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Activity of Ca^{2+}-Dependent Neutral Proteases in Tissues of Ground Squirrel during Hibernation and during Self-Warming after Induced Awakening

P. M. Nurmagomedova, M. M. Abasova, and E. Z. Emirbekov

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Cyclic changes in activity of Ca^{2+}-dependent neutral protease occur during preparation for hibernation, with an increase in September and November and decrease in October and December. During hibernation proteolytic enzyme activity decreased, while during self-warming after induced awakening, the role of Ca^{2+}-dependent processes in the tissues of ground squirrels increased according to the body temperature.

Key Words: *hibernation; induced awakening; ground squirrel; Ca^{2+}-dependent neutral proteases*

Cells of various animal and human tissues contain proteolytic enzymes with maximum activity at near neutral pH. These enzymes include Ca^{2+}-dependent neutral proteases (calpains), cysteine proteases (caspases), and metalloproteases. Substrates of these enzymes are proteins of the cytoskeleton and nuclear matrix, substance P, opioid peptides, neurotransmitters, protein kinase C, phospholipases, *etc*. [1]. The regulatory function is very important function of neutral proteases [8]. Calpains are involved in apoptosis, either directly by cleaving cytoskeleton proteins in certain types of apoptosis, orindirectly, through regulation of caspase activity [7,12]. Participating in limited proteolysis, they are involved in adaptation of cell metabolism to changing environmental conditions, *e.g*. during hibernation [10], probably via activation of hibernation triggers.

The decrease in body temperature during hibernation widely varies. The body temperatures of hibernating ground squirrels are maintained by a few degrees above ambient air temperature [6]. In the time course of hibernation, the mechanisms of thermoregulation remain unchanged. In this case, biochemical processes are rearranged so that homeostasis is maintained even at very low body temperature [4,14]. During hibernation, the degree of inhibition of metabolic processes varies in different brain structures. However, at low body temperature, the energy deficit leads to calcium ion excess in cells, which affects activity of Ca^{2+}-dependent processes.

Here we studied activity of Ca^{2+}-dependent neutral proteases in tissues of ground squirrels during hibernation and in the course of self-warming after induced awakening.

MATERIALS AND METHODS

The experiments were carried out on ground squirrels (*Citellus pigmaeus Pallas*) weighting 200-250 g, typical representatives of hibernating animals. Ground squirrels were caught in the region of Buinaksk pass

Department of Biochemistry and Biophysics, State University of Dagestan, Makhachkala, Russia. ***Address for correspondence:*** miasat1981@mail.ru. M. M. Abasova

0007-4888/11/1515-0575

(Dagestan Republic) in spring and summer by flooding of their holes and kept in vivarium conditions on a mixed vegetable and grain diet until cold season. For induction of hibernation, the rodents were placed in a dark and unheated rooms with air temperature corresponding to that outside the vivarium, and transferred to dry food. After reducing air temperature to 8-6°C, the animals were placed in glass tanks with prepared bedding. Animal activity was examined daily by the "sawdust test". For evaluation of the processes occurring during awakening two months after the beginning of hibernation in the middle of the period of hibernation, the animals were placed in a refrigerator and after attaining body temperature 2.5°C were transferred to a room with a temperature of 25°C for self-warming. After reaching body temperature of 10, 20, 25, 30 and 38°C, ground squirrels were taken to experiments. Ca^{2+}-dependent neutral protease activity was determined by the methods of Baundry [5] and Nilsson and Karlsson [13]. The protein content was determined after Lowry [11].

RESULTS

Table 1 shows the data on activity of Ca^{2+}-dependent neutral proteases in the tissues of ground squirrel during hibernation and in the course of self-warming after induced awakening.

In September, in awake ground squirrels, activity of Ca^{2+}-dependent neutral proteases in the brain cortex and brainstem structures increased by 79 and 92%, respectively, and in the heart by 1.8 times ($p<0.01$). Taking into account the fact that preparation for hibernation starts in September, this significant increase in activity of neutral proteases can be explained by renovation of tissue proteins, as well as the appearance of new peptides [7,8], probably, due to limited proteolysis.

Thus, activity of Ca^{2+}-dependent neutral proteases in the examined tissues increased during the period preceding hibernation. This may reflect the involvement of these enzymes in preparations for hibernation, taking into account the fact that calpains perform a regulatory or signaling function, rather than degrading. Apparently, calpains play an important role in limited proteolysis of precursors of such peptides, as kyotorphin and neokyotorphin. Kyotorphin is considered as an endogenous regulator of hibernation responsible for the transition from active wakefulness to sleep in hibernating animals, while neokyotorphin participates in the opposite process. Injections of kyotorphin reduce heart rate and body temperature in non-hibernated animals [3].

In October, activity of Ca^{2+}-dependent neutral proteases in the brain cortex decreased by 75% and in stem structures by 59%, which was accompanied by a twofold increase in enzyme activity in the heart ($p<0.01$). In November, however, activity of Ca^{2+}-dependent neutral proteases increased by 40% in the brain cortex and by 1.3 times in brainstem structures and heart ($p<0.01$).

Probably in November, activity of Ca^{2+}-dependent neutral proteases decreased, which was preceded by its reduction to zero, observed in hibernation. This cyclic activity of Ca^{2+}-dependent neutral proteases in the brain reflect preparation for saving energy before winter hibernation. In published reports, proteins synthesis and breakdown in the brain are suppressed during the first month of hibernation [2,14].

The rate of protein synthesis in the brain of hibernated ground squirrels was only 0.04% of its level in active animals [9]. The intensity of protein synthesis was lowered due to not only a decrease in the rate of this process, but also complete cessation of the synthesis of some individual proteins [14]. It is believed that the decrease in the intensity of protein synthesis during hibernation is associated with the need to save energy resources under conditions of reduced blood flow rate, low substrate content, and oxygen consumption [9,14]. Synthesis and breakdown of proteins are interrelated and interdependent processes. If the synthesis were reduced, the breakdown would be certainly reduced too. Cellular proteolytic system must be strictly regulated, because uncontrolled breakdown of proteins essential for life, as well as blockade of degradation of regulatory proteins (or peptides) with short half-lives can seriously affect cell functioning.

After two months of hibernation, activity of Ca^{2+}-dependent neutral proteases remained reduced by 78% in the brain cortex and by 87% in brainstem structures ($p<0.01$), while in the heart it exceeded the level observed in awake ground squirrels by 2.3 times ($p<0.001$). During the third month of hibernation, activity of Ca^{2+}-dependent neutral proteases in the brain cortex and brainstem increased by 58 and 26%, respectively, and in the heart remained 2.5-fold elevated ($p<0.01$) compared to control values.

The preparation for the exit from winter hibernation starts in advance. Even in a state of hibernation, RNA is actively synthesized, accumulated, and released into the cytoplasm of hippocampal neurons; protein synthesis dramatically increases. Intensification of protein synthesis in tissues of awakening ground squirrels is aimed at compensation for functional and structural proteins lost or altered during hibernation or replacement of some enzymes to specific isoforms needed for a given temperature.

The process of synthesis of new cell proteins or modification of structures and functions of existing ones may play an important role in adaptation of heterothermal animals cells for functioning under different temperature conditions [2].

TABLE 1. Activity of Ca^{2+}-Dependent Neutral Proteases (μmol tyrosine/mg protein) in Tissues of Ground Squirrels during Hibernation and Self-Warming (*M*±*m*; *n*=4-7)

Terms of wakefulness and hibernation		Brain		Heart
		cortex	brainstem structures	
Wakefulness (July), body temperature 38°C		18.21±0.52	25.43±1.38	18.45±0.32
Wakefulness (September), body temperature 38°C		32.67±1.01*	48.82±1.95*	52.21±1.04*
Before entrance in hibernation (October), body temperature 16-19°C		4.64±0.20*	10.43±0.38*	55.88±1.11*
Beginning of hibernation (November), body temperature 10-13°C		25.52±0.22*	57.33±1.05*	41.55±0.62*
Two months of hibernation, body temperature 3-4°C		4.07±0.02*	3.21±0.08*	60.07±1.07*
Three months of hibernation, body temperature 5-8°C		28.87±0.81*	32.09±0.92*	63.67±0.92*
Before awakening, body temperature 6-7°C		33.39±0.63*	41.47±2.20*	71.70±0.54*
Self-warming up to body temperature	10°C	14.79±0.63*	17.89±0.63*	61.53±2.18
	20°C	23.10±0.95*	31.27±0.98*	77.25±2.53*
	25°C	26.42±0.84*	37.54±1.32*	76.52±2.96*
	30°C	24.76±1.21*	35.54±1.27*	76.49±3.01*
	38°C	31.32±1.09*	38.39±0.94*	70.44±2.55*

Note. $p<0.05$ compared to July (normothermia 38°C).

Before awakening, activity of Ca^{2+}-dependent neutral proteases in the brain cortex and brainstem structures increased by 83 and 63%, respectively, compared to the level in alert animals. Activity of Ca^{2+}-dependent neutral protease in the heart increased most significantly during this period (by 2.9 times).

Large amounts of proteins characteristic of active animals were synthesized in the tissues of ground squirrels during waking up from hibernation. Specific proteins also appear, which are not present in active and dormant animals. Thus, the awakening process in ground squirrels requires the presence of specific cell proteins that are actively synthesized [2].

At self-warming to 10°C, activity of Ca^{2+}-dependent neutral proteases considerably increased: by 3.6 times in the brain cortex and by 5 times in the stem structures.

Even more pronounced increase in protease activity was observed during self-warming to 20-25°C: during self-warming to 20°C and 25°C enzyme activity in the brain cortex increased by 4.7 and 6.5 times ($p<0.01$), respectively; in brainstem structures, this parameter increased by 8.9 and 10.6 times ($p<0.001$), respectively, compared to the level observed during hibernation.

Self-warming to 30°C did not change activity of Ca^{2+}-dependent neutral proteases of ground squirrels compared to that observed at body temperature 25°C.

However, after self-warming to 38°C activity of Ca^{2+}-dependent neutral proteases increased again compared to the level during hibernation: by 7.7 times ($p<0.001$) in the brain cortex and by 11 times in brainstem structures ($p<0.001$). In the heart, activity of enzymes of calpain system at 20, 25, 30, and 38°C remained practically unchanged.

Different temperature dependence of activity of Ca^{2+}-dependent proteases in various tissues suggests that every tissue of hibernating animals has its own neutral proteases, working in peculiar mode, and with peculiar substrates.

Awakening after long-term hibernation can be viewed as a process of adaptation to new conditions associated with the intensification of metabolism, elevated oxygen consumption, and increased body temperature. Thus, changes in enzyme activities at different stages of awakening in ground squirrels are aimed at metabolism maintenance. We observed a significant increase in activity of Ca^{2+}-dependent neutral proteases in the heart during hibernation relative to the period of self-warming, whereas in the brain activation of Ca^{2+}-dependent neutral proteases during waking probably played a leading role in adaptive processes of restoration of body temperature to normothermia.

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