovosyldiacylglycerol is not required for photosynthetic electron transport in Rhodobacter sphaeroides but enhances growth under phosphate limitation. *PNAS*. 90: 1561 – 1565.

[8] Kawai G., Ikeda Y. (1985) Structure of biologically active and inactive cerebrosides prepared from *Schizophyllum commune*. *J. Lipid Res*. 26: 338 – 343.

[9] Kawai G. et al. (1986) Stimulatory effect of certain plant sphingolipids on fruiting of Schizophyllum commune. *J. of Biol. chem*. *261*: 779 – 784.

[10] Chung N. et al. (2001) Phytosphingosine as a specific inhibitor of growth and nutrient import in *Saccharomyces cerevisiae*. *J. of Biol. chem*. 276: 35614 – 35621.

[11] Cheng J. et al. (2003) Induction of Apoptosis by Sphingoid Long-Chain Bases in *Aspergillus nidulans*. *Mol. Cell. Biol.*. 23: 163 – 177.

[12] Aridor-Piterman O., Lavie Y., Liscovitch M. (1992) Bimodal distribution of phosphatidic acid phosphohydrolase in NG108-15 cells. Modulation by the amphiphilic lipids oleic acid and sphingosine. *Eur. J. Biochem*. 204: 561 – 568.

[13] Lavie, Y., Piterman, 0., Liscovitch, M. (1990) Inhibition of phosphatidic acid phosphohydrolase activity by sphingosine. Dual action of sphingosine in diacylglycerol signal termination. *FEBS Lett*. 277: 7 – 10

[14] Wu W.-I. et al. (1993) Carman G.M. Regulation of Phosphatidate Phosphatase Activity from the Yeast *Saccharomyces cerevisiae* by Sphingoid Bases. *The Journal of Biological Chemistry*. 268: 13830 – 13837.

[15] Guan X. et al. (2009) Functional Interactions between Sphingolipids and Sterols in Biological Membranes Regulating Cell Physiology. *Mol. Biol. Cell*. 20: 2083 – 2095.