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EXPERIMENTAL ARTICLES

Malate–Aspartate Shunt in Neuronal Adaptation to Ischemic Conditions: Molecular–Biochemical Mechanisms of Activation and Regulation

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Abstract—Acute or chronic brain ischemia induces a cascade of pathobiochemical reactions that finally result in the development of focal neurological deficit, dyscirculatory encephalopathy, or the death of a patient. We studied the effects of ischemia at different time points, including 1, 6, 24, 48, 72, and 120 h, and 21 days. During the period of the strongest ischemia-induced disturbances (24–72 h), we found lactate overproduction associated with inhibition of hexokinase, an enzyme that catalyzes the first "trigger" reaction of glycolysis. An increase in the malate content associated with increasing activities of mitochondrial and cytosolic malate dehydrogenases within the first hours of cerebral ischemia indicates the activation of the malate–aspartate shuttle, which is responsible for the transportation of reduced equivalents to mitochondria. The inhibition of malate production and activity of NAD-dependent malate dehydrogenase correlates with a decrease in the contents of ATP, HSP70, and hypoxia-induced factor-1a (HIF-1a) and the severity of neurological disturbances. We believe that in response to brain ischemia, HIF-1a is expressed, which induces compensatory mechanisms of energy production.

Keywords: brain ischemia, compensatory shunts of energy production, mitochondrial-cytosolic energy transmission, heat shock proteins

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INTRODUCTION

Vascular brain diseases are one of the most important causes of human deaths and disabilities all over the world. The occlusion of vessels that supply the brain is the starting point in a chain of negative events that result in the severe deterioration of neuronal metabolism and structural and functional modifications that often lead to neuronal death. Acute or chronic brain ischemia induces a cascade of pathological biochemical reactions that finally result in the development of a focal neurological deficit, circulatory encephalopathy, or the death of a patient [1, 2]. The close relationship between disturbances of energy and plastic turnovers and, frequently, their effects on the course and prognosis of disease are not taken into account during the development of treatment schemes; the restoration of the blood supply is used as a basis for pathogenetic therapy. Impairments of energy metabolism and the possibility of their correction are a subject of many recent studies [3, 4]. Many researchers believe that metabolic therapy at the early stage of a stroke and during the period of restoration is a potent preventive factor for repeated strokes and patient disability and death [5, 6].

Fundamental studies on energy production under ischemic conditions have demonstrated that energy is produced due to anaerobic glycolysis, which results in the formation of two ATP molecules and lactate accumulation [7, 8]. At the early stage of cerebral ischemia of various etiologies the rate of aerobic oxidation in mitochondria decreases. This is associated with a decrease in the ATP content and an increase in the levels of ADP and AMP, which results in a decrease in the [ATP]/([ADP] + [AMP]) ratio. A low [ATP]/([ADP] + [AMP]) ratio leads to the activation of hexokinase (HK), which substantially increases the capacity of anaerobic glycolysis reactions. Under these conditions, the cell uses glycogen for energy production via anaerobic glucose cleavage [9, 10]. At this stage, adaptation to hypoxia and the stabilization of energy turnover may be observed. However, this stabilization is short lived and is followed by a rapid exhaustion of the glycogen store. Anaerobic glycolysis cannot meet the energy demands of the brain completely and longitudinally. The end product of glycolysis is lactate, whose accumulation results in intracellular acidosis. During the early stages of ischemia, cellular acidosis may be considered as a protective response, because a pH decrease stabilizes

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cellular membranes. However, the progression of acidosis induces the denaturation of some proteins and the formation of granules in the cytoplasm, which is expressed via the appearance of cytoplasmic opacity known as "opaque swelling" or "granule dystrophy" [11]. An enhanced lactate release during hypoxia results in metabolic lactic acidosis, which blocks gene activities and limits adaptation. At this stage of hypoxia, a true ATP deficit is formed in the cell because aerobic and anaerobic mechanisms do not function due to oxygen deficit and acidosis, respectively [7, 9, 12]. Thus, resistance to hypoxia is formed via modification of energy pathways involving mobilization of the proton supply for oxidative phosphorylation and the economical use of oxygen under conditions of its failure. The pathway that is mediated by succinate oxidase is considered to be the principal pathway; multiple compensatory mechanisms result in anaerobic succinate synthesis, including Kondrashova's transaminase cycle, the Roberts cycle and others. Thus, in the 1980s and 1990s, metabolic therapy was considered to be an important preventive factor of repeated strokes, the disability of patients, and their death during the acute or restorative periods after stroke [13].

However, clinical trials with drugs containing succinic acid, such as reambirin, yantavit, polisar, tsitoflavin, and others, had a low level of therapeutic efficacy against acute cerebral ischemia. Recent breakthroughs in the field of molecular biology shed light on the importance of regulatory proteins in the functioning of many stages of energy metabolism. Thus, it has been shown in several experimental studies that during ischemia the genes that code the hypoxia-inducible factor-1 (HIF-1) and specifically its 120 kD HIF-1a subunit are activated. Under ischemia conditions, HIF-1a is responsible for the expression of the erythropoietin gene and approximately 60 more genes whose products are involved in proliferation, apoptosis, angiogenesis, and stabilization of protein molecules during oxidative stress. Furthermore, according to recent data, heat shock proteins (HSPs) are involved in the stabilization of HIF-1a during cerebral ischemia, which is followed by intensification of free radical oxidation, a shift in thiol-disulfide balance, development of nitrosative stress, and glutamate excitotoxicity [14–16]. HSPs are induced in cells of all living organisms in response to various stress factors, including heat shock, hypoxia, ischemia, metabolic disturbances, viral infections, and pharmacological agents. The genes of these proteins are activated, not only under stress conditions, but also during basic processes of the vital activity of cells, proliferation, differentiation, and apoptosis [17, 18].

A predominating role of the succinate oxidase-mediated mechanism was proposed on the basis of the data from experiments with isolated organs and tissue cultures that were subjected to ischemia and hypoxia of different severities. Keeping this fact in mind, we decided to study the state of the systems that limit energy turnover, compensatory metabolic shunts, and the molecular mechanisms that control these processes in brain ischemia. This simultaneous study of various metabolic processes and the levels of heat shock protein 70 (HSP 70) and HIF-1a subunit provided us with data on the direction and rate of changes in these processes. In this study, we examined the indices of the transportation system of reducing equivalents and oxidation substrates for mitochondria, the Krebs cycle, tissue respiration, the content of macroergic phosphates (ATP), the activity of enzymes that regulate mitochondria–cytosol energy transport, and contents of HSP 70 and HIF-1a in mitochondria of the brains of Mongolian gerbils that were subjected to acute cerebral ischemia.

MATERIALS AND METHODS

The disturbance of brain circulation was modeled by an irreversible unilateral occlusion of the carotid artery in Mongolian gerbils (*Meriones uniculatus*) weighing 65–70 g. According to the literature data, these animals are often used for modeling disturbances of brain circulation, because their systemic circulatory system is separated, while the system of collateral circulation is less developed [19].

All experimental procedures were performed in accordance with the regulations on the use of animals for biomedical research. The animals were killed by intraperitoneal injection of sodium thiopental at a dose of 40 mg/kg.

Biochemical studies were performed in the brain tissue taken 1, 6, 24, 72, 120 h, and 21 days after ischemia induction. For this purpose, the fraction that was enriched with neurons was divided into cytosolic and mitochondrial fractions using differential centrifugation. Centrifugation was performed at 60000 *g* using a 5804R refrigerated centrifuge (Eppendorf, Germany). We studied the activities of mitochondrial and cytosolic NAD- and NADP-dependent malate dehydrogenases (mMDH and cMDH, respectively), succinate dehydrogenase (SDH), mitochondrial aspartate aminotransferase (AST), cytochrome oxidase, and hexokinase in the cytosolic and mitochondrial fractions using spectrophotometry. The activities of mitochondrial and cytosolic creatine phosphokinase (mCPK and cCPK, respectively) were measured after separation on DEAE-sephadex A-50 using Warburg's optical test. The contents of lactate and malate in the brain were estimated using the method of Hohorst. The isocitrate level in the tissue was measured accordingly to the method of Siebert [20].

The levels of the HIF and HSP proteins were assayed by Western-blot analysis. Proteins were sepa-

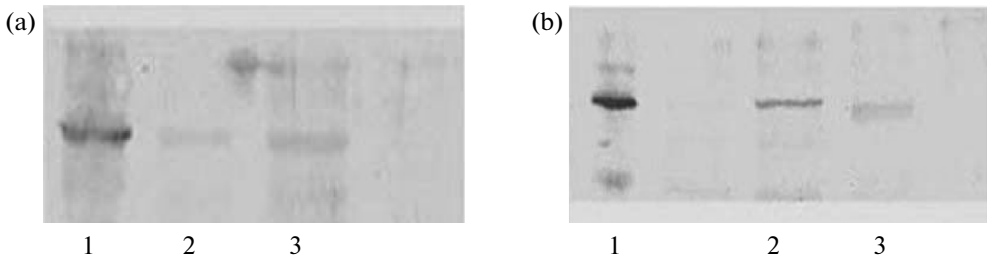

Fig. 1. Immunodetection of HIF-1a (a) and HSP 70 (b) proteins. Groups 1, 2, and 3 of Mongolian gerbils with low, severe, and moderate neurological disturbances, respectively.

rated on a 10% polyacrylamide gel (PAAG). Protein transfer from PAAG to a nitrocellulose membrane was performed using electroelution for 45 min. Western-blot preincubation was performed in 5% skimmed milk dissolved in Tris-buffered saline containing Tween-20 (TBST). The blots were then incubated for 1 h with primary monoclonal anti-HIF and anti-HSP antibodies (Santa Cruz Biotechnology) diluted at 1 : 1000. After washing, the blots were incubated for 1 h with secondary antibodies (Santa Cruz Biotechnology) conjugated with horseradish peroxidase diluted at 1 : 2000. Densitometric measurements were performed using Adobe Photoshop software [21, 22].

The neurological deficit was estimated according to the scale of McGrow [23]. The severity of the state was calculated as the sum of the points using the following point scale: less than 3 points, 3–7 points, and over 7 points for low, moderate, and severe deficits, respectively. We also recorded paresis, limb palsy, tremor, circling behavior, ptosis, lying in a side position, and locomotion.

The normality of the distribution was estimated using the Kolmogorov–Smirnov, Lilliefors, and Shapiro–Wilk tests. When the distribution was not normal or we analyzed ordinal variables, we used the Mann–Whitney *U*-test for two independent samples or the Kruskal–Wallis *H*-test followed by Games–Howell comparison for higher numbers of samples. Qualitative indices were compared using the χ^2-test with analysis of contingency tables. The data are presented as mean values with standard errors. The interrelationships between the variables were estimated using binary regression analysis. All calculations were performed using SPSS 16, Microsoft Excell 2003, and STATISTICA® for Windows 7.0 (StatSoft Inc.) software. The differences were considered as statistically significant at a level of significance less than 0.05 [24].

RESULTS AND DISCUSSION

The data from the experiments performed at different time points after ischemia induction demonstrated that during the period of the strongest ischemic disturbances, e.g., 24–72 h, lactate was overproduced, whereas hexokinase, which catalyzes the first trigger reaction of glycolysis, was inhibited (Table 1). During the time course of the changes in oxidative metabolism, we observed a strong inhibition of SDH by 77–85% and a decrease in the isocitrate level by 56–70%. Restoration of these indices started only 21 days after ischemia induction. A remarkable increase in the activity of mMDH and cMDH, which was observed in 1–24 h after ischemia induction, led to a 20–50% accumulation of malate, which was then followed by a moderate depression of their activity by 10% at 48–72 h; this was associated with a respective decrease in the malate content by 16–38% (Table 1). Thus, we observed expressed inhibition of the tricarboxylic acid cycle in the citrate–succinate stage. This strong inhibition of the SDH activity aggravated functioning of the succinate oxidase-mediated pathway of the proton supply to the respiratory chain. Malate accumulation associated with increased mMDH and cMDH activities during the first hours after ischemia induction in the brain demonstrated the activation of the malate–aspartate shuttle mechanism for the transportation of reducing equivalents to mitochondria. Very interesting changes in the bioenergetical indices were observed during the acute period of ischemia up to 24 h. The strongest changes were revealed in the activities of mitochondrial and cytosolic NAD- and NADP-dependent malate dehydrogenases, as well as in the contents of HSP 70 and HIF-1a (Fig. 1).

We found parallel changes in the level of malate and the activity of NAD-dependent mitochondrial MDH, cytoplasmic AST, and the contents of HSP 70 and HIF-1a. We also revealed a significant correlation between changes in the levels of malate, NAD-MDH, and HSP70 ($m_r = 0.821$; $T = -2.94$). The general trend to a decrease in malate content was associated with restoration of NADP-MDH and HIF-1a ($m_r = 0.839$; $T = -3.09$). Mathematical analysis revealed the direct dependence of the activity of MDH on the content of HSP70. These results demonstrated a close association between the level of expression of the heat shock proteins, the independent parameter, and the MDH activity in mitochondria (Fig. 2). This dependence had direct transcendent character and may be

statistically approximated by a logarithmic regression model. The approximation error was 0.19; the value of the residual variance showed the high accuracy of the linear model. Thus, we can consider that the task of regression analysis was solved ($R = 0.93$; $R^2 = 0.86$; normalized $R^2 = 0.84$ at $F = 53.25$; standard error, 0.604; $p = 0.00082$) (Fig. 2).

In order to clarify the role of the malate shunt in the mechanisms of compensatory energy production and the molecular mechanisms of its regulation, we randomized animals with acute disturbances of their cerebral circulation in accordance with their resistance to ischemia as estimated using McGrow's scale 24 h after ischemia induction. We found that the animals with higher point totals according to McGrow's scale (a clear neurological deficit) had the lowest contents of malate, HSP70, and HIF-1a (Fig. 1, Table 2). Moreover, the decrease in the malate content in mitochondria was significantly correlated with a reduction in the levels of HSP70 ($m_r = 0.899$; $T = -11.4$), NAD-mMDH ($m_r = 0.976$; $T = -6.3$), mitochondrial AST ($m_r = 0.997$; $T = -9.1$), and ATP ($m_r = 0.994$; $T = -9.3$) (Table 2, Fig. 2). These data show that under conditions of acute brain ischemia energy production depends on the functioning of the malate–aspartate shunt. Taking the data from multiple experimental studies into account, we can suggest that the malate–aspartate shunt has several advantages. Firstly, it is more resistant to hypoxia and, second, it supports the electron transport chain in ischemic conditions and thus partially replaces the succinate oxidase-mediated mechanism of proton supply. In addition, Roberts' shunt function is limited by the content of gamma-aminobutyric acid (GABA) in the brain [7, 10]. Moreover, the accumulation of malate is an index of the efficiency of this shuttle system and the level of HIF-1a determines the probability of activation of this compensatory energy shunt, whereas the content of HSP70 determines the possibility of its long-term functioning. This conclusion is supported by the data from other studies. Dery and Huang demonstrated that chaperone HSP90 can bind to the PAS domain (Per–Arnt–Sim) of B-factor and stabilize it [16, 25]. Another cellular chaperone, HSP 70, recognizes the other structural motif of the HIF-1a molecule, the so-called oxygen-dependent degradation domain (ODD) [25]. The role of these protein–protein interactions is not clear. It has been proposed that they are important for HIF-1a stabilization under normoxia conditions. In hypoxia, at least one HSP 70 chaperone is replaced in the HIF-1a complex with the aryl hydrocarbon receptor nuclear translocator protein (ARNT), which protects the structure of the factor from directed proteolysis for 20–30 min of hypoxia. Thus, HSP 70 may prolong the lifetime of HIF-1a under conditions before and after hypoxia and it is necessary for an appropriate cellular response to oxygen deprivation [25].

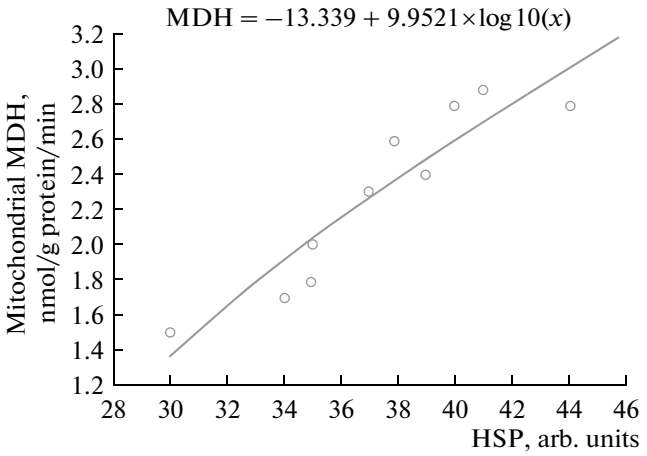

Fig. 2. Data from regression analysis of the relationship between HSP and MDH.

We can make several conclusions from the results of our study. Bilateral occlusion of the common carotid arteries is associated with disturbances that are typical of ischemia, including activation of glycolysis with lactate overproduction, inhibition of the enzymes of the Krebs cycle and the electron transport chain, ATP deficit, and attenuation of HSP 70 and HIF-1a expression. However, studying the time course of their development and dividing them in accordance with the severity of neurological disturbances helps one to estimate the molecular-biological mechanisms of adaptation and to evaluate the severity of disturbances at specific metabolic stages, the compensatory pathways of energy supply, and regulatory proteins, including HSP 70 and HIF-1a. The degree of inhibition of SDH, which provides protons to the FAD-dependent step of the electron transport chain, was substantially higher as compared to cytochrome oxidase, which determines electron flow along the entire chain. Moreover, we observed a substantial decrease in the content of isocitrate, an intermediate of the tricarboxylic acid cycle. These data suggest that the Krebs cycle, which is under control of citrate synthase and α-ketoglutarate dehydrogenase, is strongly inhibited. In addition to this, the functioning of the compensatory succinate oxidase mechanism is hindered. In spite of these facts, the respiratory chain works and ATP is produced, although at a lower level. We believe that other compensatory mechanisms are involved in proton supply to the respiratory chain. The increase in the malate content and mitochondrial NAD-MDH activity, which correlates with the HSP 70 level, both during the first minutes of ischemia and in animals that are resistant to ischemia, seems to be very important. These data may be explained by the activation of malate–aspartate mechanisms for the transport of reducing equivalents to the mitochondria and the involvement of the HSP 70 and HIF-1a adaptation

Table 1. The energy turnover and contents of the HIF and HSP proteins in the brains of Mongolian gerbils at different time points after the induction of cerebral ischemia

Indices	Intact	1 h	6 h	24 h	48 h	72 h	120 h	21 days
Malate, μmol/g of tissue	0.44 ± 0.022	0.56 ± 0.012*	0.87 ± 0.032*	0.78 ± 0.014*	0.37 ± 0.034*	0.27 ± 0.011*	0.25 ± 0.023*	0.43 ± 0.021
Isocitrate, μmol/g of tissue	0.48 ± 0.021	0.67 ± 0.027*	0.54 ± 0.044*	0.21 ± 0.027*	0.18 ± 0.033*	0.14 ± 0.018*	0.20 ± 0.015*	0.27 ± 0.031*
Mitochondrial NAD-MDH, μmol/g tissue/min	1.23 ± 0.071	1.87 ± 0.016*	2.47 ± 0.033*	2.11 ± 0.041*	1.87 ± 0.037*	1.12 ± 0.016*	1.11 ± 0.019*	1.44 ± 0.012*
SDH, μmol/g tissue/min	5.4 ± 0.21	7.8 ± 0.7*	5.2 ± 0.3	1.2 ± 0.3*	1.2 ± 0.5*	1.0 ± 0.2*	1.2 ± 0.5*	3.77 ± 0.5*
ATP, μmol/g of tissue	2.94 ± 0.085	1.84 ± 0.074*	1.5 ± 0.068*	1.43 ± 0.081*	1.32 ± 0.047*	1.25 ± 0.057*	1.23 ± 0.074*	1.13 ± 0.053*
Hexokinase, μmol/g tissue/min	10.45 ± 0.79	18.65 ± 1.23*	25.3 ± 1.3*	16.2 ± 0.65*	12.32 ± 1.0*	7.43 ± 0.68*	6.97 ± 0.36*	7.06 ± 0.6*
Lactate, μmol/g of tissue	2.65 ± 0.36	3.96 ± 0.31*	4.13 ± 0.41*	5.4 ± 0.28*	6.37 ± 0.42*	6.4 ± 0.36*	5.8 ± 0.33*	5.1 ± 0.27*
HSP 70, arb. U/g of protein	15.4 ± 0.31	22.5 ± 0.48*	25.3 ± 0.31*	23.6 ± 0.51*	22.7 ± 0.33*	20.6 ± 0.5*	20.3 ± 0.42*	18.7 ± 0.4*
HIF-1a, arb. U/g of protein	18.5 ± 0.65	31.5 ± 0.48*	32.6 ± 0.5*	27.9 ± 0.43*	21.7 ± 0.62*	20.4 ± 0.41*	19.7 ± 0.37*	19.2 ± 0.52*
Cytochrome oxidase, μmol/g tissue/min	14.8 ± 0.5	13.5 ± 0.3*	12.0 ± 0.3*	9.2 ± 0.5*	7.8 ± 0.5*	7.7 ± 0.5*	8.4 ± 0.3*	12.4 ± 0.5*

Note: *, $p \leq 0.05$ as compared to intact group.

Table 2. Indices of energy turnover and contents of HSP and HIF-1a in the brains of animals with different severities of neurological impairment after the induction of cerebral ischemia

Indices	Intact	Low deficit	Moderate deficit	Severe deficit
ATP, μmol/g of tissue	2.98 ± 0.092	1.65 ± 0.11*	1.312 ± 0.12*	1.12 ± 0.077*
Malate, μmol/g of tissue	0.44 ± 0.022	0.51 ± 0.052*	0.37 ± 0.052*	0.20 ± 0.032*
Laktate, μmol/g of tissue	2.78 ± 0.32	3.77 ± 0.47*	5.12 ± 0.52*	6.58 ± 0.21*
Cytosolic NADP-MDH, μmol/g tissue/min	5.23 ± 0.21	5.44 ± 0.17*	4.91 ± 0.81*	4.12 ± 0.87*
Mitochondrial NADP-MDH, μmol/g tissue/min	6.27 ± 0.12	7.82 ± 0.32*	4.32 ± 0.23*	3.00 ± 0.21*
Cytosolic NAD-MDH, μmol/g tissue/min	1.57 ± 0.052	1.55 ± 0.055	1.12 ± 0.032*	0.98 ± 0.043*
MitochondrialNAD-MDH, μmol/g tissue/min	1.77 ± 0.11	2.65 ± 0.12*	1.12 ± 0.10*	0.82 ± 0.032*
Mitochondrial AST, μmol/g tissue/min	3.67 ± 0.22	3.98 ± 0.54	2.34 ± 0.43*	1.21 ± 0.20*
Cytosolic CPK, μmol/g tissue/min	0.97 ± 0.021	1.11 ± 0.033	0.82 ± 0.021*	0.61 ± 0.023*
Mitochondrial CPK, μmol/g tissue/min	0.82 ± 0.012	0.98 ± 0.027*	0.78 ± 0.017*	0.65 ± 0.026*
Cytochrome oxidase, μmol/g tissue/min	14.2 ± 0.8	11.3 ± 0.7*	5.7 ± 0.8*	4.0 ± 0.7*
HSP 70, arb. U/g protein	15.4 ± 0.31	27.5 ± 0.37*	23.2 ± 0.4*	16.5 ± 0.28*
HIF-1a, arb. U/g protein	18.5 ± 0.65	31.6 ± 0.42*	26.4 ± 0.3*	19.2 ± 0.27

Note: *, $p \le 0.05$ as compared to intact group.

proteins in its activation and control. Inhibition of malate production and the activity of mitochondrial NAD-MDH was correlated with the losses of ATP, HSP70, and HIF-1a, as well as the severity of neurological disturbances. We believe that HIF-1a is expressed in response to cerebral ischemia and it triggers the compensatory mechanisms of energy production. HSP70 then begins to control these processes. HSP70 "prolongs" the effects of HIF-1a and maintains the activity of mitochondrial NAD-MDH and the activity of the malate–aspartate shuttle mechanism.

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