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PLATELET CHEMILUMINESCENCE. APPLICATION OF CHEMILUMINESCENCE METHOD IN PLATELET ACTIVITY DEFINITION

ABSTRACT. Platelets both in intact and in activated condition are capable of generation of active forms of oxygen (AFO). Production of AFO by platelets of humans and rats is proved. Intensity of AFO generation can be registered with the help of chemiluminescence method (HL). The method is based on addition of luminol into platelet-rich plasma (PRP) and chemiluminescence registration with chemiluminometer HL-003 with a graphical display of chemiluminogram on the computer monitor.

Addition of the physiological inducer of aggregation ADP (adenosine diphosphate) in the dose used for laboratory evaluation of platelet aggregation more than twice increases emission.

For rats, due to the specific nature of platelet aggregation, it is necessary to add $CaCl_2$ (1,0 mM) into the sample together with ADP. Thus, the platelet chemiluminescence method allows to estimate the functional condition of intact (HL in the presence of only luminal) and activated platelets (HL in the presence of luminal and ADF) according to their ability to produce active metabolites of oxygen.

KEY WORDS. Platelets, chemiluminescence, free radical oxidation.

Introduction. At the present stage the platelet is viewed as a blood cell taking part in homeostasis, inflammation, reparation and immune reactions. Platelet activation is a consequence of development of a number of complicated interrelated reactions leading to the change of blood platelets metabolism and their ultrastructural reorganization. Ion mobilization and movement, hydrolysis of inositol phospholipids, release and oxidation of arachidonic acid, change of metabolism of cyclic nucleotides, plasma membrane rebuilding, etc. belong to these mechanisms [1-2]. Platelet activation is regulated and modulated by numerous factors including ADP, serotonin, thromboxane A_2, thrombin, collagen, adrenalin etc [3]. The internal and external processes of reorganization finish with realization of specific thrombocytic functions such as haemostatic, reparative, protective etc. [4-5]. The intracell signalization necessary for the reorganization of the cytoskeleton and granular secretion occurs via phosphoinositides [6-7] due to eicosanoid metabolism [8]. Production of eicosanoids from arachidonic acid is catalyzed by cyclooxygenase enzymes 1 and 2 (COG1; COG2). COG1 is involved in thrombocytic function, COG2 is mainly involved in inflammation. It is supposed that active oxygen forms (AOF) are new modulators of thrombocytic activity. It has been shown that their exogenous or intra-thrombocytic production influences on the platelet function [9].

Various AOF of thrombocytic origin including O_2 -, HO, H_2O_2 act as intermediaries of platelet activation after their stimulation with collagen [10].

The processes of free radical oxidation which pass with formation of radicals RO and RO_2 can be evaluated with measurements of chemiluminescence (CL). The biochemiluminescent method is not a direct qualitative method of definition of free radicals; CL method directly defines not the concentration of radicals but the speed of reaction in which they are formed. The method of chemiluminescence has an advantage: first of all it is usually not connected with the alteration of the course of processes in solutions, cells and even whole tissues where luminescence can be registered; secondly, it is rather sensitive to detection of exactly highly reactive oxygen radicals. As a rule natural chemiluminescence accompanied by biochemical reactions in cells and tissues has a very low intensity and received a name of "superweak luminescence". Therefore special substances accelerating the processes of chemiluminescence are used. Luminol is used as an accelerator – it is a compound entering into reactions either with active oxygen forms or organic free radicals in the course of which molecules of products in excited electronic state evolve. The luminescence observed during this process is linked with the transition of the molecules into the ground state which leads to the illumination of photons [11].

The aim of the research: to develop a method of definition of platelet chemiluminescence and estimate the intensity of chemiluminescence at ADP-induced platelet aggregation.

Materials and methods. We have carried out examinations of blood of healthy people – donors (Tyumen regional blood donation center) and blood of intact outbred white rats. The tests on animals have been carried out in accordance with the "Rules of work performance with the use of test animals" approved by the order of the Ministry of Health Care USSR No. 755 dating 12.08.1977. Blood was stabilized with 3.8% sodium citrate solution (9:1). Platelet rich plasma (PRP) was recovered by centrifugation of citrate blood at 200 g for 15 minutes at room temperature [12]. Platelet poor plasma (PPP) was obtained after sampling of PRP from test tubes and further centrifugation of the blood samples at 700 g for 30 min. The number of platelets in PRP was raised till $3x10^8$ c/mcl by addition of PPP. We judged about generation of active oxygen forms by platelet chemiluminescence intensity. The study was made with the apparatus CHL-003. The results were registered on the computer and displayed graphically. The paired Student's t-test was used to calculate significance of differences.

Results. At the first stage of the study we set the task to detect the natural luminescence of platelets with the method of chemiluminescence (CHL). We took PRP (platelet content 300000c/mcl) of donors as a test sample; PPP samples were used as control samples. PRP volume was equal to 5 ml. To enhance CHL we added 1 ml working luminol. Luminol (m.Sv. 177) is prepared with dimethyl sulphoxide in an amount of 10^{-4} and kept in the fridge. The working solution is prepared from mother liquor by a 1000-times dilution in sterile saline solution (pH 7.0-7.2). CHL registration time is 30 minutes while slow stirring and at the temperature in the apparatus chamber 37°C [13].

PRP and PPP chemiluminescence without luminol did not differ significantly (table 1). Luminol addition did not lead to significant increase of CHL in PPP but significantly increased CHL in PRP: maximum luminescence rose by more than 16

times and the light sum of luminescence increased by more than 50 times. CHL in the presence of luminol in PRP is considerably higher in all parameters of the analogous values for PPP. Thus, increase in the light sum and maximum luminance with luminol addition is stipulated by the free-radical processes in platelets.

Table 1

PRP and PPP chemiluminescence values

Values	Basic luminescence		Luminescence with luminol	
	PRP	PPP	PRP	PPP
	M ± m	M ± m	M ± m	M ± m
Light sum c.u. • min.	1,72 ± 0,42 p1 > 0,05	1,73 ± 0,46	84,6 ± 21,1 p2 < 0,01	3,20 ± 0,56
Max. lum. c.u.	0,55 ± 0,06 p1 > 0,05	0,43 ± 0,07	8,97 ± 2,56 p2 < 0,01	0,60 ± 0,07

Note: n (number of observations) – 10; p_1 – differences between PRP and PPP CHL without luminol; p_2 - differences between PRP and PPP CHL with luminol

Functional activity of platelets continues 1.5 hours after the incubation at 37°C, intensity of CHL cells did not change significantly. The light sum values (before incubation 84.60±21.1 c.u. • min, after incubation 75.93±14.6 c.u. • min. $p<0.05$); maximum luminosity (before incubation 8.97±2.56 c.u., after incubation 7.18±1.90 c.u. $p<0.05$).

To define production of oxygen free radicals in the process of platelet aggregation we used ADP as aggregation inductor. All the conditions of the sample preparation and registration were the same. 0.5ml ADP was added in a concentration of 1mg/ml. Platelet chemiluminescence was recorded in the presence of luminol – control, then we added ADP and registered the values; after that the samples were incubated for 60 min. at 37°C, CHL values were recorded again (table 2).

At platelet activation with ADP there is a significant increase in the light sum. Thus, ADP-induced aggregation accompanied by activation of the processes of free radical oxidation in platelets. Incubation of activated platelets causes significant decrease in CHL both as maximum luminance and light sum of luminosity.

Table 2

ADP-induced CHL values of platelets in the presence of luminol

Value	CHL control	ADP-induced CHL	60 min. after incubation
	M ± m (n=10)	M ± m (n=10)	M ± m (n=10)
Light sum c.u. • min.	84,6 ± 21,1	267,7 ± 47,7 p < 0,01	26,05 ± 6,07 P< 0,05
Max. lum. c.u.	8,97 ± 2,56	14,38 ± 2,8 p > 0,05	3,07 ± 0,66 P< 0,05

Note: p – differences with the control group

To prepare 5 ml PRP one needs 10 ml whole blood which complicates the application of this method in everyday use and experiments on rats. Therefore another

method for 1 ml PRP has been developed. 1ml donor PRP with a platelet content of 300.000 cl/mcl was added into the luminol solution; working luminol solution – 0.1 ml; ADP – 0.1 ml. the registration time is 30 minutes, without stirring, temperature 37°C. CHL values in the presence of luminol and ADP-induced platelet CHL values (light sum and maximum luminance) are significantly higher than the analogous values of basic platelet luminance (table 3).

Table 3

Platelet CHL values in 1 ml sample

Value	Basic luminescence	Luminescence with luminol	ADP-induced platelet CHL
	M ± m (n=10)	M ± m (n=10)	M ± m (n=10)
Light sum c.u. • min.	1.20 ± 0,19	7,70 ± 1,20 P1 < 0,01	15,57 ± 3,23 P1< 0,01 P2 <0.05
Max. lum. c.u.	0,39 ± 0,03	0,88 ± 0,08 p < 0,01	1,96 ± 0,99 P1< 0,01 P2>0.05

Note: p1 – differences with the control group; p2 – differences between CHL with luminol and ADP-induced CHL of platelets

During the approbation of the method on rats we use similar procedures for obtaining platelets, platelet concentration in the sample and luminol dose. But PRP is diluted to the necessary cell concentration with buffered saline; 0.1 ml CaCl2 (1.0 mM) is added to the sample together with ADP [14]. The temperature in the apparatus chamber is 37°C; registration time is 30 minutes. It should be noted that without luminol there are no significant CHL differences between PRP and PPP. These are the light sum values (PRP 0.68 ±0.11 c.u. • min.; PPP 0.49±0.05 c.u. • min.; p>0.05) and maximum luminance values (PRP 0.53 ±0.04 c.u. • min.; PPP 0.45±0.03 c.u. • min.; p>0.05). CHL values for PPP remain constant and do not depend on luminol application. With luminol platelet CHL significantly increases. ADP activation of platelets raises CHL values even more (table 4).

Table 4

Platelet CHL for rats

Value	Basic luminescence	Luminescence with luminol	ADP-induced platelet CHL
	M ± m (n=20)	M ± m (n=20)	M ± m (n=20)
Light sum c.u. • min.	0.68 ± 0,11	12,98 ± 1,6 P1 < 0,01	40,64 ± 5,33 P1< 0,01 P2 <0.05
Max. lum. c.u.	0,53 ± 0,04	3.23 ± 0,35 p < 0,01	9,40 ± 1,17 P1< 0,01 P2<0.05

Note: p1 – differences with the control group; p2 – differences between CHL with luminol and ADP-induced CHL of platelets

Discussion of the research findings. It is well-known that ADP is the basic physiological inductor of aggregation. In the process of ADP-induced aggregation of platelets there occurs a change in the cell shape, exposition of the fibrinogen binding sites, primary reversible aggregation in the presence of Ca^{2+} concentrations and final desynthesization [15]. After the binding of ADP with its receptors a signal "inside to outside" is transmitted which promotes accessibility of the receipting sites for binding in the complex GPIIb-IIa (integrin $\alpha_{IIb}\beta_3$) and their ability to bind with fibrinogen. There exist three types of ADP-receptors: $P2X_1$, $P2Y_1$ and $P2Y_{12}$. Through various secondary messengers these receptors lead to platelet activation, change of their shape, secretion and aggregation. The secondary aggregation is mediated by the sum of several subsequent reactions of the intramembrane and intracell activation signal transmission. These ways are different for different receptors with which ADP can bind. After ADP binding with $P2X_1$ receptor of the thrombocytic plasma membrane there follows a quick income of Ca^{2+} into the cell, indirect stimulation of A_2 phospholipase and transmission of the activation signal through the prostaglandin-thromboxane system which can be one of the ways ensuring secretion development and secondary aggregation. P2Y1 ADP receptor is bound with G protein which mediates mobilization of the intracell Ca^{2+}, change of the blood platelets shape and reversible aggregation through the activation of the phosphatidylinositol path. P2Y12 ADP receptor is bound with G_{12} subunit, which is responsible for the inhibition of the stimulated adenylate cyclase, which by itself does not cause aggregation of blood platelets but creates a background favorable for the development of other activation reactions [1-2], [16]. The presence of microperoxis in the platelets insuring endogenous synthesis of hydrogen peroxide and its release into blood in the course of the release reaction [17] indicates at the important role of ADP in the regulation of aggregation-disaggregation of platelets. Nowadays the cytotoxic influence of ADP is not considered to be the only possible influence on the body cells. There are many data on the regulatory role of the active oxygen forms. It has been stated that intact platelets are capable of constant generation of superoxide radicals [18] and the superoxide-dependent platelet activation is important in the physiological and pathological haemostatic reactions [19]. It has been proved that changes in the platelet APD concentrations are linked with their functional activity [20]. Platelet function can be disturbed with the change of their oxidative status in presence of antioxidants. It has been indicated that antioxidants, except for non-specific radically suppressed mechanisms, inhibit free radical oxidation due to interaction with specific proteins. Such regulation is marked as receptor-redox regulation [12], [20]. Thus, platelets both in rest and at ADP-induced aggregation produce free radicals which can be registered with the method of chemiluminescence.

Conclusion. The registration of the platelet proper luminescence with the method of chemiluminescence in the presence of luminol lets evaluate the initial condition of the platelets. The ADP-induced platelet activation enhances chemiluminescence and lets estimate the functional condition of the platelets.

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