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EXPERIMENTAL ARTICLES

# Lipid Metabolism in *Aspergillus niger* under Conditions of Heat Shock

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**Abstract**—The processes of lipid synthesis and decomposition in *Aspergillus niger* under conditions of heat shock (HS) were studied in a pulse-chase experiment with $^{14}C$-labeled sodium acetate. HS (60 min) resulted in the synthesis of phospholipids and sphingolipids intensified compared to the control, as was evident from incorporation of the labeled substrate. The same pattern was observed for neutral lipids, especially for triacylglycerides, while incorporation of the label into sterols remained almost the same. Further cultivation for 3 h in the medium without the labeled substrate resulted in a significant decrease of the label content in the membrane lipids of both the control and the experiment, although under HS conditions this decrease was much more pronounced, especially for phosphatidylcholines and phosphatidylethanolamines. A threefold increase of the label content in phosphatidic acids was observed only under HS conditions. These results indicate more intense metabolism of the membrane lipids under heat shock and suggest the degradation of the major cell phospholipids as the factor responsible for the increased level of phosphatidic acids in *A. niger* mycelium.

*Keywords: Aspergillus niger*, heat shock, lipid metabolism, phosphatidic acids

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The response of fungal cells to heat shock (HS) received much attention of researchers since an organism acquired resistance to both HS and lethal HS as the result of metabolic reorganization. There are two main hypotheses concerning membrane protection against heat impact. The first one, the hypothesis of "homeoviscous adaptation" [1] postulates the maintenance of membrane viscosity by altering the unsaturation level of the phospholipid acyl chains; the second one, of "homeophasic adaptation" [2] emphasizes the greatest importance of maintaining a certain balance between "bilayer" lipids with cylinder-shaped molecules and "nonbilayer" (cone-shaped) lipids. However, none of these hypotheses takes into account the differences between the temperature impacts within the tolerance range, which result in growth retardation, and those under HS, which results in growth cessation. We have earlier shown the fundamental differences in cell response to these two impacts. Characteristic changes in fungal metabolism, such as an increase in the levels of phosphatidic acids (PA) and trehalose, occurred only under HS (40°C, 1–6 h) and were not observed under heat influence within the tolerance zone (35°C, 1–6 h) [3]. As opposed to the aforementioned hypotheses, in our experiments carried out with three fungal species belonging to different genera, we did not observe a decrease in the unsaturation level of fatty acids of the major phospholipids or an increase in the amount of bilayer lipids after a 6-hour HS impact. The observed increase in the sterol and sphingolipid contents of the membrane lipids cannot be considered as a general response to HS since it is not characteristic of all fungal species.

No marked changes in the composition of membrane lipids were revealed under a 1-hour HS impact. An increase in HS duration up to 6–9 h was accompanied by a decrease in the share of the major membrane phospholipids, phosphatidylethanolamines (PE) and phosphatidylcholines (PC); at the same time, the level of phosphatidic acids increased considerably, and they became the major phospholipids in the cells.

The amount of trehalose under HS changed in the opposite direction: its accumulation in the cytosol of fungal cells reached 3% of dry weight after a 1-hour HS and was as low as 0.1% in the control. Under prolonged HS, the level of trehalose stabilized at 3–5%. Based on these results, we proposed a novel hypothesis concerning membrane protection under heat shock by means of membrane- stabilizing compounds (trehalose, sterols, and glycolipids). However, the role and origin of high PA amount under HS remain unclear. Since PA are the key intermediates of lipid metabolism, three pathways of their accumulation may be suggested: (1) *de novo* synthesis; (2) phospholipid deg-

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radation; (3) decreased synthesis of phospholipids and di- and triacylglycerols from PAs.

To clarify the reasons for an increase in PA content of mycelium under HS, in this work, we studied lipid metabolism in *Aspergillus niger* by determining the intensity of $^{14}$C-labeled sodium acetate incorporation into and release from the fractions of membrane-bound and neutral lipids in pulse-chase experiments.

## MATERIALS AND METHODS

The study was carried out with the ascomycetous fungus *Aspergillus niger* VKM F-34, which was grown on wort agar slants at 29°C for 5–6 days. The spore suspension was used as inoculum ($5 \times 10^5$–$10^6$ spores/mL of medium). Submerged cultivation of the fungus was performed in 250-mL flasks containing 50 mL of the Blumental–Roseman medium [5] on a KE-12-250T electromagnetic shaker (150 rpm) at optimal temperature of 29–30°C (control) for 24 h (trophophase stage); then $^{14}$C-labeled sodium acetate was added into both the control and experimental variants (8–10 kBq/mL). Cultivation of the control variants (C-variants) was continued at the optimal temperature, whereas the experimental variants (HS-variants) were transferred to 40–41°C. The variants C-1 and HS-1 were grown at appropriate temperatures for 1 h. In the variants C-2 and HS-2, the labeled substrate was removed by quickly filtering the culture through a Nylon mesh, washing the mycelium with distilled water, and transferring it into fresh medium; then the cultivation was continued at appropriate temperatures for 3 h. Washing off of the mycelium from labeled acetate was performed at appropriate temperatures.

By the end of cultivation, in all variants the mycelium was washed with distilled water of appropriate temperature and homogenized with isopropanol to stop the incorporation of the radioactive label. Lipid extraction was continued for 30 min at 70°C; then the biomass residue was removed by filtration and extracted twice with isopropanol–chloroform (1 : 1) under the same temperatures [6]. The combined extract was dried on a rotor evaporator; the residue was dissolved in 3 mL of chloroform–methanol (1 : 1), and the water-soluble compounds were removed by addition of 2.5% sodium chloride (4 mL). The chloroform layer was separated, dehydrated by passing through anhydrous sodium sulfate, dried in a rotor evaporator, and vacuum-dried to a constant weight. The obtained residue was dissolved in chloroform–methanol (1 : 1) and stored at –21°C.

The composition of neutral lipids (NL) was analyzed by ascending thin-layer chromatography (TLC) on glass plates (10 × 10 cm) with silica gel 60 (Merck, Germany). The separation of NL was carried out in the hexane–diethyl ether–acetic acid (85 : 15 : 1) solvent system [7]. The separation of phospho- and sphingolipids was performed by two-dimensional TLC on glass plates (Merck, Germany) in the following solvent systems: chloroform–methanol–water (65 : 25 : 4) (first direction) and chloroform–acetone–methanol–acetic acid–water (50 : 20 : 10 : 10 : 5) (second direction [8]. The amount of lipids applied to a plate was 100–200 μg. The chromatograms were developed by spraying with 5% sulfuric acid in ethanol with subsequent heating at 180°C. The lipids were identified using the individual markers and qualitative reactions with ninhydrin (for the presence of amino groups), the Dragendorff reagent (for choline), and α-naphthol (for glycolipids). The sphingolipid nature of glycolipids was determined by the saponification method [7]. Neutral lipids were identified using the individual markers: mono-, di-, and triacylglycerols; free fatty acids; sterols (ergosterol); and hydrocarbons (Sigma, United States). Quantitative analyses of the lipids were carried out using the following standards: phosphatidylcholine (Sigma, United States) for phospholipids; glycoceramide mixture (Larodan, Sweden) for sphingolipids; and ergosterol Sigma, United States) for sterols. Quantitative densitometric analysis of the lipids was performed with the aid of the Dens software package (Lenkhrom, Russia) in the linear approximation regime using the calibration curves constructed with the standard solutions.

To determine the incorporation of $^{14}$C-labeled sodium acetate, the lipids were visualized with iodine vapor; the silica gel zones containing certain lipid classes were collected from two plates after discoloration (iodine evaporation) and placed into vials with 5 mL of a ZhS-106 scintillation liquid. The radioactivity of the lipid fractions was measured on a Tri-Carb 2800 TR scintillation counter (Perkin Elmer, United States) and expressed as imp/min/g dry mass.

The paper presents typical results of the experiments carried out in three replicates. Standard deviations did not exceed 10%; the main patterns were in close agreement.

## RESULTS AND DISCUSSION

The submerged culture of *A. niger* grew in the form of small pellets (1–3 mm in diameter). Biomass grown in the Blumental–Roseman medium reached 3–4 g/L by the middle of the trophophase (24 h). Under HS, in variant HS-1, deceleration of apical hyphal growth in colonies was observed; in variant HS-2, active hyphal branching was also revealed.

It has been earlier shown that the fungus *A. niger* exposed to HS (40°C, 1 h) acquired resistance to the lethal HS impact (55°C, 20 min) [4]. Therefore 1-hour incubation at 40°C was chosen to investigate the effect of HS on lipid metabolism by measuring incorporation of labeled acetate into the lipid fractions. The mycelium was washed off from labeled substrate after 1-hour incubation under HS, transferred into fresh medium, and cultivated for 3 h under the

**Table 1.** Composition of the membrane lipids under heat shock (μg/g dry biomass)

| Lipids | Variants | | | |
|---|---|---|---|---|
| | C-1 | HS-1 | C-2 | HS-2 |
| PE | 18.6 | 16.6 | 19.7 | 14.4 |
| PC | 29.3 | 34.6 | 30.6 | 25.1 |
| CL | 17.0 | 14.5 | 11.2 | 11.9 |
| PA | 4.2 | 5.5 | 6.1 | 14.3 |
| X | 0.8 | 1.3 | 1.7 | 1.3 |
| SL | 1.6 | 1.0 | 1.8 | 1.8 |
| PS | 3.8 | 3.3 | 4.6 | 5.2 |
| PI + LPE | 3.8 | 4.6 | 6.2 | 8.5 |
| LPC | 1.9 | 4.6 | 2.9 | 2.8 |
| St | 19.0 | 14.0 | 15.2 | 14.7 |
| Total lipids, μg/g dry biomass | 21260.4 | 20827.3 | 16912.6 | 21570.7 |

PC, phosphatidylcholines; PE, phosphatidylethanolamines; CL, cardiolipins; PA, phosphatidic acids; PS, phosphatidylserines; PI, phosphatidylinositols; LPE, lysophosphatidylethanolamines; SL, sphingolipids; LPC, lysophosphatidylcholines; St, sterols.

**Table 2.** Incorporation of $^{14}C$-labeled acetate (imp/min/g dry biomass × $10^3$) into the membrane lipids under heat shock

| Lipids | Variants | | | |
|---|---|---|---|---|
| | C-1 | HS-1 | C-2 | HS-2 |
| PE | 1783.6 | 1831.6 | 1355.8 | 881.9 |
| PC | 1757.7 | 2285.6 | 1602.1 | 1371.4 |
| CL | 147.4 | 282.4 | 432.7 | 267.5 |
| PA | 243.4 | 330.0 | 190.3 | 608.4 |
| SL-1 | 113.7 | 147.1 | 74.1 | 20.0 |
| SL-2 | 55.3 | 132.2 | 66.0 | 158.4 |
| PS | 312.4 | 340.9 | 142.2 | 183.8 |
| LPE + PI | 328.2 | 524.9 | 152.4 | 284.4 |
| LPC | 81.8 | 124.7 | 71.6 | 59.8 |
| St | 1888.0 | 1843.2 | 1271.7 | 1320.9 |

appropriate temperature. This approach made it possible to evaluate the rate of lipid turnover by measuring incorporation of the label into individual lipid classes (biosynthesis) and the label release (catabolism).

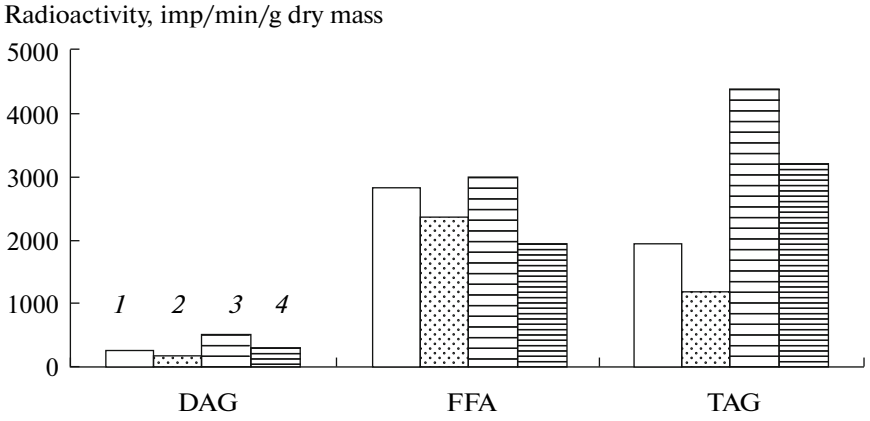
The fungal membrane lipids were represented by phospholipids (PL), sphingolipids (SL), and sterols (St) (Table 1). Among nine classes of PL revealed in *A. niger*, phosphatidylethanolamines (PE), phosphatidylcholines (PC), and cardiolipins (CL) were the major components, while phosphatidic acids (PA), phosphatidylserines (PS), phosphatidylinositols (PI), lysophosphatidylethanolamines (LPE), lysophosphatidylcholines (LPC), and phosphatidylglycerols (PGs) were the minor components. This fungus also contained two classes of sphingolipids (SL-1 and SL-2) and sterols, minor- and major membrane lipids, respectively. Among neutral lipids, triacylglycerols (TAG) and free fatty acids (FFA) dominated, whereas diacylglycerols (DAG) were the minor components (figure).

Composition of the membrane lipids changed slightly under a 1-hour HS impact (HS-1 variant), although their total amount decreased as compared with the control (C-1 variant) (Table 1). Prolongation of HS impact up to 3 h (HS-2 variant) resulted in 20–25% decrease in the amount of the major phospholipids, PC and PE, and in a twofold increase in PA content as compared with the HS-1 variant, although the total amount of membrane lipids was not changed.

While the amount of the membrane lipids changed but slightly, the label incorporation into the lipids under a 1-hour HS (HS-1 variant) slightly increased, especially for the PC and CL fractions, although no reliable differences was revealed in the label amounts in the St, PE, and PS fractions as compared with the control (C-1 variant) (Table 2). Under these conditions, incorporation of labeled acetate into TAG increased twofold and was insignificant in the case of DAG and FFA (figure).

When the fungus was cultivated at optimal temperature (C-1 and C-2 variants), in three hours after removal of the labeled substrate, radioactivity of TAG, DAG (figure), PE, PA, PS, SL-1, and St (Table 2) decreased 1.5-fold. Fractions of PC, LPC, and SL-2 were the most inert; the rate of FA turnover was not different from that for other lipids.

Under HS, after removal of the labeled substrate, radioactivity of PE and PC in the HS-2 variant decreased 2- and 1.7-fold, respectively, as compared with those in the HS-1 variant (Table 2). These changes were more evident than those observed in the control variants. Fractions CL and SL-2 were the most inert ones. A fivefold washing out of the label was observed in SL-1. Unlike the control, where LPC was an inert phospholipid, under HS, a twofold decrease in radioactivity of this fraction was observed. It should be noted that only in one class of phospholipids, namely PA, a twofold increase in label incorporation was observed, rather than a decrease. Radioactivity of the




Incorporation of $^{14}$C-acetate (imp/min/g dry biomass × $10^3$) into neutral lipids (DAG, FFA, and TAG) in the variants: C-1 (*1*), C-2 (*2*), HS-1 (*3*), and HS-2 (*4*).

neutral lipids decreased 1.5-fold (figure). These data indicate that phospholipid turnover in the fungus was more intense under HS than at optimal temperature.

Thus, washing out of the label from the major phospholipids (PC and PE) under HS was considerably higher than that in the control, whereas label incorporation into PA increased twofold. Theoretically, intense cleavage of PC and PE under HS may indicate an increase in activities of (1) phospholipase D or (2) phospholipase A2 and a group of nonspecific hydrolases. According to obtained results, phospholipase D plays a key role in intensifying of the hydrolytic processes. This conclusion is confirmed by increased incorporation of radioactive acetate into PA, active hydrolysis of PC and PE (Table 2), and by a twofold increase in PA amount (Table 1), whereas label incorporation into the fractions FFA, lyso-PL, DAG, and TAG did not increase (figure).

It has been shown that HS impact on submerged culture of this fungus in the trophophase stage resulted in accumulation of PA, with its share among the membrane lipids increasing with HS duration [4]. These results are confirmed by the studies on the labeled acetate incorporation into membrane lipids; after a 1-h HS impact, label incorporation was revealed in almost all lipid classes, including PA. After removing the labeled substrate and subsequent 3-h cultivation of the fungus in fresh medium at the same temperature, the label accumulation in PA was observed, whereas the label amounts in PE and PC decreased 2- and 1.5-fold, respectively. These results make it possible to suggest that PA is produced, most probably, at the expense of cleavage of the major phospholipids, PE, and, especially, PC, which are the substrates for the membrane-bound phospholipase D [9]. It should be noted that under optimal conditions, PC is a rather inert component, whereas under HS, its turnover increases considerably.

It has been earlier shown that a 6-h HS impact on the middle-trophophase culture of *A. niger* caused marked changes in the composition of membrane lipids; the share of PA reached the value (about 20%) comparable with the content of the major phospholipids (PC and PE) [4]. It is remarkable that the level of total phospholipids among the membrane lipids decreased insignificantly and comprised 75%. On the contrary, in the idiophase under HS, relative content of total phospholipids decreased to 42%; the level of sphingolipids increased considerably, whereas sterol content remained unchanged. The general pattern of the HS effect on lipid composition was that the level of PA increased independently of the growth phase, and it became the major component of the membrane phospholipids, whereas the amounts of PC and PE after a 6-h HS impact decreased to 6 and 4% of the total membrane lipids, respectively. These data confirm our conclusion concerning PA formation under HS impact at the expense of cleavage of the major PL.

The question arises as to the role of PA under HS impact. This class of phospholipids is characterized by a unique ability to exhibit the properties of either bilayer- or nonbilayer lipids depending on the physiological conditions [10, 11]. Thus, at neutral pH and in the absence of bivalent ions, PA possess the properties of bilayer lipids, whereas under slightly acidic conditions and in the presence of ions, for instance, in the Golgi apparatus, they form II type micelles. Moreover, it is considered that the PA aggregation leads to formation of microdomains, which are involved in negative membrane bending that may result in the vesicle formation and, therefore, may participate in the regulation of vesicular transport from the Golgi apparatus, as well as endo- and exocytosis. Since intracellular pH is known to decrease under HS [12], existence of PA in a form of nonbilayer phospholipid may be expected under these conditions. It can be assumed that acceleration of lipid turnover under HS provided for an increase in vesicular transport from the Golgi apparatus and the endoplasmic reticulum. Moreover, the reorganization of the protein apparatus in the cell

requires the isolation of defective proteins within the endosomes for their subsequent degradation. The results obtained indicate that due to their large content and peculiar metabolism, PA may play an important role in the cell adaptation to HS impact.

The effect of HS on phospholipase D activity has not been studied. It is known that the membrane-located enzyme is involved in phospholipid signaling and regulates the level of PA, which are known as secondary messengers of many kinases [11, 13]. Thus, phospholipase D controls such processes as reorganization of the cytoskeleton, cell reproduction and survival, vesicular and intracellular transport, and endo- and exocytosis. For instance, a mutation in the *Spo14* gene encoding phospholipase D was shown to prevent membrane formation during meiosis in *Saccharomyces cerevisiae* [14]. The localization of phospholipase D in the membranes, its ability to hydrolyze various phospholipids, including the major PL, phosphatidylcholine, to which this enzyme exhibits the highest specificity, together with the results of our study make it possible to suppose important roles of PA and phospholipase D in the cell metabolism under HS impact.

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