We have studied biochemical changes occurring in brain cell mitochondria of white rats on the background of stress induced by 30-day isolation and disruption of circadian rhythms. It was ascertained that due to long-lasting stress, there occurs activation of oxidative processes in mitochondria as well as inhibition of anti-oxidant system activity, causing development of energy deficiency in brain cells. The above-mentioned biochemical processes become the reason for activation and opening of the mitochondrial permeability transition pore (MPTP), which, in its turn, signals the start of neuroapoptosis and various neurodegenerative processes.

Key words: Long-lasting stress, brain, mitochondria, oxidative stress, enzymes

INTRODUCTION

Multiple investigations have shown that stress causes damage to living cells, and, as a result, leads to development of various pathologies (Takeuchi et al. 2000, Cenci and Sitia 2007). Nervous tissue proves to be especially sensitive to damage of this type, and this is particularly true for the brain. It is already an established fact that stress serves as a cause of a wide range of neurodegenerative diseases (Smith et al. 2005, Masood et al. 2006, Reddy and Beal 2008, Ilieva et al. 2007). Stress factors act as a stimulus for response reactions in cells, namely, free radical oxidation, infringement of the intracellular homeostasis of ions, disturbances of energy metabolism, destruction of the hormonal status of the organism, loss of activity in DNA, protein molecules and enzymes, and so forth (McCord and Edeas 2005, Ando et al. 2008). Among the processes resulting from stress, activation of lipid peroxidation (LPO) is especially remarkable. This process results in oxidation of membrane phospholipids and development of oxidative stress, which serves as a cause of various pathologies of the nervous system (Giordano 2005, Cao et al. 1988).

Various biologically-active molecules, among them nitric oxide (NO), are involved in stress-related processes. NO activates the process of lipid peroxidation (LPO), which, in its turn, may frequently become the cause of cell death (Giordano 2005). NO, as a cell messenger, also takes part in the processes that determine cell viability and functional activity. The nature of NO action depends on metabolic conditions, namely, on the oxidation/reduction potential, pH and balance in the nascence of NO and active forms of oxygen in cellular compartments, which becomes the cause for development of oxidative stress (Thomas et al. 2003, Paolocci et al. 2007). It has been established that NO can do both: increase the viability of cells, and exert cytotoxic influence thereon. For instance, it has a positive effect on astrocytes, meanwhile causing death of neurons (Brorson et al. 1999). Cytotoxic properties of NO are mainly directed towards mitochondria, e.g., in the case of neurodegenerative diseases. Presumably, a mitochondrion represents the target of cytosolic NO. The cause of this effect is the following: high concentrations of NO inhibit oxidative phosphorylation, disrupt membrane potential and permeability, enhance outflow of Ca²⁺ ions and pro-apoptotic proteins from
mitochondria, and switch the tissues onto the anaerobic pathway of metabolism (Lacza et al. 2006, Navarro 2008). It is widely accepted that mitochondrial respiration brings forth nascent of O$_2^-$ particles rendered harmless by Mn-SOD. Through its action NO enhances creation of O$_2^-$ and, hence, superoxide that reacts with residues of mitochondrial proteins and alters activity of aconitate, creatinkinase, Mn-SOD and glutathione peroxidase (Mateo et al. 2003). Under the influence of NO, Ca$^{2+}$ ions get liberated from mitochondria, thus causing the transition of the cell into the stressed state characterized by the synthesis of the transcriptional protein CHOP (Szabó et al. 1996). Thus, it is evident that moderate amounts of NO contribute to viability of cells and possess cyto-protective properties; yet, in cases of considerable increases in its concentration, conditions are created that stimulate activation of oxidative stress in cells.

Some data suggest that structural changes of mitochondria and their results contribute to the process of creating free radicals and the events following their creation. This is related to an alteration of mitochondrial membrane permeability (Dolder et al. 2003). To a great extent, such alterations depend on MPTP formed at the sites of the junction of internal and external membranes and representing a complex of protein molecules and channels. As the complex binds with Ca$^{2+}$ ions, its conformation changes, followed by impairment of the membrane potential and swelling of the mitochondrial matrix, as well as disruption of the mitochondrial membrane and outflow of apoptotic proteins from the intermembrane space into the cytosol (Zamzami and Kroemer 2001, Dolder et al. 2003). A whole range of signaling proteins takes part in this process that finally results in the degradation of the DNA molecule in the nucleus. One of such protein molecules is Ras-protein. Our early experiments demonstrated that this protein is actively involved in the alteration of metabolism as a result of the stress caused by isolation of the animals and disruption of their circadian rhythms, and has a certain role in the structural changes occurring in mitochondria (Zhuravliova et al. 2009). In the scientific literature there exist some references to the fact that NO directly causes activation of the opening of the mitochondrial pore (Lin et al. 1997, Alano et al. 2002, Kern and Kehrer 2005). The mentioned effect is presumably caused by the enhancement of lipid peroxidation and by oxidation of thiolic groups of mitochondrial membrane proteins.

The aim of our project was to determine the quantitative changes of nitric oxide under the conditions of psycho-emotional stress caused by isolation of the animals and disruption of circadian rhythms, and to assess the functional state of mitochondria against the afore-mentioned background.

**METHODS**

**Animals and social conditions**

The experiment was conducted on 50 adult male Wistar rats (348 ± 5 g) divided into 2 groups, with 25 rats in each group. Rats in group 1, i.e., socially isolated rats (SI rats), were placed into individual cages in the dark (dark to light ratio, 23.5/0.5 h) during 30 days respectively. The control group contained 25 animals kept in a common cage under natural conditions (dark to light ratio, 14/10 h). During the experiments the rats were given water and a standard laboratory chow ad libitum. The experiment was repeated four times.

**Subcellular fractionation**

Fractionation of brain tissue was performed as per Whittaker (1969). Briefly, rat brains were homogenized in 0.32 M sucrose and centrifuged at 1000× g for 10 min. The supernatant (S1) was collected and centrifuged at 17 000× g for 55 min, resulting in the S2 supernatant and the P2 pellet (crude mitochondrial fraction). S2 was centrifuged at 100 000× g for 1 h to generate the P3 pellet (microsomal fraction) and supernatant (cytosol fraction). P2 was re-suspended in 0.32 M sucrose and layered onto a 1.2 M, 0.8 M block sucrose gradient. Following centrifugation at 53 000× g for 2 h, fractions A (0.32/0.8M boundary), B (0.8/1.2 M boundary) and C (pellet below 1.2 M) (pure mitochondrial fraction) were collected and centrifuged at 100 000× g 1 h to isolate membranes. Pure mitochondrial and cytosol fractions were used for further experiments.

The experiments were conducted in full accordance with the legal and statutory acts applicable in Georgia and the international agreements ratified by the country, such as the Law of Georgia on Health Care and European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Quantity of calcium in the samples was measured by means of test-system Calcium AS FS (Germany) manufactured by the company “DiaSys.”
Biomarkers of oxidative damage

Amount of NO was measured by determination of the product (NaNO₂) of reaction between NO and molecular oxygen (O₂) (Pahan et al. 2000). Concentration of active products of thiobarbituric acid, including malondialdehyde was determined in experimental samples by means of thiobarbituric acid test at the wavelength of 532 nm (Michara and Uchiyama 1978). Diene conjugates of non-saturated fatty acids were determined by spectrophotometry (Skornyakov et al. 1988). The method for determination of catalase activity was based on the ability of hydrogen peroxide to form a colored complex with salts of molybdenum, and intensity of their coloring was measured at the wavelength of 410 nm. The principle used to determine superoxide dismutase (SOD) proceeds from the ability of the enzyme to compete with tetrazole nitro blue for superoxide anion radicals (Tasset et al. 2008).

Energy metabolism enzyme activity

In order to determine activity of aconitase and fumarase, freshly isolated mitochondria were suspended in 0.5 ml of buffer containing 50 mM Tris–HCl (pH 7.4) and 0.6 mM MnCl₂ and sonicated for 2 s. Aconitase activity was measured spectrophotometrically by monitoring formation of cis-aconitate from added iso-citrate (20 mM) at 240 nm and 25°C. One unit (U) was defined as the amount of enzyme necessary to produce one micromole cis-aconitate per minute (ε₂₄₀=3.6 mM⁻¹ cm⁻¹). Fumarase activity was determined by measuring the increase in absorbance at 240 nm at 25°C in the reaction mixture to which 30 mM potassium phosphate (pH 7.4), and 0.1 mM L-malate were added. One unit (U) was defined as the amount of enzyme necessary to produce 1mM fumarate per minute (ε₂₄₀=3.6 mM⁻¹ cm⁻¹). Hydrazine assay was used to measure aldolase activity using d-fructoso-1,6-diphosphate (Sigma, St. Louis, USA) as substrate and hydrazine sulfate as detection reagent for the 3-phosphoglyceraldehyde formed. One unit (U) is described as absorbance (A) change per minute at 25°C, pH 7.5, and 240 nm wavelength (unit/mg=(A₂₄₀,test - A₂₄₀,blank)/mg enzyme per ml reaction mixture) (Miyadera et al. 2003). In order to determine the activity of succinate dehydrogenase, freshly isolated mitochondria were suspended in 0.5 ml of buffer containing 50 mM Tris–HCl (pH 7.4) and sonicated for 2 s. Mitochondria were incubated with 2% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in a solution of 0.05 M Tris, 0.5 mM MgCl₂, 2.5 mM CoCl₂ and 0.25 M disodium succinate at 37°C for 30 min. Mitochondria were solubilized by adding 150 µl of 6.35% dimethyl sulfoxide (prepared in 0.1 N NaOH) and colored formazan product was measured using a microplate reader at wavelength of 560 nm. One unit (U) was defined as the amount of enzyme necessary to produce one micro-

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria</th>
<th>Cytosol</th>
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<tbody>
<tr>
<td></td>
<td>Control animals</td>
<td>Stressed animals</td>
</tr>
<tr>
<td>NO (µM)</td>
<td>0.43 ± 0.02</td>
<td>0.95 ± 0.09**</td>
</tr>
<tr>
<td>Ca²⁺-ions (nM)</td>
<td>56.95 ± 2.36</td>
<td>115.53 ± 15.09*</td>
</tr>
<tr>
<td>Malondialdehyde (nM/mg protein)</td>
<td>0.40 ± 0.06</td>
<td>1.45 ± 0.20**</td>
</tr>
<tr>
<td>Diene conjugates (nM/mg protein)</td>
<td>0.57 ± 0.04</td>
<td>2.57 ± 0.33**</td>
</tr>
</tbody>
</table>

*P≤0.01; **P≤0.001

Table I

Changes in concentrations of NO, Ca²⁺ and products of lipid peroxidation in rat brain mitochondrial and cytosolic fractions under the conditions of 30-day stress
mole formazan per minute ($e_{560}=17 \text{ mM}^{-1}\text{cm}^{-1}$) (Zhuravliova et al. 2009). Activity of creatine kinase was determined by NADH formation following absorbance at 340 nm at 25°C. The assay medium contained 0.1–0.15 mg/ml of the mitochondrial protein, 50 mM Tris/HCl, pH 7.4, 10 mM glucose, 5 mM MgCl$_2$, 2 mM ADP, 1 mM NAD$^+$, 5 U/ml yeast hexokinase, and 1 U/ml glucose-6-phosphate dehydrogenase. The reaction started when 5 mM creatine phosphate was added. The change in absorbance over time at 340 nm was measured, and NADH generation rate was determined. One unit (U) was defined as the amount of enzyme necessary to produce one micromole of NADH per minute at 25°C (Ueda and Wada 1970).

**Assessment of the solubility of MPTP**

The solubility of MPTP was measured spectrophotometrically at the wavelength of 540 nm at room temperature. The incubation area was made by mitochondrial suspension (~0.5 mg/ml protein), and the solution (120 mM KCl, 3 mM KH$_2$PO$_4$, 5 mM sodium succinate) made in the area of 10 mM Tris-HCl (pH=7.4) and by 250 mM sucrose. In order to stimulate opening of the pore, we used CaCl$_2$ (200 µM), and to block the pore, cyclosporin A (0.42 µM) was applied. The degree of opening of the pore was assessed by means of corresponding light extinctions (Dolder et al. 2003).

All the reagents were purchased from Sigma–Aldrich (Sigma–Aldrich Inc., St. Louis, USA) unless otherwise specified.

**Data analysis**

Significance for tests was set at $P<0.01$. The data from each biochemical experiment were analyzed separately and treated by ANOVA. Experiments were repeated four times with triplicate samples for each experiment. When the significant effect was observed by ANOVA, Student’s $t$-test was also used to compare the samples.

**RESULTS**

**Study of intensity of lipid peroxidation in brain mitochondria**

Investigations carried out at the initial stage have revealed that there is a marked increase in the quantity of nitric oxide in mitochondrial and cytosolic fractions of rat brain mitochondria, as a result of 30-day isolation and disruption of circadian rhythms (Table I). For instance, in mitochondria, in comparison with the control data, this value increased by approximately twice, and in cytosol 1.8 times.

It is known that nitric oxide, as a signal molecule, is linked with a whole range of cellular processes, e.g., alteration of ion homeostasis in cells, amongs others; this is also true for calcium ions, Ca$^{2+}$. Hence, quantitative changes in Ca$^{2+}$ concentration in mitochondrial and cytosolic fractions were determined. The obtained results are presented in Table I. It becomes evident that due to stress in both fractions there is a marked increase in the concentration of calcium ions.

In view of the processes developing as a result of the increase in the concentration of nitric oxide, the course of the process of lipid peroxidation occurring in mitochondria was studied. For this purpose, the quantities of the products of lipid peroxidation were determined. This concerned malondialdehyde and diene conjugates in brain mitochondrial fraction under 30 days of psycho-emotional stress. The obtained data are presented in Table I where it becomes obvious that due to the 30 days of stress, the LPO process is activated in mitochondria. This is indicated by the increase in the products of lipid peroxidation (malone dialdehyde increases 3.5 times, and diene conjugates increase 4.5 times).

**Fig. 1. Ca$^{2+}$-induced opening of MPTP in brain cell mitochondria under the conditions of isolation and disruption of circadian rhythms.** Along the ordinate axis – swelling of mitochondria expressed in changes of optical density (OD); Along the abscissa axis: time of the experiment, in minutes.
It is known that lipid peroxidation products, namely, malone dialdehyde, interacting with proteins and nucleic acids, cause creation of intermolecular bonds. This is followed by structural changes of various receptors, ionic channels, enzymes and nucleic acids, ultimately resulting in the death of the cell (Bounous and Molson 2003). The cellular anti-oxidant system reacts to changes of this type. It contains many components, such as many enzymes, among them SOD and catalase. In view of this fact, the dynamics of the enzyme activity in brain mitochondria (Table II) was determined against the background of stress.

As it becomes evident from the table below, due to 30-day stress to which the animals are subjected, the activity of both enzymes in brain cell mitochondria shows a considerable drop. Namely, the activity of SOD amounts to 63% of the control value, and of catalase equals even less – only 34%. The obtained results point to the accumulation of excess amounts of superoxide radicals in brain mitochondria, and to the activation of oxidative stress as a consequence.

### Table II

<table>
<thead>
<tr>
<th>Fraction Type</th>
<th>Enzyme</th>
<th>Control</th>
<th>30-day stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>Superoxide dismutase (units/mg protein)</td>
<td>14.93 ± 0.25</td>
<td>9.41 ± 1.05**</td>
</tr>
<tr>
<td></td>
<td>Catalase (units/mg protein)</td>
<td>12.67 ± 1.04</td>
<td>4.32 ± 2.42**</td>
</tr>
<tr>
<td></td>
<td>Creatine kinase (Pi μM/mg protein)</td>
<td>21.30 ± 1.45</td>
<td>12.37 ± 1.33*</td>
</tr>
<tr>
<td></td>
<td>Succinate dehydrogenase (units/mg protein)</td>
<td>0.75 ± 0.005</td>
<td>0.25 ± 0.11*</td>
</tr>
<tr>
<td></td>
<td>Aconitase (units/mg protein)</td>
<td>0.48 ± 0.04</td>
<td>0.19 ± 0.02**</td>
</tr>
<tr>
<td></td>
<td>Fumarase (units/mg protein)</td>
<td>0.25 ± 0.07</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Cytosol</td>
<td>Aldolase (units/mg protein)</td>
<td>0.57 ± 0.01</td>
<td>0.82 ± 0.06*</td>
</tr>
</tbody>
</table>

*P ≤ 0.01; **P ≤ 0.001

Changes in the activity of energy metabolism enzymes under stress

It has been accepted that oxidative stress in a mitochondrion is accompanied by a reduction in the intensity of energetic metabolism in mitochondria, which is caused by a decrease in the activity of the enzymes participating in this process. The obtained data point to the fact that, as a result of isolation and disruption of the natural circadian rhythms of the animals, there occurs a decrease of functionality of the antioxidant system of rat brain mitochondria, and, accordingly, oxidative processes are activated. The latter, in its turn, has a negative impact on the state of mitochondria. Such negative impact is also expressed in the decrease in the activity of mitochondrial enzymes including those participating in energy metabolism. Table II shows the characteristics of activity of succinate dehydrogenase, mitochondrial creatine kinase, fumarase and aconitase after the 30-day psycho-emotional stress. From the table it becomes evident that
activation of oxidative processes and decrease in the function-ality of the anti-oxidant system in mitochondria is also accompanied by a reduction in the activity of the enzymes involved in energy metabolism.

It is widely accepted that the active centre of aconitase is presented by Fe-S cluster, due to which it reveals sensitivity to the active forms of oxygen and so it can be possibly used as a sensitive and specific marker for oxidative stress (Zhuravliova et al. 2009). It can be seen from the table that on the 30th day of psycho-emotional stress activity of this enzyme in the brain mitochondria diminishes about 2.5 times in comparison with the control group. Unlike aconitase, the enzyme fumarase, proceeding from its structure, does not reveal sensitivity to the oxidative agents and its activity does not practically change during the period of stress.

The next enzyme to study was succinate dehydroge-nase (Table II). It was found that its enzymatic activity was diminished 3 times, which also pointed to the decrease in oxidative phosphorylation taking place in mitochondria, and, respectively, to the reduction in ATP generation and enhancement of oxidative stress. Changes in creatine phosphokinase activity are other good indices for this process. The enzyme carries an important function in the process of ATP regeneration (Comim et al. 2011). It can be seen from the table that the activity of creatine phosphokinase in mitochondria decreases by about 40% in comparison with the control value.

In order to establish how anaerobic transformation of carbohydrates functions in such conditions we studied activity of aldolase, the glycolitic enzyme. The obtained data are presented in Table II. As we can see activity of aldolase under the conditions of the studied stress increased by about 44% in comparison with the control data.

**Determination of the functional state of MPTP of mitochondria of brain cells**

The obtained results have shown that due to long-lasting psycho-emotional stress in cells, and, namely, in mitochondria, oxidative processes are activated, which can be caused by Ca\(^{2+}\) ion-induced alterations in the messenger system (hormones, nitric oxide). The processes taking place under stress conditions, presumably, affect viability of cells, linking the process with apoptosis. To attest such hypotheses, we observed the functional status of the mitochondrial permeability transition pore (MPTP). It is known that MPTP is influenced by various factors, among them, by oxidative stress, which causes damage to the mitochondrial membrane, followed by a reduction in the membrane potential, disturbances in Ca\(^{2+}\) deposition, influx of water molecules into the mitochondrial matrix and a resulting mitochondrial swelling, finally ending with the outflow of mitochondrial components into cytosol and with the cell death (Dolder et al. 2003).

It is known that functioning of MPTP depends on various substances that interact with the protein components included in pore composition and alter the character of their action. In all cases Ca\(^{2+}\) ions alone, without the help of any additional factors, mediate activation and opening of the pore. A peptide cyclosporin A has an effect that is different from Ca\(^{2+}\) ions, and inhibits MPTP activation.

Proceeding from the above-said, the further aim of our work was to determine the link between the brain mitochondria under the conditions of stress and activation of oxidative processes and energy deficiency.

For this purpose we determined the functional state of MPTP of brain mitochondria of the stressed and control animals by studying how the extent of its activation is linked with inhibition of Ca\(^{2+}\) ions and cyclosporin A.

As shown on Figure 1, brain mitochondria of control animals, in comparison with those of the stressed animals, are more susceptible to the addition of pore activator, Ca\(^{2+}\) ions into the reaction area; whereas sensi-
tivity of mitochondria of the stressed animals to the ions of calcium is relatively low. The obtained result, presumably, must be caused by the increase in the concentration of $\text{Ca}^{2+}$ ions against the background of stress, which serves as a cause of the increase of MPTP permeability and its opening. The mentioned effect can possibly cause reduction in the quantity of intact mitochondria. The same fact is also attested by a lower index of light absorption (optical density, OD) of the stressed mitochondria as compared to the control mitochondria, which can be explained by the damages to mitochondrial membranes and by the presence of open MPTP.

A similar conclusion can be made by the data obtained in the next experiment, where we studied the influence of cyclosporin A on the changes in mitochondrial MPTP permeability in cases of prior addition of the ions of calcium into the reaction area (Fig. 2).

As it becomes evident from Figure 2, addition of cyclosporin A to the activated control mitochondria causes inhibition of the opening of MPTP. A different picture is observed in the case of stressed mitochondria. Specifically, addition of cyclosporin A does not reveal any protective effect on the MPTP in mitochondria.

It is seen from the obtained data that long-lasting psycho-emotional stress is largely characterized by damage to mitochondria. This is revealed by disturbance of normal functioning of MPTP, though for this stage it is difficult to establish the exact molecular mechanism of the damage and it has been set as a goal of our future research.

Thus, the obtained results enable us to suppose that long-term isolation and disruption of circadian rhythms of the animals represents a powerful factor, due to which certain processes are activated in brain mitochondria, which ultimately lead to activation of the mechanisms of cell death.

**DISCUSSION**

It is known that structural and functional damage are the main cause of development of degenerative changes in the nervous tissue. This is expressed in the reduction of ATP synthesis, production of active radicals, creation of energetic deficiency in nervous cells, damage to membranes, etc. These processes can be caused by various factors, stress being the most important of them (Friedlander 2003, Sierra et al. 2007, Kilbride et al. 2008, Schmidt et al. 2008).

Factors, such as long-lasting social isolation and disruption of circadian rhythms, cause psycho-emotional stress in animals, which, in its turn, conditions various behavioral disturbances, increased aggressiveness, etc. (Maekawa et al. 2010). Accordingly, social isolation and disruption of circadian rhythms can be discussed as one of the most important psychological stress factors. Here we should also mention the results of our previous research. In particular, we also determined the status of locomotor activity parameters among the rats after the stress period by open field test. We found that socially isolated rats were significantly hypoactive, but showed no significant difference in center/total distance ratios. These results indicate that isolated rats have similar levels of basal anxiety relative to normal rats (Zhuravliova et al. 2009). At the same time, multiple research studies attest that there is a link between social isolation, disruption of life cycle rhythms and various pathologies, among them, neuroapoptosis (Bottino et al. 2004). Among the triggers of neuroapoptosis, the creation of active molecules is of key importance. Nitric oxide is considered to be one of such molecules, the important source of which is provided by mitochondria themselves. The intensity of the process increases, especially, under the conditions of growing calcium concentrations in mitochondria (Atlante 1997). According to the data obtained by us, under the conditions of stress caused by isolation and disruption of circadian rhythms, the quantities of both nitric oxide and calcium ions show a considerable increase in rat brain mitochondria (Table I). It is known that the quantitative increases in the concentration of endogenous $\text{Ca}^{2+}$ as of the co-factor of nitric oxide synthase represents a factor in augmented synthesis of intracellular NO. The latter serves as a source of active radicals, promoting oxidation of membrane components (Sinner et al. 2001, Pshennikova et al. 2002). These results can presumably cause the processes occurring in mitochondria, meaning activation of oxidative stress. The data obtained by us point to the activation of the process of lipid peroxidation against the background of stress. Increases in the concentration of malone dialdehyde and diene conjugates serve as an index of this kind of activation (Table I). It is known that malone dialdehyde, interacting with proteins and nucleic acid molecules, causes creation of intermolecular bonds. Such an action of this substance serves as a cause of the changes in the activity of cell receptors, ionic channels, proteins of the cytoskeleton.
and enzymes (Dubinina et al. 2002). We can consider the decrease in the activity of the cell antioxidant system to be the cause of the increase in the quantities of lipid peroxidation products; namely, there is reduced activity of enzymes, such as catalase and SOD (Table II). The data presented by us show that the long-term or 30-day isolation and disruption of biological rhythm among animals considerably reduces their activities, which, in its turn, becomes the cause of activation of oxidative processes.

One of the results of oxidative stress is the development of neurodegenerative pathologies of various types. For instance, it is known that amyotrophic lateral sclerosis develops due to changes occurring in the gene coding superoxide dismutase, accordingly implying changes in its activity. Changes in the catalase and glutathione peroxidase systems can cause development of diseases such as Alzheimer’s, Huntington’s and Parkinson’s (Perez Nievas et al. 2011, Schapira and Jenner 2011). The obtained results reveal that psycho-emotional stress represents factor that changes the function of the antioxidant system acting in mitochondria.

It is a widely accepted opinion that enzymes of the respiratory chain represent one of the principal targets of oxidative processes (Gopalakrishna and Jaken 2000, Pastorino and Hoek 2003, Da-Silva et al. 2004, Zhuravliova et al. 2009). The data presented in Table II indicate that, due to long-lasting stress activity of succinate dehydrogenase and aconitase, enzymes are considerably reduced, which, in its turn, is an indicator of a decrease in the oxidative phosphorylation and ATP generation, and, accordingly, in energy deficiency.

There exist data in scientific literature suggesting that mitochondria indeed represent the source of free radicals, which is reflected in the damage of its membranes and changes in their permeability. This fact finds a good reflection in the processes of formation and functioning of the specific mitochondrial pore MPTP (Albensi et al. 2000, Fiskum 2000, Sullivan et al. 2000, Alano et al. 2002).

It is known that this structure has a complex construction and is formed at the sites of junction of internal and external mitochondrial membranes. Its opening causes disturbances of the membrane potential, outflow of calcium ions into the cytosol and increase of the permeability of the mitochondrial membrane. All of the above-listed are accompanied by the appearance of apoptotic proteins in cytosol and inhibition of the systems of respiratory chain. Due to this process, the potential difference across the membrane decreases, causing reduced ATP synthesis and formation of energy deficiency in the cell (Fiskum 2000, Alano et al. 2002, Doczi et al. 2011).

Our data show that the psycho-emotional stress in animals takes place against the background of the increase in the amount of nitric oxide and increased intensity of accumulation of Ca²⁺ ions in mitochondria (Fig. 1). It is known that disruption of the process of accumulation of calcium ions represents one of the principal causes for opening of the mitochondrial pore and outflow of apoptotic proteins as well as of cytochrome C into the cytosol (Pastorino and Hoek 2003). Appearance of cytochrome C in cytosol is also supported by a decrease in pH and oxidative modification of mitochondrial proteins and lipids. Various signaling proteins are also involved into this process, e.g., Bcl-2 and Ras (Kroemer et al. 2007, Zhuravliova et al. 2009). There are also documented changes of Ras protein under the conditions of stress induced by isolation and disruption of circadian rhythms, namely, activation of Ras in cells of the hippocampus and its translocation from plasmatic membrane to intracellular compartments.

The data available from the scientific literature also point to the connection of MPTP and the creatine kinase enzyme that serves as a pore stabilizer under usual conditions (Wyss and Kaddurah-Daouk 2000). If we look at the data presented in Table II it is fairly probable that long-lasting psycho-emotional stress creates all the conditions for disruption of the function of the mitochondrial pore. Results of the experiments made with the aim to reveal the activities of MPTP also point to very much the same (Figs 1 and 2).

The results obtained indicate that prolonged psycho-emotional stress activates various pathological processes that can possibly affect the messenger system, which is indicated by quantitative changes in Ca²⁺ and NO (Table I). This process is accompanied by a reduced activity of the anti-oxidant system and activation of oxidative processes (Table II), which has some additional effect on the cell. The process is coupled with a drastic change in the energy metabolism and can lead the system to severe outcomes.

The processes in progress as a result of the stress are directly related to the transformations occurring in the mitochondrion, followed by an increased permeability of the mitochondrial membrane at the expense of MPTP activation, and swelling of the mitochondrion. This can explain the data presented in Figures 1 and 2.
CONCLUSION

Prolonged stress activates oxidative processes in mitochondria and inhibits activity of the anti-oxidant system, causing development of energy deficiency in brain cells.

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