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MEMBRANE RESERVE OF LYMPHOCYTES IN MIDDLE-AGED PEOPLE

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Abstract:
Age changes at the cellular level are closely related to the features of the functioning of biomembranes. In the present study the involvement of the membrane reserve of lymphocytes in middle-aged people in the mechanisms of regulation of the cell volume was studied. It was found that the functional activity of biomembranes depends on the elastic properties of its surface. It was proved that under the influence of adrenalin load in vitro the rigidity of the membrane increased and therefore the cell had limited resources for involving the surface pool of the plasmalemma in osmoregulatory reactions in the hypotonic environment. Reduction of the elasticity of the cell almost by 1.5 times led to an increase in the duration of the phase "regulatory volume increase" by 1.25 times in comparison with cells without load while the reserve of membrane structures involved to the volume regulation were decreased almost 2 times. Under the influence of the propranolol the stiffness of the membrane decreased by 1.25 times while the duration of the phase "regulatory volume increase" was decreased by 1.5 times but the percentage of additional membrane structures involved in the hypotonic environment decreased by 6 times compared to cells without load. The obtained data testify to participate of the elements of adenylate cyclases signaling pathways into the mechanisms of the lymphocyte's volume regulation in the middle-age people by changing the elastic properties of cell's surface.

Key words: *membrane reserve, lymphocytes, adenylate cyclases signaling pathways, elastic properties, middle-age people.*

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INTRODUCTION:

Cellular membranes are universal targets beginning of age-related changes and pathophysiological processes in the organism. In the microvasculature leukocytes are direct participants of intercellular interactions providing a whole range of immune responses. Functional activity of lymphocytes and their age-related changes essentially depends on the activity of receptor's apparatus of cell and membrane reserve enclosed in a folding of plasmalemma. 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 problem of the study of the volume regulation of lymphocyte in the microcirculatory bed in the middle-age people is very actual because in this age begin the forming a various pathophysiological conditions. The aim of the work was the investigation of the participation the membrane reserve of lymphocytes in the middle-age people in the mechanisms volume regulation.

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 35 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 (Vacuette K3E). 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 lymphocytes. 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 usedas 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 than from each sample 20 cells was 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 adenylate cyclases signal pathway (Kaloyianin M., Doukakis I., 2003). In particular adrenaline 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 adrenaline carried out by incubation

during 15 min at 37^0C 30 µl leukocyte suspensions into 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.,* 2012). The obtained experimental data were statistically processed. Significance of differences was evaluated using Student *t*-test.

RESULTS:

Under the influence of adrenaline load the stiffness of cells was increased by 36.4% ($p < 0.05$). In the relief of surface the number of globular protrusions with reduced height was increased (table 1).

Table 1:The properties and relief of lymphocytes in the middle-age people under influence functional loads

Samples	Young's modules, µPa	The depth of the cantilever's immersion, nm	Globular protrusions	
			height, nm	number
Plasma (control)	3.5±0.2	345.2±3.7	41.3±3.7	36.0±0.9
Adrenalin	5.5±0.4*	155.1±23.5*	17.5±0.5*	46.0±1.1*
Propranalol	2.8±0.3*	468.5±15.5*	75.8±8.8*	35.0±1.6

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

In the parallel experiment with propranolol the stiffness of lymphocytes decreased by 20% *($p <0.05$)* while the height of globular protrusions increased and their numbers remained unchanged.

In the conditions of adrenaline load the duration of the phase "regulatory volume increase" of lymphocyte in the hypotonic environment increased to 450 seconds that is 90 seconds more than in cells without load. In the hypotonic environment the lymphocyte involved a maximum membrane reserve (≈15%) at 60 second incubation. At the same time in the isotonic solution lymphocytes involved ≈2% of the reserve surface into the volume regulation. The nucleus used about 16% of the reserve at 30 second of incubation in hypotonic solution while an isotonic medium the nucleus didn't use the additional reserve of the intracellular pool. The duration and implementation of the phase "regulatory volume decrease" in the system of cell-nucleus didn't coincide in time. The change in the volume of the nucleus proceeded by 90 seconds faster than the cells as a whole. The relief of the surface during the first 60 seconds of exposure in the hypotonic environment was smoothed and the number of globular structures was reduced. In the period from 90 to 120 seconds incubation on the surface of lymphocytes the globules protrusions were enlarged and on the surface clusters were appeared. Inaddition the number of deepening was increased and their overall dimensions were decreased with compared to the same structures in isotonic solution.

Under the influence of the propranolol load the duration of phase the "regulatory volume increase" was 240 seconds. In the system cell-nucleus the changes of cell volume were 210 seconds faster than the nucleus. Length of phase the "regulatory volume decrease" both at the cell and nucleus levels was averaged 30 seconds. The maximum use of the membrane reserve (≈6%) by the cell was observed at 30 seconds of incubation in a hypotonic environment. In samples with propranalol the nucleus didn't use additional membrane structures both in the hypotonic medium and in isotonic solution. In hypotonic environment the surface's relief in the first 90 seconds of incubation was smoothed. The number and height of the globular protrusions on the surface of the membrane were reduced. In the interval from 120 to 150 seconds of incubation in the hypotonic medium the height of the globules increased but their amount remained reduced that indicates the processes enlargement of the protein structure in the membrane and their rearrangement. The number of deepening in the membrane and their overall dimensions decreased. Reducing the number of deepening on the

surface led to its expansion and increase in surface area.

DISCUSSION:

In the study the use of the membrane reserve by lymphocytes in middle-age people 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 change of stiffness lymphocyte's surface under the influence of adrenalin load 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 (Raucher *et al.,* 1999).

Under influence of propranolol the membrane's stiffness decreased by 1.25 times herewith the 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 *et al.,* 2006) and decrease cAMP formation (Anderson *et al.*, 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 lymphocytes by changing the stiffness of the cell's surface. Elastic properties of cells determine duration of the phase of" the regulatory volume increase" and the phase of "the regulatory volume decrease". The revealed regularities have an important theoretical and practical value in studying the mechanisms of aging at cellular damage and the prediction of course of the adaptation process under the conditions of an altered microenvironment at the influence of extreme factors.

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