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Chemiluminescent Analysis of Reactive Oxygen Species Synthesis by Platelets from Patients with Coronary Heart Disease

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A novel chemiluminescent method was developed to evaluate ROS generation by platelets. This method allows measuring activities of NADPH oxidase (NOX2) and enzymes synthesizing secondary ROS (superoxide dismutase, catalase, *etc*.) in resting and ADP-activated platelets (inductor of platelet aggregation and ROS generation) using a small number of cells. The method was tested in the examination of patients with coronary heart disease. It was found that platelets from patients with coronary heart disease were characterized by NOX2 activation, while cell metabolism is tuned for a long-term intensive production of superoxide anion radical. The enzymes synthesizing secondary ROS were also activated, but cell metabolism could not maintain their enhanced activity for a long time.

Key Words: *platelets; chemiluminescent activity; reactive oxygen species; coronary heart disease; NADPH oxidase*

Functional activity of platelets is an important factor of cardiovascular pathologies. In activated platelets, ROS are intensively synthesized by enzymes located both on the membrane and inside of the cell (particularly, in mitochondria) [2,12]. The key stage of ROS synthesis, generation of superoxide anion radical (primary ROS), is catalyzed by NADPH oxidase (NOX) enzyme complex primarily located on the outer membrane. Of 7 isoforms of mammal NOX differing by cell specificity, subunit composition, and some other parameters, NOX2 is the most prevalent type of Nox in human platelets [7,13]. Inhibition of NOX2 reduces platelet aggregation, while high activity of this enzyme is associated with high level platelet of aggregation and sometimes is observed in patients with coronary heart disease (CHD) [9,14].

Dismutation of superoxide radical by superoxide dismutase (SOD) yields H_2O_2 that can oxidize SH groups and peroxidize unsaturated fatty acids [9]. In platelets, two SOD isoforms are synthesized: cytoplasmic SOD1 and mitochondrial SOD2. Generation of H_2O_2 triggers a cascade of reactions resulting in the synthesis of secondary ROS. Catalase, cyclooxygenase, and glutathione peroxidase also play an essential role in ROS generation in platelets [11].

In platelets, ROS are signaling and regulatory molecules that trigger and modulate their functional activity [8,13]. All drugs modulating functional activity of platelets (specifically, antiaggregants) affect the level of ROS generation. Hence, a method is needed to assess the level and kinetic parameters of ROS production in platelets. Chemiluminescent (CL) assay is widely used in experimental and clinical studies. Different methods for assessing ROS generation in platelets by using CL analysis are known [3,10]. However, they differ in algorithms and inducers of ROS ge-

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neration. Here, we propose a method for assessing the spontaneous and ADP-induced synthesis of primary and secondary ROS in isolated platelets.

Our aim was to develop a CL method for evaluation of the intensity and kinetics of ROS generation in platelets and to test this method on CHD patients.

MATERIALS AND METHODS

Platelets were isolated from the whole blood of healthy individuals using 3.2% sodium citrate as an anticoagulant (9:1 blood:citrate ratio) [6] and counted. The sample for CL assay contained 10^6-10^8 cells. The reaction mixture for measuring the intensity and kinetics of ROS generation by the CL method included 50 µl lucigenin or 50 µg/ml luminol, 50 µl ADP in a concentration range of 0.01-0.50 M for assessment of induced ROS generation, and 250 µl (or 200 µl in case of ADP-induced ROS generation) buffer containing (in M): 0.13 NaCl, 0.02 tris-HCl, 0.03 EDTA, and 0.015 glucose (pH 7.4). Spontaneous or ADP-induced CL was recoded for 90 min on a 36-channel BLM-3607 biochemiluminescent analyzer (MedBioTekh). The time to maximum CL (T_{max}), maximum intensity (I_{max}), and area (S) under CL curve were analyzed. The increment of ADP-induced CL was assessed by activation index (AI) calculated as the ratio of the area under curve for induced CL to that for spontaneous CL (S_{ind}/S_{spont}).

ROS generation by platelets was assessed using lucigenin and luminol used as indicators of CL reaction. Lucigenin does not penetrate into the cells and interacts only with superoxide radical, i.e. characterizes activity of membrane NOX2 [1,5]. In contrast, luminol can cross the membrane and interact with all ROS [1,4,10]. In this study, both CL indicators were used in concentrations used in the analysis of phagocyte respiratory burst [4,5].

To assess validity of the proposed method of evaluation of ROS generation in platelets by CL, we used blood samples from patients with chronic CHD treated at the Department of Cardiovascular Surgery of Federal Center of Cardiovascular Surgery (Krasnoyarsk). The control group comprised healthy donors (N=26).

All participants gave informed consent for participation in the study. The experiments were carried out in strict adherence to ethical principles and regulations of World Medical Association Declaration of Helsinki (2013) and according to Order No. 266 of the Ministry of Health of the Russian Federation (On Establishing the Rules of Clinical Practice in the Russian Federation; June 19, 2003).

The results were statistically processed using Statistica 8.0 software (StatSoft, Inc.) and presented as median (Me) and interquartile range (Q_1; Q_3). Significance of differences was assessed using non-parametric Mann—Whitney test.

RESULTS

CL activity of platelets depended on the number of cells in the sample. This dependence was examined on spontaneous luminol-dependent CL. There was practically no CL reaction when platelet content in the sample ranged $(1\text{-}5)\times10^6$. In the range of 5×10^6-2×10^7 cell/sample, CL intensity attained maximum; further increase in cell concentration was not accompanied by significant increase in CL intensity, but shifted T_{max} to the left and accelerated CL drop, which in some cases decreased S_{spont}. Thus, platelet concentration 2×10^7 cells/sample was used in further CL analysis.

ATP is the classical inducer of platelet aggregation widely used in assessment of the state of thrombocytic-vascular hemostasis in clinical laboratory diagnostics. Figure 1 shows the dependence of the intensity ADP-induced luminol-dependent CL of platelets on ADP concentration. The optimal ADP concentration for inducing ROS synthesis in platelets is 0.1 M. Further increase of ADP concentration did not enhance CL intensity.

The developed method was employed to examine the patients with chronic CHD (class III-IV effort angina) prior to coronary bypass surgery. It was found that CHD was associated with an increase in T_{max}, I_{max}, S_{spont}, and S_{ind} of lucigenin-dependent CL as well as increased I_{max} of spontaneous and induced luminol-dependent platelet CL (Table 1). It should be remembered that lucigenin-dependent CL reports the level of superoxide radical generation and corresponding activity of membrane NOX2 [1,5]. In CHD patients, platelet NOX2 was activated. Importantly, this enzyme was activated both in resting and ADP-induced

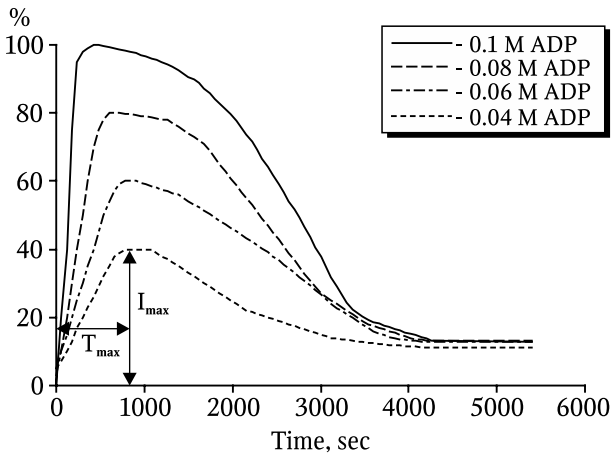

Fig. 1. CL activity of platelets depending on ADP concentration. T_{max} is the time to the maximum level of ROS synthesis; I_{max} is the maximum level of ROS synthesis.

TABLE 1. CL Platelet Activity in CHD Patients Me (Q1, Q3)

Parameter	Control (*N*=26)	CHD (*N*=44)
Spontaneous lucigenin-depended CL		
T_{max}, sec	212 (80; 450)	669* (271; 2341)
I_{max}, rel. units	80.0 (72.5; 92.5)	96.0* (78.0; 485.1)
S, rel. units×sec×10^4	22.76 (17.63; 27.52)	29.23* (17.93; 65.50)
ADP-induced lucigenin-depended CL		
T_{max}, sec	96 (49; 608)	1019** (287; 2965)
I_{max}, rel. units	85.1 (75.5; 126.5)	115.0* (82.0; 495.5)
S, rel. units×sec×10^4	27.46 (18.69; 36.47)	37.84* (23.42; 108.50)
AI	1.03 (1.00; 1.87)	1.05 (1.00; 1.35)
Spontaneous luminol-depended CL		
T_{max}, sec	71 (52; 463)	106 (45; 624)
I_{max}, rel. units	79.5 (76.5; 110.0)	112.0* (79.0; 530)
S, rel. units×sec×10^4	26.18 (22.23; 31.29)	29.67 (21.00; 81.24)
ADP-induced luminol-depended CL		
T_{max}, sec	154 (87; 471)	349 (178; 906)
I_{max}, rel. units	81.7 (77.3; 126.5)	124.0* (82.6; 568.3)
S, rel. units×sec×10^4	27.44 (22.97; 33.40)	30.48 (21.71; 85.36)
AI	1.11 (1.05; 1.26)	1.03 (1.00; 1.16)

Note. *p<0.05, **p<0.001 in comparison with the control. The chemiluminescent platelet activity is shown in relation to ADP concentration. T_{max} is time to maximal ROS generation; I_{max} is maximal level of ROS generation.

platelets, which reflects readiness of platelet metabolic system in CHD patients for enhanced synthesis of this signaling molecule. In CHD patients, the synthesis of all ROS in platelets measured by luminol-dependent CL was also changed. In this study, we observed only elevation of I_{max} relatively to the control level, which reflected capacity of platelets in CHD patients for pronounced spontaneous and ADP-induced activation of ROS generation. S_{spont} and S_{ind} were within the control ranges, which determined the total level of ROS generation during CHD that corresponded to the control one. It can be hypothesized that in platelets of CHD patients, the intracellular enzymes responsible for ROS generation (SOD, catalase, *etc*.) are rapidly activated, but the entire metabolic system in the cells does not provide long-term augmented ROS generation.

Thus, the study developed a novel CL-based method to assess ROS generation by platelets isolated from the peripheral blood. The method allows measuring activity of NOX2 and the enzymes (SOD, catalase, *etc*.) implicated in ROS generation in resting and ADP-activated platelets in samples containing relatively low number of cells (2×10^7). The method was tested during examination of CHD patients. The study revealed that in platelets of CHD patients, NOX2 is activated, and the cellular metabolic system is tuned for a long-term intensive generation of superoxide radical. The enzymes that generate secondary ROS are also activated, but the cellular metabolic resources cannot maintain long-term elevation of intracellular activity. The advanced method assesses functionality of platelets under any physiological or pathological states of human organism. In this method, any regulator molecules can be used as inducers of platelet aggregation and/or ROS generation in relation to the aim of examination.

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