In this study, we report the changes of the glycolipid levels in the mitochondria and synaptosomes from hypoxic rat brains. Twenty male Wistar rats at the age of three months were subjected to sodium nitrite-induced hypoxia. The mitochondrial and synaptosomal fractions were isolated and the glycolipid content was measured by spectrophotometry and thin-layer chromatography.

In control rats, gangliosides and cerebrosides were the major glycolipid classes in both fractions. In the brains of experimental rats subjected to hypoxia, we found increased levels of total glycolipids – 2.7-fold in synaptosomes and 4.6-fold in mitochondria. The concentration of gangliosides and cerebrosides increased markedly in both fractions. Most probably these changes are associated with impaired energy metabolism and reflect the disturbances in glycolipid turnover.

**Key words**
glycolipids, hypoxia, mitochondria, synaptosomes, rat brain.

**Ključne reče**
glikolipidi, hipoksija, mitohondrije, sinaptozomi, mozak pacova.

**INTRODUCTION**

Hypoxia is one of the major pathological events causing neuronal cell injury, neurodegeneration and cell death. In the brain, the most hypoxic vulnerable of all vertebrate tissues, low oxygen quickly results in a fall in ATP and a consequent increase in adenosine. When ATP falls below about 50% of normoxic levels the membrane depolarizes and ion gradients are lost [1]. Changes in oxygen availability can stimulate the activity of hypoxia-sensitive genes that mediate oxygen homeostasis. Their expression is upregulated during prolonged oxygen limitation.

The lipid fraction is particularly sensitive to hypoxia, as compared to other macromolecular compounds. Glycolipids constitute an important class of lipids in the neuronal membranes, in particular the mitochondrial and synaptosomal membranes. Gangliosides and cerebrosides – the main large groups of glycolipids, play crucial modulatory roles in various cellular functions, including signal transduction, regulation of cell proliferation and differentiation, cell–cell recognition, adhesion, and cell death [2]. Some glycolipids (GM1, GD1a, GD1b, and GT1b) are closely associated to synaptogenesis [3], other have impact on the mitochondrial function.

The aim of the present investigation was to establish the changes of the level of total and individual glycolipids in synaptosomes and mitochondria from hypoxic rat brains.

**MATERIAL AND METHODS**

Twenty male Wistar rats at the age of three months, each weighing 190-220 g, were subjected to sodium nitrite-induced hypoxia as we have previously reported [4]. Sodium nitrite was administered intravenously at 20 mg/kg body weight (2 ml/kg dosing volume). Hypoxic rats were killed by decapitation.

Mitochondrial and synaptosomal fractions were isolated according to the method described by Venkov [5] using two-step sucrose gradient. Lipids were extracted according to the method of Kates [6] using the following eluates: chloroform:methanol 1:2 (v/v) and chloroform:methanol:water 1:2:0.8 (v/v/v).

The total glycolipid content was measured spectrophotometrically at 490 nm [7]. The major glycolipid classes were separated by thin-layer chromatography using eluate from chloroform:methanol:water 65:25:4 (v/v/v). Perkin-Elmer
scanning spectrophotometer was used to estimate the concentration of migrated spots.

The animal experiments were performed in accordance with animal protection guidelines approved by the Ethics Committee for experimental animal use at IEMPAM – BAS.

The data were analyzed with Student’s t-test.

RESULTS AND DISCUSSION

The glycosphingolipids (glycolipids) are a large and heterogeneous family of amphipathic lipids commonly found in the outer leaflet of the plasma membrane bilayer. At the plasma membrane, they expose the sugar-containing hydrophilic portion to the extracellular space, contributing to the complexity of the glycolaxial. Cerebrosides and gangliosides, which are the main glycolipid classes, are present in high concentration in the neuronal membranes. Gangliosides are diverse and highly complex molecules characterized by containing a variable number of sialic acid residues. About 200 gangliosides differing in carbohydrate components are known today [8]. Gangliosides are known to have various biological functions depending on their ceramide and carbohydrate structures. Cerebrosides is the common name for a particular group of monoglycosylceramides.

We estimated the control content of total glycolipids in the rat brain synaptosomal and mitochondrial subcellular fractions at 0.457±0.08 mg/g/ml (mg glycolipids per g dry lipid residue per ml lipid extract) and 0.478±0.05 mg/g/ml, respectively (Fig. 1). The gangliosides and cerebrosides were the two main glycolipid classes in both fractions (Fig. 2 and Fig. 3). The ganglioside content was higher in synaptosomes and the cerebrosides were predominant in mitochondrial membranes. These observations are in accordance with literature data showing that the gangliosides are found to be highly enriched in the pre and postsynaptic membranes of the synaptic terminals [9]. Gangliosides are thought to play a role in the neurotransmitter release. They have been considered as the Ca^{2+}-binding co-factor in synaptic transmission [10]. It is reported that exogenous gangliosides reduce the activity of the synaptosomal membrane ATPase. In preincubation experiments it has been demonstrated that the interaction of the glycolipid with synaptosomal membranes itself is temperature dependent and enhanced by ATP [11]. Moreover, it is suggested that ganglioside micelles might have been incorporated by the membranes in a way comparable to a fusion process.

The biochemical functions of mitochondria strongly depend on the membrane lipids, too. Lipids influence the stability and the osmotic behavior of mitochondria; respiratory activity and energy production; permeability and transport processes across mitochondrial membranes; mitochondrial protein synthesis, import of proteins into mitochondria, and assembly of mitochondrial membranes; the membrane structure, the phase behavior, and the lipid-protein interaction, and the activity of mitochondrial enzymes in vivo and in vitro [12].

The oxygen limitation is generally considered as an impairment of mitochondrial respiration. Consequences of mitochondrial injury include metabolic failure, oxidative stress, disruption of Ca^{2+} homeostasis, promotion of apoptosis [13]. Total reduction of electron transport chain elements results in the formation of free radicals leading to the initiation of a free radical-mediated peroxidation. The peroxidation of lipids is considered to be a major mechanism of free radicals cell damage. The major phospholipid components of the mitochondrial and synaptosomal membranes are rich in unsaturated fatty acids whose double bonds are especially susceptible to oxygen radical attack.

In animals hypoxia is signaled at three levels: an immediate systemic response which involves central and peripheral chemoreceptors, an immediate/chronic gene response initiated by cellular oxygen signals and an immediate emergency or crisis response signaled by changes in energy metabolite concentrations [14]. Recent advances in the understanding of the molecular mechanism of cell injury have led to the realization that cell injury is a gradual process, which involves, in its early stages, specific biochemical responses of cells to the injury process [15].

Several experimental models have been used to recapitulate the human cerebral hypoxia syndrome. In our experiments we applied a model of sodium nitrite-induced hypoxia. Sodium nitrite is commonly used for induction of hypoxia in experimental animal models. It converts hemoglobin to methemoglobin and unlike ferrous form of hemoglobin, methemoglobin does not bind oxygen strongly. It is known that elevated levels of methemoglobin can lead to anemic hypoxia, and rats exposed to sodium nitrite achieve elevated concentrations of methemoglobin in their blood [16]. The administration of sodium nitrite in high concentrations may cause brain inflammation, ischemia and impaired cerebral energy [17].

In the brains of experimental rats subjected to hypoxia, we found increased levels of total glycolipids – 2.7-fold in synaptosomes and 4.6-fold in mitochondria (Fig. 1). In synaptosomes, the content of gangliosides and cerebrosides increased 2.9 times the control value (from 0.227±0.04 to 0.657±0.01 mg/g/ml) and 2.5 times the control value (from 0.23±0.04 to 0.567±0.02 mg/g/ml), respectively (Fig. 2). In mitochondria, the increase of gangliosides and cerebrosides was more pronounced – 6.9-fold (from 0.161±0.05 to 1.116±0.04 mg/g/ml) and 3.4-fold (from 0.317±0.02 to 1.083±0.06 mg/g/ml), respectively (Fig. 3). The glycolipid composition of rat brain synaptosomes and mitochondria following hypoxia is poorly investigated. There are data about marked increase in the intensity of lipid peroxidation in the synaptosomal and mitochondrial fractions after acute hypoxia [18]. Both mild and severe hypoxia is shown to influence the physiology of mitochondria [19]. The effect of hypoxic hypoxia on glycolipids is studied in the brain cortex and a reduction in the glycolipid content is observed [20]. It is suggested that hypoxia could have activated neuraminidase and change directly the content of ganglioside-bound sialic acid on synaptic plasma membrane. In contrast to the above studies, we explored the effect of hypoxia on glycolipids at brain subcellular level and our findings demonstrate an increase in the glycolipid content in the synaptosomal and mitochondrial fractions.

The mitochondria play a key role in the organization of death signals. They are increasingly recognized as a sensitive target for oxidative damage in hypoxia. It is known that mitochondrial oxidative phosphorylation is the primary source of high-energy compounds in the cell [21]. Recent
Evidence has accumulated pointing toward the mitochondrial membranes as the key targets for lipid and glycolipid mediators of stress-induced apoptosis. These membranes may thus act as sensors of cellular stress by quantization of the local accumulation of specific lipids and glycolipids [22]. Most probably the high content of gangliosides is associated with their neuroprotective effect although some gangliosides are highly expressed in pathological conditions and have been reported to cause mitochondrial permeability transition and release of apoptogenic factors. For example, it was found that GM3 act as a modulator of neuronal cell death, and the accumulation of GD3 can alter mitochondrial function and thereby induce cell death in a caspase-dependent manner [23]. On the other hand, another hypothesis supports the view that gangliosides may promote neuronal regeneration through modulation of trophic factors, stimulation of neuronal plasticity and reducing the injury processes. For example, GM1 ganglioside is shown to prevent in vitro neurotoxicity of glutamate, play a scavenger role, partially correct the hypoxia-induced neurotransmitter deficits in neonatal rats and reduce the vulnerability of fetal sheep brain to subsequent injuries [24]. The intraperitonial administration of mixed gangliosides decreases the accumulation of intracellular Ca\(^{2+}\) and stabilizes protein kinase activities [25].

In conclusion, sodium nitrite-induced hypoxia results in glycolipid accumulation in the brain mitochondria and synaptosomes. The high levels of gangliosides and cerebrosides indicate the energy disturbances and may represent an adaptive response to this form of brain injury.
REFERENCES


Apstrakt

U radu su prikazane promene nivoa glokolipida u mitochondrijima i sinaptosomima u hipoksično oštećenom mozgu pacova. Dvadeset muških Wistar pacova starosti od tri meseca bili su izloženi hipoksijski indukovanoj natrijumnitritom. Izolovane su mitochondrijalna i synaptosomalna frakcija a sadržaj glokolipida meren je spektrofotometrijom i tankoslojnom hromatografijom.

U kontrolnoj grupi pacova gangliosidi i cerebrosidi bili su glavni glokolipidi u obe frakcije. U mozgovima eksperimentalne grupe pacova izloženih hipoksijski, našli smo povećane nivo ukupnog glycolipid - 2,7 puta u sinaptosomima i 4,6 puta u mitochondrijima. Koncentracija gangliosida i cerebrosida znatno je povećana u obe frakcije. Verovatno su te promene povezane s oštećenim energetskim metabolizmom i odražavaju poremećaju u metabolizmu glokolipida.