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Peculiarities of RBCs resistance to acid hemolysis in hibernating mammals

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ABSTRACT

The binding of acids and alkalis, formed in tissues by metabolism, along with oxygen and CO_2 transport are recognise as the principal functions of red blood cells (RBCs). Decreases in internal environment pH may result in activation of potential endogenous cytotoxic metabolites, $OH^{\cdot}$ and oxidant formation and, as a consequence, result in oxidative damage of cell membrane leading to hemolysis. The characteristics of acid hemolysis in hibernating mammals have been determined in this study. Parameters of HCl-hemolysis, such as the average time for RBC hemolysis and the population distribution of the response, have been investigated. Measurements were performed within 40–5 °C temperature range. The resistance of hibernator RBCs to increased acidity, determined according to the acid hemolysis parameters, was found to reflect whether the animal was in the summer active period or in hibernation, with differences also apparent at different points with the hibernation season itself. It was demonstrated that hemolysis parameters of naturally cold-tolerant organisms are altered by decreases in temperature. We discuss a role of cytoskeletal–membrane interactions as a fast-acting switch of the structural and functional state of hibernator RBCs as an adaptation mechanism to acidosis arising from hypothermia and hypermetabolism.

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1. Introduction

The principal functions of RBCs are recognised to be the transport of oxygen and CO_2, as well as the binding of acids and alkali, formed in tissues by metabolism. Acidification of the internal environment can result in an elevation of potential endogenous cytotoxic metabolites such as bile salts [1]. Acidosis leads also to a release of metals with variable valency from metalloproteins, which participate in Fenton and Haber–Weiss reactions, stimulating $OH^{\cdot}$ and oxidant formation [2] that can result in oxidative damage to cell membranes and hemolysis [3,4].

Measurements of acid hemolysis kinetics [3] together with the chemical (acid) erythrogram method [5] can be used to make comparative studies of RBCs acid resistance, and to determine variations in resistance within a population of RBCs. Although the exact sequence of events leading to acid hemolyis is not certain, the fact that the acid hemolysis development involves all the main structural and functional RBCs links is considered as distinctly established. It is believed that transfer of acid equivalents by anion exchange proteins is the limiting process for acid hemolysis [3,4].

Binding of acids and alkali by RBCs is of especial significance in hibernating mammals, in which the respiratory acidosis, which results from delayed CO_2 release from tissues in the torpid state, is replaced during animal arousal by acidosis arising from the accumulation of lactic acid [6].

The ability of some mammalian species to hibernate has attracted the attention of researchers in many disciplines for more than a century. Undoubtedly, “the ability to hibernate is arguably the most dramatic example of phenotypic plasticity by mammals” [7].

Hibernation in mammals is a dramatic example of tolerant adaptation strategy, accompanied by significant changes in the organism's physiological state. Hibernating mammals in a torpor are capable of enduring prolonged periods (up to 5 weeks) of subzero body temperatures, with general metabolic levels reduced to 2–4% of the norm. Torpor is periodically interrupted by spells of rapid (lasting several hours) increases in body temperature up to 36 °C, followed by a period of euthermic metabolic activity (interbout arousal: IBA) of 12–24 h before a new period of torpor begins. In hibernators during torpor, most physiological functions are suspended, while those that persist occur at substantially reduced rates. Since hibernating mammals periodically arouse from torpor throughout the hibernating season, their ability to function under euthermic temperatures should be constantly maintained. In addition body temperature, metabolic rate, cardiac rhythm and other parameters fall during torpor to levels that are likely to be lethal for a strict homoiotherm. At the same time biochemical processes, and functions at the organ and systemic level are rearranged such that even at very low body temperatures, homeostasis is achieved. Many studies have been devoted to consider this question, and other peculiarities of the phenomenon of mammalian hibernation, including molecular aspects [7–9]. Despite this, the molecular and cellular mechanisms underlying hypothermic tolerance remain largely unclear. In particular, how the RBCs of hibernating mammals can successfully function in an organism with

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subzero body temperature, and also when temperatures increase to euthermic values is not known.

We believe that the cellular mechanisms underlying tolerant adaptation of hibernator RBCs have received insufficient attention. Obviously, mechanisms conferring tolerance for acidosis should exist in hibernating mammals. Such acidosis would be most apparent during periods of intensified metabolism associated with recovery of normal rates for physiological processes when body temperature increases towards euthermic values during the torpor–IBA transition. We believe that the research of cell mechanisms of natural acidosis-tolerance is possible in such a way. To reveal if there are the peculiarities for acid hemolysis development in hibernator RBCs, being under different physiological states.

This study compares the acid resistance of ground squirrel RBCs during hibernation, arousal and active periods in winter (January–February), spring (March) and in summer active animal and estimates the effect of a long-term (21 days) hypothermic storage of hibernator RBCs on resistance to acid hemolysis.

2. Materials and methods

RBCs of *Citellus undulatus* ground squirrels being under hibernation, arousal and active states in winter (January–February), spring (March) and under summer active state were studied. Each group numbers at least 5 animals.

Freshly drawn blood from the animal of definite group was prepared with heparin. The blood was washed three times by centrifugation (1800 ×*g*, 5 min) in isotonic saline (150 mM NaCl). Plasma and buffy coat were removed by aspiration.

RBCs acid hemolysis (HCl-hemolysis) was performed as previously described [3,5]. Given the cold-tolerance exhibited by hibernating mammals, we have modified the protocol with respect to the temperatures employed.

Washed RBCs suspension was diluted with isotonic saline so that the initial absorption at 700 nm equaled 0.7–0.8 (hematocrit 0.05%). RBCs suspension in 1.5 ml volume was mixed in the thermostated spectrophotometer cuvette at the time zero with 1.5 ml 0.004 N HCl (diluted with 150 mM NaCl), thermostated at the studied temperature. Kinetics of HCl-hemolysis (acid hemolysis) was measured by changes (decreasing) in optical density (OD) at $\lambda = 700$ nm monitored continuously with time using the Pye Unicam SP 8000 spectrophotometer. The temperature effect within the range of 5–40 °C on hemolysis was assessed using a chamber equipped with thermostatic control. An accuracy of thermostabilisation was ±0.5 °C.

To characterise RBCs acid resistance we used two parameters: an acid erythrogram (and not only a kinetic curve of optical density) and a mean hemolysis time in contrast to conventional half-hemolysis time.

An acid erythrogram (distribution of RBCs population by acid resistance) adequately characterises a qualitative composition of RBCs population. Erythrograms were obtained according to the method described and substantiated by Terskov and Getelson [5]. Namely, each erythrogram point represents a fraction of RBCs (f_n) hemolysed to definite time point (t_n). The fraction was calculated from a kinetic curve of optical density as:

$$f_n = [\mathrm{OD}(t_{n-1}) - \mathrm{OD}(t_n)/\mathrm{OD}(t_0)] \times 100\% \quad (1)$$

In Eq. (1) f_n represents the fraction of RBCs hemolysed to definite time point t_n and $\mathrm{OD}(t_0)$ refers a difference between the optical density ($\lambda = 700$ nm) at the time zero and residual one after the end of hemolysis. f_n is a relative quantity and expressed in percents. In accordance to cited paper [5] we determined f_n values for each t_n in 0.5 min ($t_n - t_{n-1} = 0.5$ min) using the spectrofotometrically obtained kinetic OD curve. In fact the acid erythrogram represents the 1st derivative of OD kinetic curve and is an adequate characteristic of RBCs population heterogeneity.

The second parameter used is a mean hemolysis time (t_m) which in contrast to conventional half-hemolysis time accounts for a weight of different RBC fractions in a population. When studying the temperature effect on RBCs acid resistance, this parameter could be more accurate, because young and old erythrocytes are supposed to have various temperature dependence of their acid resistance.

A mean acid hemolysis time is calculated as:

$$t_\mathrm{m} = \sum f_n t_n / 100\% \quad (2)$$

In Eq. (2) f_n and t_n represent the same parameters as in Eq. (1).

An acid erythrogram and mean hemolysis time were calculated using a kinetic curve of optical density for at least 5 independent experiments ($n = 5$), each performed in triplicate under studied temperature point.

3. Results and discussion

For better understanding the RBCs adaptive rearrangements, accompanying significant physiological changes in an organism of a hibernating mammal under conditions of hibernation–IBA transition and to compare with summer active animal together with studying the acid hemolysis kinetics we have investigated the distribution of RBCs population of ground squirrels by acid resistance according to the method, described in the cited paper [5].

Comparison of the acid erythrogram at 24 °C of the summer ground squirrel (Fig. 1) with that of a healthy human adult [5] (the first population distribution derived using such acid resistance methodology) shows that there are no principal differences in the population response (the pattern of acid erythrograms) between heterothermal mammals in their summer euthermic period and strict homoiotherms. Though the RBCs of the former are, on the whole, more resistant as a result of the presence of some 'high-resistance' RBCs, for which the time of acid hemolysis exceeds 7.5 min.

Figs. 2 and 3 show that the acid erythrograms for populations of RBCs from winter hibernating and active ground squirrels differ, especially at reduced temperatures (10 °C). It can be seen that there is a displacement of the erythrograms of the ground squirrel in the torpor state to the right (that is, there is a higher fraction of acid-

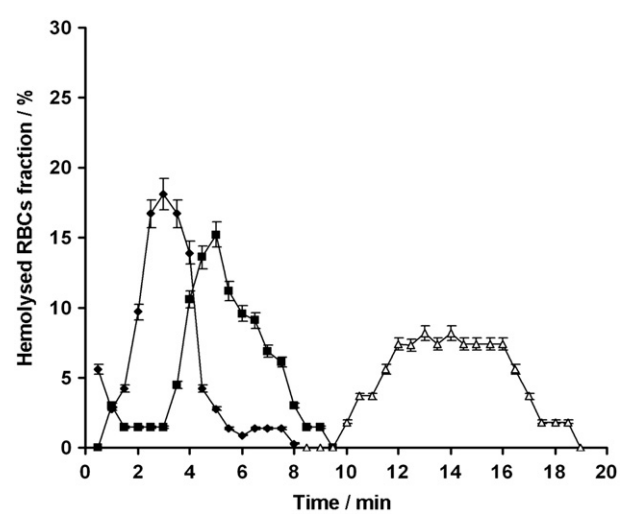

Fig. 1. Temperature sensitivity of distribution of acid resistance for a population of RBCs from summer ground squirrels: (◆) 32 °C, (■) 24 °C, (△) 10 °C. Acid erythrograms were calculated using a kinetic curve of optical density at 700 nm (as described in Materials and methods). Each erythrogram is the mean of at least five independent experiments, each performed in triplicate under studied temperature point. The error bars represent the standard deviation. Points without drawn error bar have a standard deviation that is within the size of the symbol.

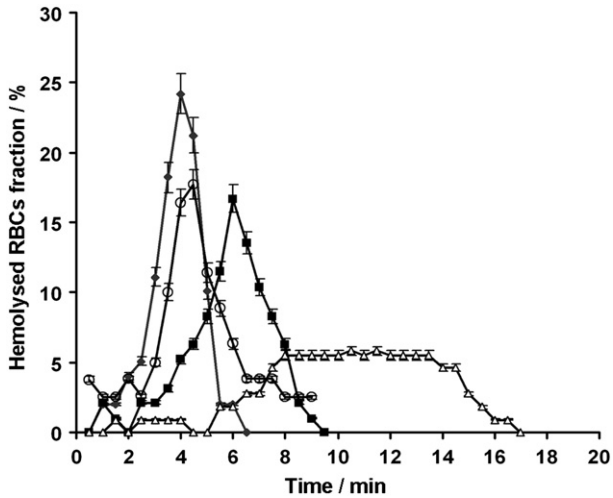

Fig. 2. Temperature sensitivity of distribution of acid resistance for a population of RBCs from winter hibernating ground squirrels: (♦) 32 °C, (■) 24 °C, (△) 10 °C, (○) 24 °C, long-term hypothermic storage. Each erythrogram is the mean of at least five independent experiments, each performed in triplicate (as described in Materials and methods). The error bars represent the standard deviation. Points without drawn error bar have a standard deviation that is within the size of the symbol.

resistant erythrocytes), with the population of RBCs being the most heterogeneous at reduced temperatures. In the torpor state, at 24 °C the presence of high-resistance RBCs (where the time of acid hemolysis exceeds 7.5 min) is observed. According to current understanding [5,10] a rightwards shift of an erythrogram represents intensive regenerative processes; for example, in anemia where there is pronounced erythropoiesis and high numbers of young, resistant RBCs. Both biochemical membrane composition (cholesterol levels) and the nature and properties of hemoglobin can significantly affect the human erythrogram pattern [5,10].

Of interest was to find-out if there were the differences in acid hemolysis parameters of RBCs, being at subzero temperatures *in vivo* (deep hibernation, body temperature 4–6 °C) and *in vitro* (long-term storage of "winter" hibernating blood at +4 °C). We have established that long-term (21 days) hypothermic storage of "winter" hibernating blood does not result in a dramatic change in RBCs acid resistance. A slight change in erythrogram pattern (Fig. 2) and a decrease in average time of acid hemolysis of stored RBCs from 5.5 ± 0.30 min down to 4.63 ± 0.25 min have been observed.

The modified temperature protocol that we have employed provides additional information about the acid resistance of hibernator RBCs, in different physiological states. Figs. 4 and 5 show the temperature dependency of the average time for acid hemolysis in hibernating, arousing, active "winter" or "spring" ground squirrels. These figures include also the temperature dependency of the average

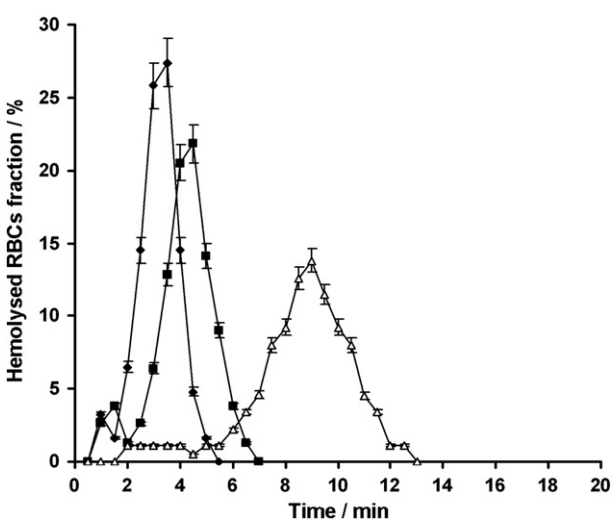

Fig. 3. Temperature sensitivity of distribution of acid resistance for a population of RBCs from winter active ground squirrels: (♦) 32 °C, (■) 24 °C, (△) 10 °C. Each erythrogram is the mean of at least five independent experiments, each performed in triplicate (as described in Materials and methods). The error bars represent the standard deviation. Points without drawn error bar have a standard deviation that is within the size of the symbol.

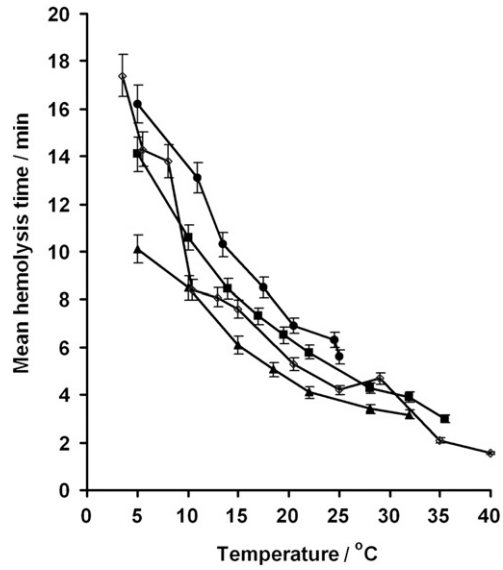

Fig. 4. The temperature dependency of mean acid hemolysis time for RBCs from hibernating, arousal and active "winter" and summer active ground squirrels: (■) hibernate ground squirrel, (●) arousal ground squirrel, (▲) active ground squirrel, (◊) summer active ground squirrel. Each point is the mean of at least five independent experiments, each performed in triplicate (as described in Materials and methods). The error bars represent the standard deviation. Points without drawn error bar have a standard deviation that is within the size of the symbol.

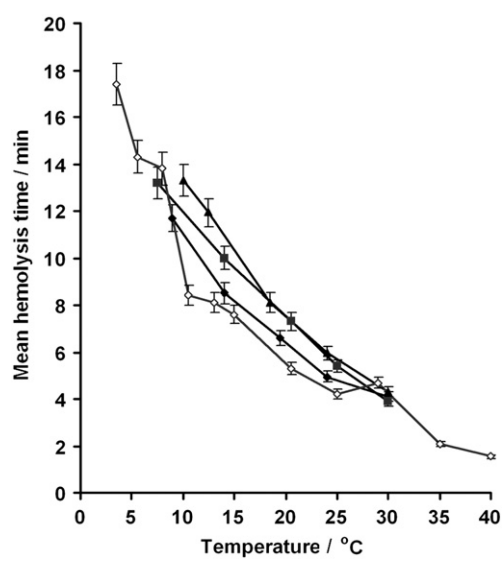

Fig. 5. The temperature dependency of mean acid hemolysis time of RBCs from hibernating, arousal and active "spring" and summer active ground squirrels: (■) hibernate ground squirrel, (♦) arousal ground squirrel, (▲) active ground squirrel, (◊) summer active ground squirrel. Each point is the mean of at least five independent experiments each performed in triplicate (as described in Materials and methods). The error bars represent the standard deviation. Points without drawn error bar have a standard deviation that is within the size of the symbol.

time for acid hemolysis for ground squirrel RBCs in the summer active period.

Fundamental differences in the profiles of the hemolysis time temperature dependence are apparent for RBCs from all squirrels tested during the hibernation season when compared with those from summer active ones. The breaks presence in hemolysis time temperature dependency curve is characteristic for the latter within the certain temperature ranges. The sharpest change in the curve run, and hence in the dependency character of acid hemolysis kinetics on temperature is observed at temperatures of about 10 °C, at which point modification of band 4.1 protein, binding spectrin with integral membrane components is known to occur [11]. This may indicate that cytoskeletal–membrane interactions play a major role in the mechanism of acid hemolysis.

RBCs from ground squirrels during the hibernation season are characterised by a shallower relation between hemolysis time and temperature, indicative of an adaptive process.

In the case of the three states of "winter" ground squirrels, acid resistance was reduced as follows: arousal > hibernate > active across the temperature range tested. The increase in resistance to acid of RBCs from ground squirrel in the arousing state relative to that seen for the torpid animal is appropriate given the additional acid production that will be associated with huge changes in physiological and metabolic activity during the arousal period.

In the case of "spring" ground squirrels, the differences are less apparent, especially between hibernating and active ones, although in general RBCs from "spring" animals are more resistant than those from "winter" ones, and the order of acid resistance is different, namely active ≥ hibernate > arousal. Such variations are probably a consequence of an altered hibernation regimen, with shortened bouts as the animal prepares for euthermia.

Thus, the resistance to acid of hibernator RBCs, determined using acid hemolysis parameters, reflects the physiological state, being determined not only by whether an animal is within the summer active period or in hibernation period, but also by its status during hibernation.

The average time for acid hemolysis provides quantification of a complex response of RBCs to increased proton concentrations. Although the transfer of acid equivalents by anion exchanger proteins into the cytosol is considered the limiting step of acid hemolysis [3,4], a change in the conformation of integral membrane proteins and their lateral aggregation are necessary links in the HCl-hemolysis mechanism [12,13]. The role of spectrin in RBCs acid resistance is not excluded, but is doubtful [4].

The strategy for tolerant adaptation of hibernating mammals must involve structural and functional rearrangement of membrane and cytosol components, which are manifested in the form of changes in acid resistance of RBCs and an altered population distribution for this parameter. As the data presented indicates, an important link to achieve this adaptation must be the modification of structural state and function of the anion exchanger — band 3 protein.

At the same time, we believe that an important role of spectrin in the mechanism of acid hemolysis cannot be excluded [4]. Lateral aggregation of integral membrane proteins is impossible when the cytoskeletal network is in its native state [14]. On the other hand, cytosol acidification to pH 6.0–4.0 can result in spectrin aggregation [14] that in turn results in modification of structural and dynamic state of integral membrane proteins [15]. A role of spectrin in the mechanism of acid resistance, at least for hibernator RBCs, is supported by our previous finding that interactions of cytoskeletal components (spectrin, band 4.1) and cytosolic (hemoglobin) components to integral membrane components are enhanced in RBCs from torpid animal [16]. This observation is consistent with the concept [7] that molecular response underlying hibernation occurs not only at the level of protein synthesis, but also through modifications to interactions between proteins.

We consider a cytoskeletal–membrane interaction to be a specialised fast-acting switch of the structural and functional state of hibernator RBCs as an adaptive mechanism in response to hypothermia, hypermetabolism and, most notably, acidosis.

The key finding of this study is that specialised structural and functional properties of cells from cold-tolerant animals can be revealed by the differences in the responses of these cells when temperature is decreased.

We believe that structural and functional rearrangements of RBCs have received comparably little attention when the considering mechanisms of adaptation in hibernating mammals. A detailed characterisation of these will facilitate an understanding of methods for adaptation to unfavourable conditions, which will be of benefit to the biomedical sciences.

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