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Lipids of Nuclear Fractions from Neurons and Glia of Rat Neocortex under Conditions of Artificial Hypobiosis

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Abstract—Lipid contents were studied in tissue and nuclei isolated from neurons and glia of neocortex of rats under conditions of normothermia and in the state of artificial hypobiosis caused by hypothermia—hypoxia—hypercapnia. Compared to the neocortex tissue, both nuclear fractions were fivefold impoverished in phospholipids and cholesterol and strongly enriched with mono- and diglycerides and fatty acids. The nuclear fractions from neurons and glia contained similar amounts of phospholipids, and only the cardiolipin content in the neuronal nuclei was lower than in the glial nuclei. The state of artificial hypobiosis in rats led to an increase in the cholesterol/phospholipids ratio (mol/mol) in the nuclei from the neurons and glia; amounts of cholesterol and sphingomyelin in the nuclei from the glia were increased. The increases in the cholesterol and sphingomyelin contents and in the cholesterol/phospholipids ratio suggest an involvement of lipid-dependent signaling systems of the nuclei in the functional response of mammalian neocortex cells to artificial hypobiosis.

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Lipids are structural components of cellular membranes that are involved in functioning of the cell signaling systems as bioeffectors. The lipid composition of membranes is determined genetically and depends on the intensity of synthesis, degradation, and transfer of lipids and on the functional state of the cell [1]. Various stimuli obtained by the cell determine specific features of metabolism and can influence the lipid composition of the membranes [2]. For a long time mechanisms of effects of low environmental temperatures on the membrane lipids of mammals attract the attention of researchers. An association was found between the transition temperature of membrane functions and the lethal temperature, and the temperature reactions of membranes were suggested to be limiting for the organism's survival under conditions of hypothermia [3, 4]. Phase transitions and physical properties of lipids are exquisitely sensitive to changes in temperature. Adaptation to low temperatures is supposed to be associated with molecular organization of the membrane lipids. The temperature restructuring of membranes is termed homeophase, or homeoviscous adaptation [5]. Increases in the fraction of unsaturated fatty acids and in the amount of phosphatidylethanolamine, a decrease in the amount of phosphatidylcholine, and also a positive correlation with temperature of the cholesterol/phospholipids ratio were observed in membrane lipids of prokaryotes, plants, fishes, poikilotherms, and hibernating mammals during temperature adaptation [6]. However, there are also data not fitting the idea that changes in the lipid composition of membranes are essential only for lowering the viscosity under conditions of low environmental temperature but suggesting that membrane lipids should be involved in adaptive changes in metabolism [7-9]. No significant changes have been found in the level of fatty acid unsaturation in phospholipids during hibernation of mammals [10, 11].

Cholesterol is the most important structural and functional component of biological membranes [12]. Studies on its involvement in the organism's adaptation to low environmental temperatures are interesting, especially in connection with its role in the structure of rafts and in functions of receptors [13].

Studies on the phospholipid composition of membranes remain urgent. Electrostatic interactions in membranes between differently charged phospholipids influence phase transitions in lipids, and the phospholipid ratios can be significant for homeoviscous adaptation

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[14]. Temperature compensation – changes in the enzyme affinities for substrates at low temperature of the body – is supposed to be due to changes in membrane lipids [15, 16]. Elucidation of the role of membrane lipids during natural (hibernation) and artificial hypobiosis of mammals is important for spatial biology and clinical medicine [4, 17].

Body temperature can be decreased in non-hibernating mammals using drugs, by cooling, and combining drugs and cooling [4]. A decrease in the body temperature of rats to 14-22°C under conditions of hypoxia/hypercapnia induces a state of the so-called cold narcosis, or hypobiosis, characterized by a sharp suppression of mobility and intensity of metabolism and by disappearance of the brain electrical activity [4, 18, 19].

For understanding the role of lipids in the adaptation of non-hibernating mammals to low temperatures, it is interesting to study the lipid composition of membranes of functionally different intracellular organelles. The brain is known to be the major regulatory organ of hibernation in hibernating mammals [20, 21], and the lipid composition of hibernants has been studied in many works [3, 6, 22-24]. However, the effect of artificial hypothermia of mammals on the brain lipids, in particular on lipids of such very important structures as nuclei of neurons, is unknown. There are only a few studies on nuclear lipids of the neocortex neurons and glia of mammals subjected to various exposures [25].

The purpose of our work was to investigate the lipid composition of nuclei isolated from neurons and glia of the neocortex of rats under normal conditions and under conditions of hypothermia–hypoxia–hypercapnia as a model of hibernation to elucidate the role of lipids of the brain cortex nuclei in the organism's response to the state of hypobiosis.

MATERIALS AND METHODS

Male Wistar rats with initial body weight of 190-230 g were obtained from the Stolbovaya nursery and maintained on food and water *ad libitum* with 12-h day-night cycle. The animals were cooled using an approach of hypoxia–hypercapnia [19]. The rats were kept in a hermetic chamber 5 liters in volume at the temperature of 1-2°C for 3.5-4 h. Upon placing into the usual gas environment, the rats recovered to normothermia and later manifested no changes in behavior. After the cooling and taking the rats from the chamber their body temperature and heart rate (HR) were determined. The body temperature was measured rectally with the accuracy of 0.2°C using an electrothermometer sensor placed into the colon at the depth of 6 cm; the HR was recorded using an EEG-4-02 electroencephalograph, with lead electrodes placed subcutaneously on the animal's left front leg and above the scapula.

All procedures with the animals were performed in accordance with requirements of the Institute's Commission on Ethics and of the European Convention for Protection of Vertebrates used in experiments and for other scientific purposes (European Communities Council Directive (86/609/EEC)). The rats were decapitated according to the rules described in [26] in the states of normothermia (body temperature 37-38°C) and hypothermia (15-20°C). Fractions of neuronal and glial nuclei were isolated by Thompson's method [27] in the modification of Baker and Chang [28]. For each determination the neocortex of five rats was combined. The brain was isolated rapidly, washed with ice-cooled saline, and then the cortex was separated and placed into a cold solution of 0.32 M sucrose supplemented with 1 mM $MgCl_2$. All procedures were performed at 0-4°C. The cortex was rapidly minced with scissors and homogenized in a glass homogenizer with a Teflon pestle at 800 rpm in four volumes of 2 M sucrose supplemented with 1 mM $MgCl_2$. The homogenate was diluted with an equal volume of the homogenization medium and filtered through nylon net. Part of the homogenate was taken for determination of lipids and protein. The remaining part was centrifuged for 30 min on a Centricon T-20-70 centrifuge in a bucket rotor at 64,000*g* and the supernatant was removed. The total precipitate of nuclei was resuspended in 4 ml of 2.4 M sucrose solution supplemented with 1 mM $MgCl_2$, and then the suspension of nuclei was placed into centrifuge tubes, 1.5 ml of 1.8 M sucrose containing 1 mM $MgCl_2$ was carefully layered above, and centrifuged for 30 min at 85,000*g*. The precipitate and interphase resulting by the centrifugation contained glial and neuronal nuclei, respectively. The upper layer of 1.8 M sucrose was carefully drawn off, the neuronal nuclei in 2.4 M sucrose were poured into another tube, diluted in two volumes of 0.32 M sucrose, and centrifuged for 3 min at 2000*g*. Then the precipitate was additionally washed with 1 M sucrose containing 1 mM $MgCl_2$ and precipitated by centrifugation at 2000*g* for 3 min. The precipitates of nuclei were resuspended in 0.32 M sucrose containing 1 mM $MgCl_2$.

According to Thompson's data, the fraction of neuronal nuclei contains 22 ± 5% of glial nuclei, and the fraction of glial nuclei has an admixture of 8 ± 2.5% of neuronal nuclei [27]. After an additional washing, aliquots were taken for determination of protein by the Lowry method [29] and for investigation of the nuclei by standard approaches of electron microscopy [23]. Lipids were extracted with a 20-fold volume of chloroform–methanol mixture 2 : 1 (v/v) according to Folch [30]. Neutral lipids were separated by TLC on silica gel L (5/40) in hexane–ethyl ether–acetic acid 73 : 25 : 2 (v/v) [31]. Phospholipids were separated on silica gel H (Merck, Germany) in methyl acetate–*n*-propanol–chloroform–methanol–0.25% KCl 25 : 25 : 25 : 10 : 9 (v/v) [32]. Phospholipids were determined by amount of inorganic phosphate after pyrolysis [33]. Quantities of choles-

terol and other neutral lipids were determined by ashing [34]. Quantities of free fatty acids and mono- and diglycerides were calculated using a calibration curve with arachidonic acid. Quantities of lipids were expressed as μg/mg protein of the fraction. The results were processed using Student's *t*-test. Mean values ± standard errors are presented.

RESULTS

The yield of protein from the nuclear fractions of neurons and glia of the rat neocortex in the state of hypobiosis was the same as in the norm (Table 1). No morphological difference from the norm was revealed by electron microscopy in either the neuronal and glial nuclei obtained from the neocortex of rats in the state of hypobiosis. Contents of cholesterol in the nuclei from neurons and glia were, respectively, four- and six-fold lower than in lipids of the neocortex; in lipids of the nuclei from neurons and glia the contents of monoglycerides were twofold, of diglycerides – four- and sixfold, and of fatty acids – fourfold higher than in the neocortex tissue (Table 1). Contents of total phospholipids and individual phospholipids in the neuronal and glial nuclei were drastically decreased as compared to their contents in the neocortex tissue. In the nuclei of neurons the cardiolipin content was lower than in the nuclei of glia (Table 2). The state of artificial hypobiosis in rats under conditions of hypothermia–hypoxia–hypercapnia did not cause changes in lipid contents in the neocortex tissue (Table 2). In the nuclei of neurons the contents of phospholipids, fatty acids, and mono- and diglycerides did not change, and the ratio of cholesterol/phospholipids (mol/mol) was increased. In the nuclei of glia the contents of cholesterol and sphingomyelin were increased, the ratio of cholesterol/phospholipids was also increased, and amounts of other lipids under study were unchanged (Tables 1 and 2).

DISCUSSION

Although the role of lipids in normal and pathological states of the brain seems to be interesting, data on the lipid composition of cells and subcellular components of the neocortex neurons are scarce. Contents of total lipids in the glial cells are 50% higher than in neurons (lipids/mg protein), but contents of cholesterol and phospholipids in the glial cells are only slightly higher than in the neurons [35]. Contents of phospholipids in the nuclei of rabbit neocortex neurons [36] and of cholesterol and phospholipids in nuclei of rat neocortex neurons and glia [25] are significantly lower than in neocortex microsomes and tissue. The nuclei contain significantly higher contents of fatty acids than of cholesterol [25]. The composition of neutral lipids in the nuclei of neurons is sharply different from that of the neocortex tissue because of a low level of cholesterol (Table 1). The difference in the cholesterol content is still more pronounced on comparison between cholesterol of nuclei and microsomal fraction (endoplasmic reticulum and dictyosomes): the content of cholesterol in rat neocortex microsomes is 7-10-fold higher than

Table 1. Contents of neutral lipids in neocortex tissue and in nuclear fractions of neocortex glia and neurons in normothermia (upper lines) and in hypobiosis caused in rats by hypothermia–hypoxia–hypercapnia (lower lines) (μg lipid per mg protein)

Lipid	Neocortex tissue	Glial nuclei	Neuronal nuclei
Cholesterol	90.8 ± 8.3 (8) 74.3 ± 14.7 (5)	21.4 ± 2.9 (7) 32.0 ± 3.4* (5)	14.5 ± 2.8 (7) 27.8 ± 8.3 (5)
Monoglycerides	11.8 ± 3.4 (7) 7.8 ± 1.8 (5)	33.0 ± 4.7 (8) 36.9 ± 9.3 (7)	23.9 ± 5.0 (7) 28.7 ± 5.4 (5)
Diglycerides	5.6 ± 0.6 (7) 7.1 ± 0.5 (4)	36.6 ± 6.0 (8) 30.0 ± 4.8 (5)	21.4 ± 5.3 (7) 24.1 ± 7.3 (6)
Fatty acids	22.6 ± 2.4 (7) 18.3 ± 1.3 (5)	105.1 ± 19.2 (6) 69.6 ± 16.0 (6)	82.9 ± 21.0 (8) 94.4 ± 21.3 (5)
Cholesterol/phospholipids, mol/mol	0.40 ± 0.08 (4) 0.36 ± 0.07 (4)	0.69 ± 0.12 (7) 1.07 ± 0.06* (5)	0.48 ± 0.11 (6) 1.0 ± 0.15* (5)
Protein, mg/g tissue	85.7 ± 4.6 (9) 73.3 ± 4.8 (6)	0.74 ± 0.15 (8) 0.77 ± 0.12 (6)	0.90 ± 0.12 (8) 0.95 ± 0.14 (6)

Note: Here and in Table 2, figures in parentheses designate number of experiments.
* Difference is significant relatively to control at $p < 0.05$.

Table 2. Contents of phospholipids in neocortex tissue and in nuclear fractions of neocortex glia and neurons in normothermia (upper lines) and in hypobiosis caused in rats by hypothermia–hypoxia–hypercapnia (lower lines) (μg phospholipid per mg protein)

Phospholipids	Neocortex tissue	Glial nuclei	Neuronal nuclei
Total phospholipid fraction	381 ± 31 (5) 392 ± 33 (4)	77.1 ± 7.5 (8) 59.0 ± 4.6 (6)	64.4 ± 2.8 (8) 80.1 ± 12.0 (4)
Phosphatidylcholine	113 ± 16 (7) 119 ± 13 (5)	41.2 ± 6.1 (7) 41.0 ± 4.3 (5)	29.0 ± 1.9 (7) 26.2 ± 1.6 (5)
Phosphatidylethanolamine	95.6 ± 4.6 (6) 101.1 ± 17.6 (5)	13.8 ± 0.7 (6) 13.0 ± 2.6 (4)	15.8 ± 2.0 (7) 14.5 ± 2.7 (5)
Phosphatidylserine	42.3 ± 4.7 (7) 52.0 ± 9.1 (4)	4.7 ± 0.2 (5) 5.8 ± 1.0 (4)	4.2 ± 1.2 (6) 4.5 ± 1.2 (5)
Phosphatidylinositol	35.9 ± 5.4 (5) 38.1 ± 3.2 (5)	9.1 ± 0.8 (5) 9.0 ± 2.1 (5)	7.5 ± 0.8 (6) 7.2 ± 1.2 (4)
Sphingomyelin	19.5 ± 1.2 (7) 26.0 ± 4.1 (4)	3.4 ± 0.3 (5) 6.0 ± 1.0* (5)	4.1 ± 0.2 (6) 3.7 ± 0.9 (4)
Cardiolipin	14.4 ± 1.7 (6) 14.5 ± 2.3 (5)	4.2 ± 0.7 (4) 4.7 ± 0.8 (4)	2.4 ± 0.6** (5) 1.8 ± 0.5** (4)

* Difference is significant relative to amount in normothermia at $p < 0.05$.
** Difference is significant relative to amount in glial nuclei at $p < 0.05$.

in the nuclei and is 148 ± 24 μg/mg protein [24]. Although the neocortex is 10-20-fold richer in cholesterol than other rat tissues, differences in its contents in the nuclei of liver, thymus, neurons, and glia are not very pronounced. Cholesterol contents in liver and thymus nuclei are 3.5 ± 0.3 and 9.1 ± 0.9 μg/mg protein, respectively [37], whereas its contents in neuronal and glial nuclei are respectively 14.5 ± 2.8 and 21.4 ± 2.9 μg/mg protein (Table 1). Cholesterol is the major neutral lipid of neocortex tissue, microsomal fraction, and synaptosomal membranes [23, 24]. In lipids of the neuronal and glial nuclei the major neutral lipids are fatty acids (Table 1).

As a signaling molecule, cholesterol plays an important role in metabolism of neurons, memory mechanisms, plasticity, and neurodegenerative processes [38]. Cholesterol and sphingolipids are necessary components of rafts and influence the activity, lateral diffusion of receptors, and their interaction with agonists [39]. In this connection it should be noted that increases in cholesterol content in glial nuclei and in cholesterol/phospholipids ratio occur due to increases in cholesterol contents in nuclei of glia and neurons of the neocortex of rats in the state of artificial hypobiosis under conditions of hypothermia–hypoxia–hypercapnia (Table 1). The chain of cholesterol synthesis is localized in the cytosol and in the endoplasmic reticulum membranes. An increase in cholesterol amount under hypothermia seems to affect mechanisms of cholesterol synthesis and its transport into nuclei.

An increase in the cholesterol/phospholipids ratio in membranes is reported on activation of specific functions of the organelles. Respiration was activated in the liver of rats subjected to very high doses of ionizing radiation along with an increase in the cholesterol amount in the liver mitochondria [40]. An increase in the amount of cholesterol in neocortex microsomes of ground squirrel was found on activation of protein synthesis during the active state in the hibernation period [23]. The activation of protein synthesis in rats subjected to acute irradiation was accompanied by an increase in the amount of cholesterol in the liver microsomes and by acceleration of its transmembrane transfer [41, 42]. Injection into rats of the apoptosis-inducing agent tumor necrosis factor-alpha (TNFα) caused 30-60 min later a slight increase in the amount of cholesterol in the neocortex [43]. The increase in cholesterol/phospholipids ratio in the nuclei of the neocortex cells can be considered a manifestation of the functional role of cholesterol in the response to hypobiosis not immediately associated with the homeoviscous adaptation of membranes [5]. The increase in the amount of cholesterol and the enhancement of the cholesterol/phospholipids ratio in the nuclei seem to be associated with a certain stage of the activation of the neocortex cells in response to the hypobiosis state under conditions of hypothermia–hypoxia–hypocapnia.

As to other neutral lipids, note the enrichment of the nuclear fractions with mono- and diglycerides. The endogenous cannabinoid monoglyceride 2-arachidonyl

glyceride represents the major part – up to 47% of all monoglycerides in rat brain [44]. Endogenous cannabinoids and their receptors are important in training, memory, locomotion, and neurodegenerative processes [45].

Contents of diglycerides in the nuclei of neurons are also significantly higher than in the neocortex tissue (Table 1). Diglycerides are thought to be a crucial element in the system of cell signaling and are intermediates of phosphatidylcholine and phosphatidylethanolamine synthesis [46]. Diglycerides are also intermediates in the action on phospholipids of phospholipase C and (in the presence of phosphatases) of phospholipase D [47]. Phosphatidylinositol is a source of nuclear diglycerides during cell signaling [46]. In the neocortex neuronal and glial nuclei phosphatidylinositol is the third phospholipids in amount, whereas in the tissue and microsomes this position belongs to phosphatidylserine. As the third major phospholipid phosphatidylinositol has been reported earlier in the nuclei of neurons and glia [25, 36] and in the nuclei of rat thymus and liver [37]. Thus, the common character of the lipid composition of nuclei of different organs and tissues is manifested by the prevalence of phosphatidylinositol amount above that of phosphatidylserine.

Both our data and the literature indicate that the lipid/protein ratios for neutral lipids and phospholipids in the nuclei of neurons are fundamentally different from such ratios for lipids in the neocortex tissue and microsomal membranes: the nuclei contain sharply increased amounts of fatty acids, mono- and diglycerides. Activities of marker enzymes and amounts of RNA and phospholipids in isolated nuclear membrane of neurons from rabbit neocortex did not differ from those in the microsomal fraction of the neocortex [36]. Consequently, nuclei of neurons are hydrophilic islets enriched with proteins, mono- and diglycerides, and fatty acids enveloped by a hydrophobic nuclear membrane.

The state of artificial hypobiosis in rats under conditions of hypothermia–hypoxia–hypercapnia induced in glial nuclei an increase in the amount of sphingomyelin (Table 2). The final stage in sphingomyelin synthesis is the transfer of phosphorylcholine by sphingomyelin synthase (SM synthase) from phosphatidylcholine onto ceramide with production of diglyceride and sphingomyelin. SM synthase has been found in different cell components, including the nuclear membrane [48]. Phosphorylcholine is detached from sphingomyelin with production of ceramide under the influence of differently located enzymes of the sphingomyelinase family. An increase in the amount of ceramides caused by apoptosis-inducing factors has been shown in numerous works [49]. The increase in sphingomyelin amount in glial nuclei under hypobiosis conditions can be due to activation of SM synthases, suppression of sphingomyelinases, or changes in the rate of sphingomyelin transfer from the endoplasmic reticulum membranes into the nuclei. The rate of transmembrane transfer of lipids is high and can be a factor for increasing lipid contents in the membrane [42]. Note the absence of an increase in diglyceride contents and of decrease in phosphatidylcholine amount in glial nuclei, i.e. no signs of SM synthase activation were observed. Inhibition of sphingomyelinase activities or a change in the rate of sphingomyelin transfer is more likely.

An injection into rats of TNFα induced in the neocortex 30-60 min later a decrease in the activity of sphingomyelinase and a slight increase in the amount of sphingomyelin [43]. Inhibition of the sphingomyelinase activity and a decrease in the ceramide production were shown to protect neurons against apoptosis and to correlate with the increase in the amount of sphingomyelin [50]. The accumulation of sphingomyelin in the nuclei is thought to be associated with activation of cell recovery and division [51]. However, opposite data obtained on blood T lymphocytes indicated that sphingomyelin accumulation and decrease in phosphatidylcholine/sphingomyelin ratio occurred during the stage of preparing for apoptosis due to activation of SM synthases along with a decrease in the amount of phosphatidylcholine [52]. We have not found a decrease in the amount of phosphatidylcholine, and the phosphatidylcholine/sphingomyelin ratio in the glial nuclei in hypobiosis decreased due to increase in the amount of sphingomyelin (Table 2).

Considering the influence of artificial hypobiosis on nuclear lipids, one has not to assign the changes only to lipids of the nuclear membranes. Thus, chronic γ-irradiation of rats led to increase in amounts of phosphatidylserine and phosphatidylinositol in both thymus nuclei and chromatin [53].

The amount of cholesterol increased in nuclei and chromatin of the liver from rats subjected to chronic irradiation at a dose shortening the average lifespan when the damage to liver nuclear structures became noticeable [37]. Having in mind the signaling role of cholesterol and sphingomyelin, the increase in their amounts in glial nuclei can be considered as a manifestation of involvement of the neocortex glia signaling systems in the response of the mammalian organism to hypobiosis under conditions of hypothermia–hypoxia–hypercapnia.

The increases in the amounts of sphingomyelin and cholesterol in glial nuclei and the increase in the cholesterol/phospholipids ratio in nuclei of neocortex of rats under hypobiosis are likely to enhance resistance to the damaging exposure. The quantity of endoplasmic reticulum membranes was shown to increase in the neocortex of rats upon 48 h of artificial hypobiosis [54]. These effects can be also important for the known radioprotective effect of artificial hypobiosis [19].

Even in early studies on the role of phosphatidylinositides in the generation of calcium waves, the localization in the cell of intracellular signaling systems was under consideration [55]. Our findings suggest that

changes in the lipid composition of the neocortex nuclei from rats under conditions of hypothermia can be caused by involvement of these signaling lipids in intracellular signaling processes. The findings seem to be promising for using hypobiosis under conditions of hypothermia–hypoxia–hypercapnia to modify the state of the neocortex cells for treatment of pathological processes in the brain.

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