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Acetylsalicylic Acid Produces Different Effects on the Production of Active Oxygen Species by Activated Platelets in Different Inflammatory Diseases

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We studied the effect of acetylsalicylic acid on ROS generation by platelets in patients after surgical interventions and in patients with bronchial asthma was studied. Platelets stimulated with platelet-activating factor are characterized by weak luminol-enhanced chemiluminescence in healthy people and patients after operations with laparoscopic incisions. Addition of platelet activation factor to platelet samples from patients after open abdominal surgery caused intensive chemiluminescence that was suppressed after platelet incubation with acetylsalicylic acid. At the same time, platelets of patients with aspirin-sensitive asthma did not respond to addition of platelet activating factor, but after incubation with acetylsalicylic acid, an intensive burst of chemiluminescence was detected with a maximum in 5-10 sec after the addition of a platelet-activating factor. In patients with bronchial asthma tolerant to aspirin, platelet activation factor did not induce chemiluminescence irrespective of incubation with acetylsalicylic acid.

Key Words: *reactive oxygen species; acetylsalicylic acid; platelets; abdominal surgery; asthma*

Luminol-enhanced chemiluminescence (CL) reflects the production of different highly active metabolites of oxygen, such as superoxide radical, H_2O_2, and hydroxyl radical [4]. ROS are traditionally considered harmful, cytotoxic and highly aggressive metabolites that can inflict cell damage. At the same time, recent studies showed that ROS possessing signaling functions and acting as second messengers can also serve as components of signaling pathways [1,6,9].

In the absence of activating agents, human platelets are characterized by weak spontaneous CL. Intensification of CL in the presence of luminol after addition of arachidonic acid or metabolites of arachidonic acid to platelets was described [10]. In our previous studies we have demonstrated intensive ROS generation by platelets stimulated with platelet activating factor (PAF) in some coronary heart disease patients receiving acetylsalicylic acid (ASA) [2]. However, the effect of ASA on the production of ROS by activated platelets in non-cardiac patients has not been studied.

Here we analyzed the effect of ASA on luminol-enhanced CL of PAF-stimulated platelets in patients after surgical interventions and patients with bronchial asthma.

MATERIALS AND METHODS

The study included 26 patients after abdominal surgery (21 patients after laparoscopic interventions and 5 patients after open abdominal surgery), 15 patients with bronchial asthma, and 25 healthy volunteers.

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Ten patients with bronchial asthma were sensitive to ASA (patients with aspirin bronchial asthma) and 5 patients were tolerant to ASA. Exclusion criteria were age over 75, coronary heart disease, diabetes mellitus, congestive heart failure, hypertension that required medication or serious co-morbidity the use of anti-inflammatory agents including NSAID within 1 month before the study. The blood was taken after receiving informed consent from patients and volunteers.

For CL measurement in platelet-rich plasma, the blood was collected and stabilized with 3.8% sodium citrate (1:9 anticoagulant:blood ratio). The blood was taken from the cubital vein in the morning on an empty stomach after a 12-h fasting. Platelet-rich plasma was prepared by centrifugation at 150*g* for 10 min. The supernatant was collected in separate tubes and stored at room temperature for no more than 4 h.

Luminol-enhanced CL of platelet-rich plasma in the presence of PAF was measured by ROS generation by activated platelets. To this end, 900 μl reagent containing 200 nM PAF and/or 50 μM luminol was added to a cuvette with 100 μl platelet-rich plasma, gently stirred and promptly transferred into a Sirius chemiluminometer (Berthold Detection Systems GmbH). CL was recorded over 10 min, the mean CL intensity was presented in relative units. For studying the effect of ASA on platelets, the samples were incubated with ASA in a final concentration of 1 mM for 1 min. ASA (Sigma-Aldrich) was dissolved at a concentration of 50 mM in equimolar ratio with sodium bicarbonate ($NaHCO_3$) in the presence of 10 mM phosphate buffer.

Statistical analysis of the results was conducted with the software package SPSS Statistics 23.0 (SPSS Inc.). The data are presented as the median (LQ; UQ). The Shapiro—Wilk *W* test was used to check statistical hypothesis on the form of data distribution. For comparison of the two groups, the Mann—Whitney *U* test was used; in some groups, nonparametric Kruskal—Wallis test for independent samples was used. Wilcoxon's matched pairs test for was used to analyze the differences between the two samples of paired measurements. The differences were significant at $p<0.05$.

RESULTS

Luminol-enhanced CL of PAF-activated platelets was measured in platelet-rich plasma samples from 41 patients and 25 healthy volunteers. The patients were divided into 4 groups. Group 1 consisted of 21 patients after laparoscopic abdominal surgery. Group 2 included 5 patients after open abdominal surgery. Groups 3 and 4 consisted of 10 patients with bronchial asthma tolerant to NSAID and 5 patients with aspirin asthma. The background level of spontaneous luminol-enhanced CL of platelet-rich plasma did not surpass 30-50 rel. units. Addition of 200 nmol PAF to the platelets induced only a slight increase in the intensity of luminol-enhanced CL in samples of platelet-rich plasma from healthy volunteers, patients after laparoscopic operations, and patients with bronchial asthma tolerant to ASA. Medians of the luminol-enhanced CL of PAF-activated platelets in the group of healthy volunteers and these two groups of patients did not differ significantly ($p=0.90$; Table 1). Addition of 1 mM ASA to platelet-rich plasma samples from healthy volunteers and from patients of these two groups induced no significant changes in intensity of CL ($p=0.88$; Table 1).

Addition of 200 nM PAF to platelet-rich plasma samples from 5 patients after open abdominal surgery induced intensive CL with amplitude of more than 1000 rel. units. The median of intensity of CL in this group was 1335 (1150, 1800) rel. units and was significantly higher than in patients after laparoscopic interventions ($p<0.001$; Table 1). Incubation of platelet-rich plasma samples of these patients with 1 mM ASA significantly reduced the intensity of PAF-induced CL up to 157 (53; 250) rel. units ($p=0.043$, Fig. 1).

However, none of the 5 samples of platelet-rich plasma received from patients with aspirin bronchial asthma showed intensive luminol-enhanced CL after

TABLE 1. Intensity of Luminol-Enhanced CL of PAF-Activated Platelets from Healthy Volunteers and Patients in the Study Groups

Group	PAF	ASA+PAF
Healthy volunteers (*N*=25)	105 (77; 192)	103 (63; 179)
Laparoscopic interventions (*N*=21)	90 (50; 153)	81 (51; 140)
Open abdominal surgery (*N*=5)	1335 (1150; 1800)*	157 (53; 250)
Patients with bronchial asthma tolerant to ASA (*N*=10)	87 (43; 145)	48 (37; 158)
Patients with aspirin asthma (*N*=5)	188 (105; 226)	2160 (1254; 2487)+

Note. $p<0.001$ in comparison with patients *after laparoscopy, +with bronchial asthma tolerant to ASA (Mann—Whitney *U* test).

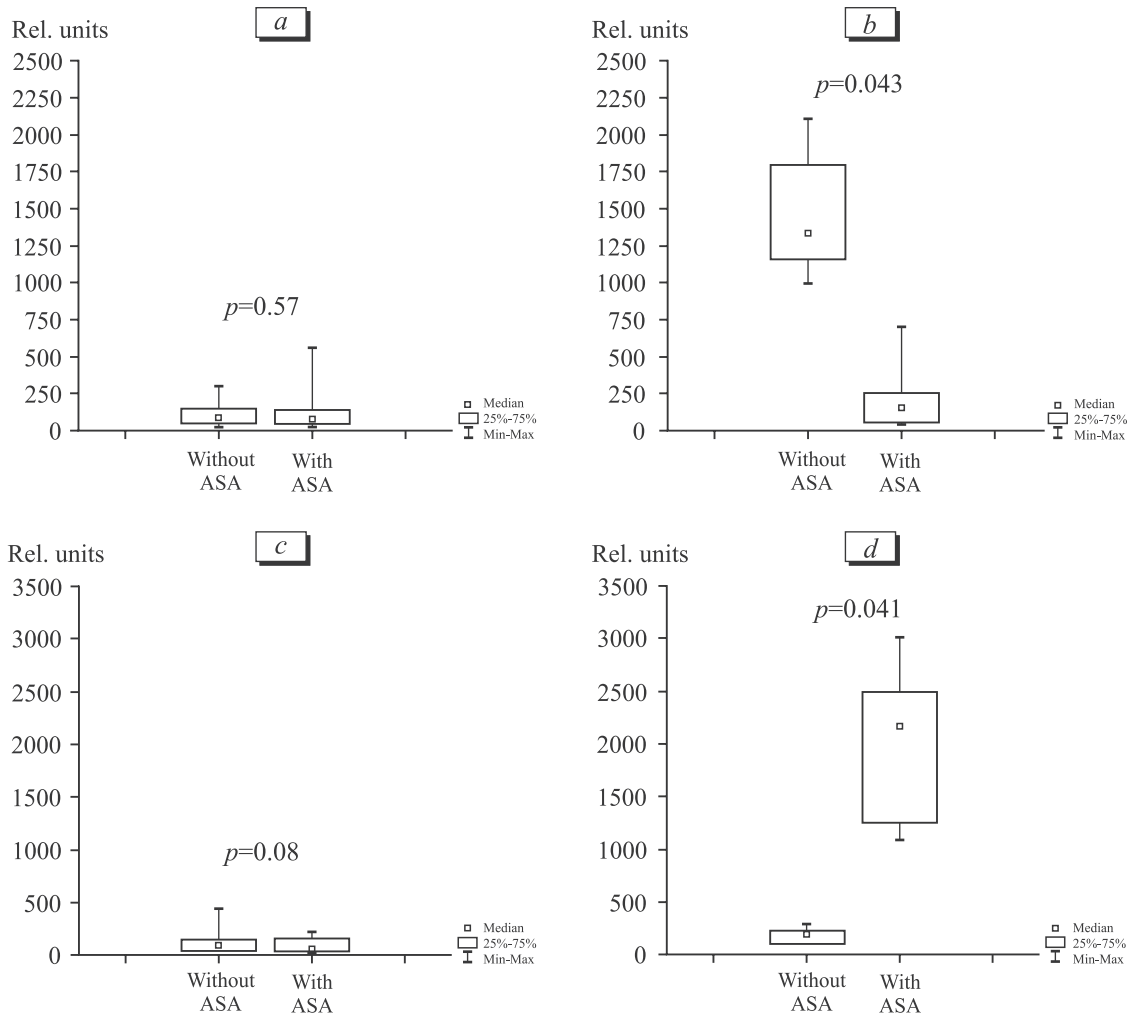

Fig. 1. Intensity of luminol-enhanced CL of platelet-rich plasma samples in the presence of PAF. *p*: Wilcoxon's test for related samples. Here and on Fig. 2: *a*) Patients after laparoscopic operations; *b*) patients after open abdominal surgery; *c*) patients with bronchial asthma tolerant to ASA; *d*) patients with aspirin asthma.

activation of platelets with PAF (Fig. 1). An intensive CL burst after addition of 200 nmol PAF to platelets was observed after incubation of these samples with 1 mM ASA. CL intensity increased to 2160 (1254, 2487) rel. units and was significantly higher than in patients with ASA-tolerant bronchial asthma ($p<0.001$, Table 1), in plasma samples in patients with aspirin bronchial asthma without the addition of ASA ($p=0.041$; Fig. 1).

Plasma samples from patients after open abdominal surgery showed intensive growth of luminol-enhanced CL, the maximum value was attained in 2 to 4 min after addition of PAF (Fig. 2). Incubation of samples with 1 mM ASA almost completely inhibited PAF-induced CL increase in this group. Plasma samples of patients with aspirin asthma did not show significant luminol-enhanced CL after addition of 200 nm PAF. Nevertheless, after incubation of platelet -rich plasma samples of this group with 1 mM ASA, they showed intensive and fast burst of CL with maximum intensity within 5-10 sec after addition of PAF.

It is known, that PAF can stimulate a wide range of biological reactions, from activation and aggregation of platelets and neutrophils to different cellular effects associated with stimulation of chemotaxis, generation of superoxide, protein phosphorylation, arachidonic acid synthesis, and activation of phosphoinositide metabolism. The PAF signaling system can trigger inflammatory and thrombotic cascades, activate these cascades, act in concert with other mediators, and generally mediate molecular and cellular relationship between inflammation and thrombosis [13]. Our study revealed differences in ROS production by PAF-activated platelets from patients who underwent different surgical procedures: production of ROS was low after laparoscopic interventions, but significantly increased after open abdominal surgery. Incubation of platelets with 1 mM ASA almost completely abolished ROS

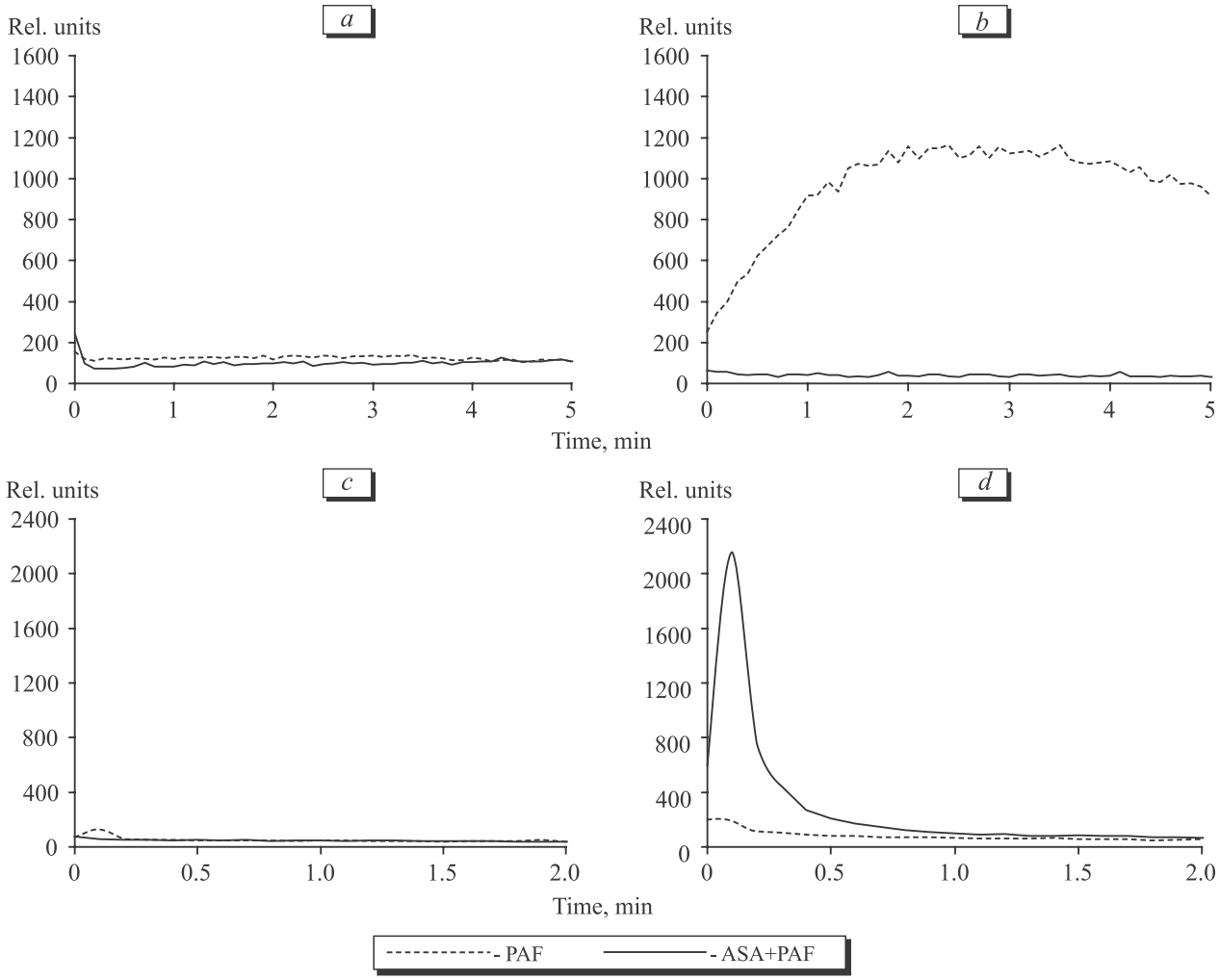

Fig. 2. Typical curves of luminol-enhanced CL in samples of platelet-rich plasma from patients.

overproduction by PAF-activated platelets in these patients. This finding suggests the presence of specific proinflammatory cellular responses to different surgical procedures and provides support to the concept of a putative crucial role for platelets in inflammation.

We also detected abnormal *in vitro* response of platelets to ASA in patients with aspirin bronchial asthma. Aspirin bronchial asthma is severe chronic inflammatory syndrome characterized by a triad of symptoms: asthma, polyposis rhinosinusitis, and asthma attacks in response to administration of ASA or other NSAID. It was hypothesized that enhanced platelet activation in response to NSAID is associated with abnormal leukotriene metabolism: more intensive production of cysteinyl leukotrienes in ASA-sensitive patients in comparison with ASA-tolerant individuals. In this case, ASA challenge led to further increase in the synthesis of cysteinyl leukotrienes and exacerbation of the symptoms [5]. Cysteinyl-leukotrienes (C4 and D4) are among the most powerful bronchoconstrictors, they are more than 10,000 times more potent than histamine. Their bronchoconstrictor activity was shown both on isolated fragments of airways and *in vivo* [3]. Previously, it was suggested that platelet activation could play an important role in the pathogenesis of aspirin asthma. However, previous studies revealed no differences in platelet aggregation in patients with aspirin asthma and ASA-tolerant patients except for slight increase in the synthesis of prostaglandin F2α in response to ASA. As platelets are potent producers of eicosanoids, these results were somewhat unexpected. Some studies have shown significant differences in the response of stimulated platelets in patients with aspirin asthma in comparison with patients tolerated for ASA. Platelets in patients with aspirin asthma released almost twice as much ATP during their activation by PAF or collagen [7]; in addition, only platelets from patients with aspirin asthma demonstrated significant enhancement of thromboxane B2 synthesis in response to PAF in comparison with platelets from healthy vol-

unteers [12]. It is important to note that both experimental models and case studies show that platelets play a key role in pulmonary defense and integrity, but also can be effectors of injury in lung diseases [11]. Our studies demonstrated abnormal platelet activity in response to ASA in patients with aspirin asthma in comparison with patients tolerant to ASA, which can contribute significantly to the pathogenesis of the disease. The molecular mechanisms that are the basis of this disturbed activity are not clear. Nevertheless, the differences in platelet behavior are significant and the results of the study show that modified platelet functions could be a relevant feature of inflammation related diseases.

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