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PARTICIPATION OF SURFACE MEMBRANE STRUCTURES OF LEUKOCYTE'S PLASMALEMMA IN REGULATION OFCELL VOLUME

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Abstract:

The membrane reserve enclosed in a folding of plasmalemma is a morphological basis involving in the mechanism regulation of the cell's volume as it to participate in the degree of biomembrane's tensions. In the performed study have been investigated the participation of the surface membrane's structures of plasmalemma in the mechanism of cell's volume regulation. In the tests with osmotic loadingin vitroit was established that the use of the relative membrane reserve in the volume regulation of leukocytes depends on stiffness of cell frame and is accompanied by a change in the "pattern" of the plasmalemma. The increase stiffness of the leukocyte's surface by 36.4% ($p<0.05$) under the influence of epinephrine contributed to elongation in time the phase of regulatory volume increase by 1.25 times, herewith the percent of the relative membrane reserve involved in volume regulation decreased almost by 2 times in comparison with leukocytes without load. The obtained data indicate the direct participation of the elements adenylate cyclases signaling pathways in the mechanisms of volume regulation of leukocytes by changing the stiffness of the cell surface. The duration of phases the regulatory volume increase and decrease depends on the stiffness of cell frame. These data are of great practical importance in the assessment of adaptive cellular reactions under the changes of the medium osmolarity in the microvasculature.

Key words: *membrane reserve, the regulatory volume increase, the regulatory volume decrease, the leukocyte's stiffness.*

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INTRODUCTION:

In the microvasculature leukocytes are direct participants of intercellular interactions providing a whole range of immune responses. Elements of plasmalemma are represented by receptors apparatus and membrane reserve enclosed in a folding of plasmalemma is a morphological basis supporting the functional activity of leukocytes. Membrane reserve is a mechanism for regulating the degree of biomembrane's tensions (Groulx*et al*., 2006)involved in such physiological reactions as vesicular transport (Apodaca, 2002), cell's mobility (Houk*et al.,* 2012), the reactions of volume regulation (Skorkina, Muravyov, 2013) and passage of leukocytes through exchange capillaries whose diameter is smaller than the cell's size (Fedorova, Levin, 2001).In this connection the issue of studying the volume regulation of leukocytes in the microvasculature on the basis of adequate cooperation of regulatory subsystem one of the key links of which are the elements of the cytoskeleton has a particular urgency (Diz-Muñoz*et al*., 2013). The concept of work is basis on the idea about involved in the volume regulation of leukocytes the membrane reserve of plasmalemma using of which closely related with the stiffness of cell frame. Elements of cytoskeleton are simultaneously the morphological base and central mechanism of the subsystem regulation of volume on the cell's level. In particular, on the one hand the cytoskeleton proteins participate in molecular mechanisms of intracellular signal transmission and regulation of the gene expression(Kreuk*et al*., 2011), monitor the functioning of membrane channels and transporters (Mazzocci C. *et al*., 2006), provide cell contact with the substrate and intercellular interactions(Kellouche S. *et al.*, 2010), movement(Tooley*et al*., 2009),ecto- and endocytosis (Gauthier *et al*., 2011) and maintain the shape of the cell (Egea G. *et al*., 2006). On the other hand, the certain rigidity of the sub-membrane framework allows cells to use the stocks of membrane material in order to maintain the morphological integrity (RaucherD., SheetzM., 1999). In connection with the above the purpose of the work was the investigation of the participation the membrane reserve of leukocytes in the mechanisms of volume regulation in loading tests*in vitro*.

MATERIALS AND METHODS:

In experiments was used peripheral blood from donors (woman and man, n=100). Blood was collected on the basis of the Regional Clinical Hospital. St. Joasaf, Belgorod. The age of donors was from 25 to 45 years. The study was approved by the local ethic committee of Medical Institute of Belgorod State National Research University and informed consent of all subjects were obtained according to the recommendations of the Declaration of Helsinki (The International Response to Helsinki VI, The WMA's Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects, as adopted by the 52th WMA General Assembly, Edinburg, October, 2000).

The blood was obtained by means of venepunction and was stored in was stored in vacuum vials (VacuetteK3E). The blood was then centrifuged (1500 rpm) during 5 minutes in order to extract suspension of leukocytes with further washing off and resuspension in RPMI-1640 medium. It was used osmotic tests *in vitro* as load tests allowing studying the participation of the membrane reserve in the volume regulation of leukocytes. In the hypoosmotic tests the relative membrane reserve used by cells in the processes of volume regulation was evaluated in accordance with the technique published in an earlier work (Skorkina, 2014). Functional activity of leukocytes was assessed by the duration of period of the regulatory volume increase and the ability of the cell to restore its volume to the initial values. The duration of the hypoosmotic load *in vitro* was 60 min. The relative membrane reserve was calculated as the difference of cell's surface area in hypo- (0.4% NaCl) and isotonic (0.9% NaCl) medium relative to the cell's volume in autologous plasma.

During the osmotic tests the morphology of cell's membrane was studied by atomic force microscopy (AFM) in the semi-contact mode. For this purpose, fixed samples was prepared. From each sample 10 µl cells suspensions was dispensed into eight Eppendorf tubes each containing 50 µl hypotonic solution (0.4% NaCl). Eight Eppendorf tubes with 10 µl cell suspension and 50 µl isotonic solutions (0.9% NaCl) were used as a control. In each of tubes successively after 30, 60, 90, 120, 150, 180, 300 and 900 sec was added 10 µl 25% glutaraldehyde solution (MERCK, Germany). From each sample was prepared the smears. Finished preparations were placed into the atomic force microscope INTEGRA VITA (configuration on the basis of inversed optical microscope Olympus IX-71 (NT-MDT, Zelenograd, Russia, 2009) and then from each sample 20 cells were scanned in semi-contact mode (frequency sweep – 0.6…0.8 Hz, NSG03 cantilever with stiffness of 1.1 N/m and curvature radius of 10 nm). On scans the surface profile size 3.5X3.5 µm were constructed and measure dimensions and calculated the number of globular protrusions by software Nova.

In this study was made an attempt to find the relationship between the stiffness of the cell

membrane and the use of a relative membrane reserve in the volume regulation reactions in hypo-osmotic stress test *in vitro*. It was used the functional load in the form of incubation with epinephrine and propranolol for modification of elastic properties of cell's surface. The choice of these substances is related to their influence on the elements of universal adenylatecyclases signal pathway (Kaloyianin M., Doukakis I., 2003). In particular epinephrine and propranolol influence to the adrenergic receptors and modulate elastic properties of cell's membrane through phosphorylation of cytoskeleton proteins (Tuvia S. et al., 1999). It is known that the main subtype the adrenergic receptors presenting on the leukocyte's surface is β-adrenergic receptors (Landmann R., 1992). Their density on the leukocyte's membrane is 1000-3000 (Tuvia S. et al., 1999).

The load with epinephrine carried out by incubation during 15 min at 37^0C 30 µl leukocyte suspensionsinto 150 µl Hank's medium containing 10^{-9}mM/l of epinephrine. In another part of experiment, the propranolol was used as an antagonist of epinephrine blocking β-adrenergic receptors. The blockade of β-adrenergic receptors was done by incubation during 15 min at 37^0C 30 µl leukocyte suspensions into 150 µl Hank's medium containing 10^{-9}mM/l of propranolol. After incubation the samples was centrifuged at 1500 rpm 5 min and then supernatant was removed. The one part of leukocyte suspension was subjected to osmotic stress *in vitro* as described above. The other part of suspension was used for preparation of samples and measuring the Young's module characterizing the stiffness of membrane. The measuring of Young's module was performed on AFM INTEGRA VITA according to algorithm described in an earlier work (Skorkina*et al.,* 2011). The obtained experimental data were statistically processed. Significance of differences was evaluated using Student *t*-test.

RESULTS:

The volume regulation of leukocytes in the osmotic load test in vitro in norm

In the osmotic tests the alternation of periods the regulatory volume increase (duration 360 sec) and the regulatory volume decrease (duration 60 sec) of leukocyteswas observed. In hypotonic medium the synchronous reactions in changing of both the cell and the nucleus were found. The leukocytes used gradually the additional membrane structures beginning with the first second of incubation. The maximum relative membrane reserve (≈36%) involved in the volume regulation was noted at 240-270 sec and then from 300 sec to the end of the incubation the alternation of periods the increase and decrease using of membrane reserve was observed. Nucleus also involved the membrane reserve in the volume regulation and maximum (≈33%) was in the interval 240-270 sec.

Considering that the substantial part of membrane reserve in the form of a folding can unfold in the current study the features of surface's morphology accompanying reactions of osmoregulation were investigated. For this purpose, the semi-contact mode of AFM was used. According to the obtained data during the phase of regulatory volume increase in the first 30 sec in the hypoosmotic medium on the cell's surface the number of globular protrusions was reduced by 44% ($p<0.05$) and was increased their height by 37.5% ($p<0.05$) as compared with isotonic medium. In the next 60 sec of incubation in the hypoosmoticsolution the tendency to increase of the number of globular protrusions with reduces their height was persisted. Starting from 180 sec and to the finish of incubation the number of globular protrusions increased but their height decreased (table 1).

Table 1: Size and number of globular protrusions on the surface of the donors' leukocytes

Time incubation, s	Hypoosmotic solution (0.4% NaCl)		Isotonic solution (0.9% NaCl)	
	height, nm	number	height, nm	number
30	35.82±2.45*	47±1.2*	22.39±1.90	84±2.9
60	20.58±1.08*	130±3.1*	36.39±4.05	22±0.4
90	20.45±1.61*	71±2.5*	82.67±7.16	34±1.9
120	21.08±2.75*	48±0.9	44.36±4.48	49±1.0
150	41.70±3.31	32±1.4*	38.27±2.70	154±5.2
180	27.86±2.09*	61±2.8*	46.17±5.27	25±1.3
300	28.92±1.76*	71±3.7*	50.05±6.59	32±4.6
900	24.28±2.06	76±3.1*	29.25±4.96	23±1.7

*Statistically reliable differences in hypotonic solution as compared with the isotonic solution at $p < 0.05$

Morphophysiological reactions of leukocytes after functional tests in the osmotic load tests in vitro

Under influence of epinephrine load the stiffness of leukocytes increased by 36.4% ($p<0.05$) compared with the control. An abundances of globular protrusions with reduced height appeared in the relief of cell's surface. In the parallel series with propranolol the stiffness of cells was reduced by 20% ($p<0.05$) but the height of the globular protrusions increased and their number practically didn't change as compared with the control (table 2).

Table 2: Young's module and surface's morphology of donors' leucocytes under influence the functional loads

Samples	Young's module, μPa	Surface's morphology	
		height of globules, nm	number
Control (plasma)	3.5±0.2	41.3±3.7	36.0±0.9
Epinephrine	5.5±0.4*	17.5±0.5*	46.0±1.1*
Propranalol	2.8±0.3*	75.8±8.8*	35.0±1.6

*Statistically reliable differences in experiment as compared with the control at $p < 0.05$

The increase of cell's stiffness under the influence of epinephrine loading led to an elongation of the duration of the phase the regulatory volume increase to 450 sec in the hypoosmotic tests which is 90 sec longer than in the cells without load. At the same time the relative membrane reserve involved in the regulation of cell volume decreased. In hypotonic medium the leukocytes involved a maximum of the relative membrane reserve (~15%)at 60 sec of incubation. The nucleus used 16% of the membrane reserve at 30 sec incubation in the hypoosmotic solution. The duration and implementation of periods of the regulatory volume decreased in the system "cell-nucleus" didn't coincide in time. The change in the volume of nucleus was by 90 secs faster than the cell as a whole (Figure1). Under influence of epinephrine in hypotonic medium in surface's relief the number of globular protrusions was reduced but their height increased as compared with isotonic solution.

In the conditionsof blockade β-adrenoreceptors by propranolol the phase of the regulatory volume increase of leukocytes was 240 sec. In the system of "cell-nucleus" the changing of cell volume was faster by 210 sec compared with nucleus. The maximum use of the relative membrane reserve (~6%) by the cell was observed at 30 sec incubation in hypotonic medium while the nucleus didn't participate in the osmoregulatory reactions (see Figure 1).

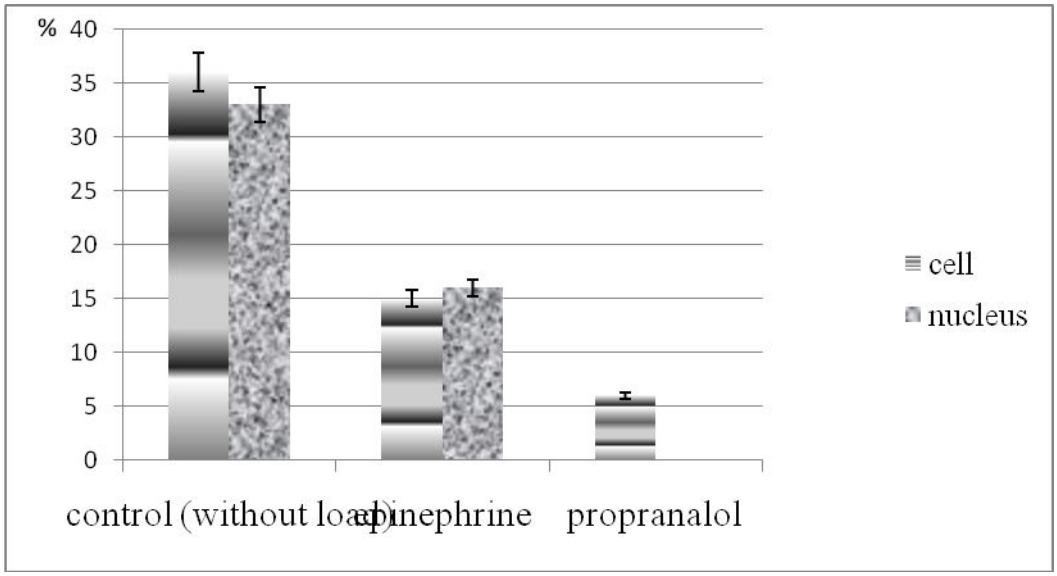

Figure 1. The relative membrane reserve (%) using by the system "cell- nucleus" in the hypotonic medium under influence the functional loads

Expressed changes in the surface's relief under the influence of propranolol were noted at 60 and 90 sec of incubation when the number of globular protrusions in the region of the membrane sharply increased and their height first increased and then decreased in comparison with isotonic solution.

DISCUSSION:

In the study the use of the membrane reserve by leukocytes in osmotic stress tests *in vitro* have been investigated. It is established that the use of the relative membrane reserve in the volume regulation is determined by stiffness of cell's frame and is accompanied by a change in the "pattern" of the plasmalemma. The osmoregulatory reactions of leukocytes from donors in norm have a cyclic character and consist of alternating periods of the regulatory volume increase and decrease. The regulatory volume increase period is accompanied by reduce the number of globular protrusions on the surface membrane and increase in the size of globules themselves. The reducing of the globular protrusions on the surface is the compensatory response of the cell aimed at using the relative membrane reserve for the remove the lytic tension in the membrane. In this case the increase of height the individual globules point at the rearrangement of highly dynamic sub-membrane cytoskeleton regions containing actin-rich structures (Tall *et al*., 2000). It has been proved that the spatial configuration of the dynamic regions of cell's membrane is regulated by the phosphoinositide signaling pathway (Sechi, Wehland 2000) whose trigger ligand is the endogenous Ca^{2+}emerging from the intracellular depot as a response on the decrease of medium osmolality (Light*etal.,* 2002).

The change of stiffness leukocyte's surface under the influence of epinephrine and its increase by 1.5 times contributed to elongation in time the phase of regulatory volume increase by 1.25 times herewith the percent of the relative membrane reserve involved in volume regulation decreased almost by 2 times in comparison with leukocytes without load. In the mechanisms of volume regulation the cell involved the maximum its reserve structure (≈15%) at 60 sec incubation while the nucleus involved about 16% intracellular membrane reserve at 30 sec. Obviously the epinephrine load increasing the stiffness of cytoskeleton was a limiting factor in the use by cells significant part of the membrane reserve presenting on the surface. In the scientific literature there are data on the effect of the stiffness of the cytoskeleton on the size of the membrane reserve. It has been shown that as the density of microtubules increasing the stiffness of cytoskeleton the amount of available for using the membrane reserve is reduced (Rauche*retal.,* 1999).

Under influence of propranolol the membrane's stiffness decreased by 1.25 times herewiththe duration phase the regulatory volume increase was reduced by 1.5 times but the volume of the reserve membrane structures involving in the volume regulation was about 6% and a cell used this structures at 30 sec incubation while the nucleus didn't participate in the volume regulation in hypoosmotic solution. The observed changes in the rate of the cell response are most likely due to the affinity of propranolol to the lipid bilayer of membrane which has membrane-stabilizing effect changing its properties (Ferreire*etal.,* 2006) and decrease cAMP formation (Anderson*etal.*, 1996).

CONCLUSION:

The obtained data indicate that the elements of adenylate cyclases signaling pathways take a direct participation in the mechanisms of volume regulation in the leukocytes by changing the stiffness of the cell's surface. Elastic properties of cells determine duration of the phase the regulatory volume increase and decrease. However, a direct relationship between the part of the membrane reserve involving in the regulation of cell volume and the degree of stiffness of cell's surface has not been established. The revealed regularities have an important theoretical and practical meaning in the evaluation of the cellular responses and the prediction of the during the adaptation process under the conditions of an altered microenvironment at the influence of extreme factors.

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