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ATP-consuming processes in hepatocytes of river lamprey *Lampetra fluviatilis* on the course of prespawning starvation

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ABSTRACT

The work was performed to establish which of the major ATP-consuming processes is the most important for surviving of hepatocytes of female lampreys on the course of prespawning starvation. The requirements of protein synthesis and Na^+-K^+-ATPase for ATP in the cells were monitored by the changes in mitochondrial membrane potential (MMP) in the presence of corresponding inhibitors from the peak of metabolic depression (January–February) to the time of recovery from it (March–April) and spawning (May). Integrity of lamprey liver cells was estimated by catalytic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in blood plasma. In January–February, the share of ATP necessary for protein synthesis was 20–22%, whereas before spawning it decreased to 8–11%. Functioning of Na^+-K^+-pump required 22% of cellular ATP at the peak of metabolic depression, but 38% and 62% of ATP in March–April and May, respectively. Progression of prespawning period was accompanied by 3.75- and 1.6-fold rise of ALT and AST activities in blood plasma, respectively, whereas de Ritis coefficient decreased from 2.51 ± 0.34 to 0.81 ± 0.08, what indicates severe damage of hepatocyte membranes. Thus, the adaptive strategy of lamprey hepatocytes to develop metabolic depression under conditions of energy limitation is the selective production of proteins necessary for spawning, most probably vitellogenins. As spawning approaches, the maintenance of transmembrane ion gradients, membrane potential and cell volume to prevent premature cell death becomes the priority cell function.

1. Introduction

Many vertebrate species of different phylogenetic levels, which living cycle includes seasonal oxygen deprivation, hypothermia, hibernation, aestivation, anhydrobiosis, etc., are able to survive the natural phenomenon of reversible metabolic depression (Boutilier, 2001; Hochachka and Lutz, 2001; Storey and Storey, 2004). Although the defense strategies are divergent and broad array of adaptations were evolved by different species, a key mechanism of survival is an ability to fundamentally suppress ATP utilization and O_2 demand to such hypometabolic state which balances the reduced capacity of cells to produce ATP. The intensity of ATP-demanding processes such as biosynthesis, protein turnover, membrane ion transport and secretion are reduced or almost suspended, leading to overall reduction in ATP consumption.

Among the species able to reorganize their cellular metabolic rate, lamprey *Lampetra fluviatilis* is a unique example. Anadromous migration of these monocyclic animals is accompanied by prolonged (7–8 months) synchonia, genetically programmed and hormonally regulated behavior phenomenon of natural starvation (Larsen, 1980). The digestive system is atrophied, and the vitality of lampreys during all phases of prespawning period is maintained due to mobilization of glycogen, lipids and proteins mainly from muscle tissue (Emelyanova et al., 2004; Emel'yanova et al., 2007). However, the supply of hepatocytes with substrates is limited, because blood enters the liver through portal vein which undergoes degenerative changes. Hepatocyte metabolism is exclusively aerobic and based on oxidation of fatty acids, but the lipid drops accumulated in hepatocytes are not used until spring (Savina and Gamper, 1998; Gamper and Savina, 2000; Emel'yanova et al., 2007). As a result, to the middle of winter the hepatocytes develop deep metabolic depression, with endogenous respiration rate, oxidative phosphorylation and ATP content exhibiting considerable decline in comparison to values observed at the beginning of spawning migration (Savina and Gamper, 1998; Gamper and Savina, 2000; Gamper et al., 2001). When the spawning approaches, hepatocyte metabolism is activated again, the rates of endogenous respiration and ATP synthesis are restored and almost reach autumn values. In spite of metabolic depression, liver cells of female lampreys synthesize vitellogenins, precursors of the yolk proteins, which titer in the blood was confirmed to be constant throughout the spawning period (Mewes et al., 2002).

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Therefore, the strategy of lamprey hepatocytes to develop metabolic depression is not associated with unfavorable environmental factors (hypoxia or hypothermia), but serves to preserve energy resources for spawning.

The goal of present work was to reveal the cellular processes being the most important for surviving of lamprey hepatocyte until spawning under conditions of energy limitation induced by prespawning starvation. For this purpose, we made an attempt to evaluate the sensitivity of major ATP-consuming processes to seasonal decrease in ATP production. In the cells of mammals and some other studied vertebrate species, major ATP-demanding processes under standard metabolic rate are protein synthesis (consuming 24–30% of ATP coupled to oxygen consumption) and activity of Na^+-K^+-ATPase (19–28%). The remaining is spent by Ca^{2+}-ATPase activity, gluconeogenesis, protein degradation, ureagenesis, mitochondrial proton leak, mRNA synthesis, etc. (Buttgereit and Brand, 1995; Hochachka et al., 1996; Rolfe and Brown, 1997; Wieser and Krumschnabel, 2001). Biosynthesis of proteins and activity of sodium pump are also most sensitive to energy deprivation. Taking this into account, we monitored the requirement of protein synthesis and Na^+-K^+-pump in lamprey hepatocytes for ATP on the course of prespawning starvation, from the peak of metabolic depression (January–February) to the time of recovery from it (March–April) and spawning (May). We also evaluated the possible gluconeogenic capacity of liver cells at different energy states. Next, we measured the catalytic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in lamprey blood plasma to monitor the integrity of liver cells at different seasons of prespawning period.

The share of ATP necessary for the maintenance of examined cellular processes were evaluated by the rate of their inhibition measured as the changes in potential on inner mitochondrial membrane - MMP ($\Delta\psi_m$). MMP is a key parameter controlling mitochondrial respiration and ATP synthesis, exerting a significant effect on the maximal ATP-generating capacity. MMP accounts for the majority of the protonmotive driving force, which is in turn the central intermediate of aerobic energy production. Resting MMP reflects the balance between energy supply and ATP consumption. The study was based on two assumptions. First, acute inhibition of individual ATP-consuming processes causes a consequential drop in ATP demand and triggers a proportional drop in MMP due to decrease in the rate of electron transport by respiratory chain after administration of inhibitor. Second, an inhibition of one ATP-demanding process does not alter the rates of other processes. Since alterations of MMP values in lamprey hepatocytes on the course of prespawning starvation completely follows the dynamics of ATP production (Zubatkina et al., 2011; Savina et al., 2011), each of these parameters can be reliable indicator of cell energy state and enables studying the relationships between energy metabolism and activities of cellular processes requiring ATP.

2. Material and methods

2.1. Animals

Lampreys (*Lamperta fluviatilis* L.) were caught in the Neva river in November–December after the beginning of their prespawning migration and maintained until May in the tanks with dechlorinated and permanently aerated water at 2–6 °C. The experiments were performed on females.

2.2. Solutions and chemicals

The standard medium used for isolation and incubation of hepatocytes contained (mM): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.44 KH_2PO_4, 0.33 Na_2HPO_4, 5 $NaHCO_3$, 10 HEPES, 5 glucose, pH 7.6.

All buffer salts, cycloheximide, emetine hydrochloride, sodium phenylpyruvate, alpha-cyano-4-hydroxycinnamate, ouabain, collagenase (type VIII), HEPES, FCCP (carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone), DMSO (dimethyl sulfoxide) were purchased from Sigma (USA). FCCP was dissolved in ethanol, emetine and sodium phenylpyruvate - in distilled water. Tetramethyl rhodamine methyl ester (TMRM) was from Fluka. Stock solution of TMRM was prepared on DMSO, aliquoted and stored at −20 °C in the dark.

2.3. Preparation of hepatocytes

Hepatocytes were isolated by a modified collagenase method (Mommsen et al., 1994). The liver was perfused for 2–3 min in situ with the standard medium through the ventral hepatic vein, then carefully minced with a razor blade and incubated for 1 h at room temperature in the same medium supplemented with 0.1% collagenase type VIII. After incubation, the digested tissue was passed through nylon mesh and the cells were washed three times by centrifugation in the standard medium supplemented with 1.5 mM $CaCl_2$ and 0.5% bovine serum albumin (BSA). The final cell pellet was suspended in the same medium at concentration of 4–6 millions of cells per ml and stored on ice. Cell viability of hepatocytes, evaluated by Trypan blue, was 90–95%.

2.4. Determination of mitochondrial membrane potential

Mitochondrial membrane potential was determined by flow cytometry using fluorescent dye tetramethyl rhodamine methyl ester (TMRM). TMRM is a lipophilic cationic compound accumulating in the mitochondrial membranes space in inverse proportion to mitochondrial membrane potential according to the Nernst equation. The hepatocytes were incubated for 30 min in the standard medium with tested inhibitors at room temperature and then loaded for 30 min with TMRM (500 nM) in the dark. After loading the cells were washed twice by centrifugation for 3 min at 1000*g* with fresh medium. Because TMRM is rapidly lost from the cells in the absence of external probe, the hepatocytes were placed in incubation medium containing 50 nM TMRM to allow continuous reequilibration of fluorophore across the plasma membrane and immediately subjected for flow cytometry. An analysis of orange TMRM fluorescence was performed on EPICS-XL (Beckman Coulter, USA) at wavelength of 575 ± 15 nm. The intensity of TMRM fluorescence was set on logarithmic scale. The data were first plotted using SSC (side scatter) vs FSC (forward scatter) to reveal cell size and complexity, and the gate was applied to isolate the population of cells with normal volume and remove the debris along the axes. Then the data were re-plotted using FSC and TMRM and the gate was used to remove dying cells with low TMRM fluorescence. Finally, the data were collected and gated so that only hepatocytes with high TMRM fluorescence were used for analysis. 30,000 events were collected for each sample. Mean fluorescence intensity in arbitrary units obtained for control samples (without inhibitors) in each experiment were taken as 100%. The fluorescence of samples in the presence of inhibitors was expressed as the percentage of control.

According to the Nernst equation, intracellular distribution of cationic mitochondrial probe (TMRM) reflects the differences in the transmembrane potential across both the plasma membrane and inner mitochondrial membrane, i.e., besides mitochondria, TMRM is accumulated in the cytoplasm as well. The specificity of TMRM fluorescence for MMP was assessed using protonophore FCCP. FCCP completely depolarized the mitochondria, thus causing a collapse in MMP and, respectively, loss of TMRM signal. After determination of TMRM fluorescence, 40 μM FCCP was added to each sample, and the fluorescence was determined again in 10 min. TMRM fluorescence in the presence of FCCP (negative control) was quite similar in all samples and did not exceed 14–18 arbitrary units, whereas without FCCP it was 200–300 units. For analysis, TMRM signal in the presence of FCCP was subtracted from the total TMRM signal in the sample.