Oxidative metabolism of neutrophils in acute coronary syndrome

Elena Proskurnina, Tatiana Danilova, Madina Sozarukova, Artem Snitsar, Anatoly Baranov

Abstract

Background: Inflammation in acute coronary syndrome (ACS) involves neutrophil activation and oxidative stress. Here, we studied the production of reactive oxygen species (ROS) by neutrophils in ACS.

Methods: The study included 42 patients, men and women aged 46–91 years with ischaemic heart disease (IHD), non-ST-segment elevation ACS and ST-segment elevation ACS. Neutrophil-derived ROS were quantified with double-step stimulated luminol-enhanced chemilumimetry.

Results: The specific indices of spontaneous and double-step stimulated chemiluminescence did not differ in the subgroups of IHD, non-ST-segment elevation ACS and ST-segment elevation ACS. The total double-step stimulated ROS production by neutrophils was significantly higher in ST-segment elevation ACS than in non-ST-segment elevation ACS and IHD.

Conclusions: In ACS, special activation mechanisms of peripherical neutrophils were not triggered in our study. The significant increase in free radical production by neutrophils in acute myocardial infarction was presumably a consequence of an increase in their number.

Keywords: neutrophils, reactive oxygen species, acute coronary syndrome

Different types of the acute coronary syndrome (ACS) such as unstable angina and myocardial infarction must be differentiated diagnostically for selection of the best personalised treatment strategy. Despite the progress achieved, diagnosing ACS is often a complex process, in which inflammatory and oxidative markers might be useful.1 However, to date, relevant biomarkers of cardiovascular inflammation useful for stratifying risk and prognosis or guiding therapy for patients with ACS have not been found.2

Inflammation substantially contributes to the pathogenesis of atherosclerosis and ACS; both innate and adaptive immunity regulate the progression of atherosclerosis, plaque stability and thrombus formation.13 Clinical studies have demonstrated the activation of neutrophils, lymphocytes and monocytes, increased concentrations of pro-inflammatory cytokines and acute-phase reactants in patients with unstable angina and myocardial infarction.2

Following plaque rupture, activated platelets will induce a subsequent inflammatory process, including neutrophil recruitment via a mechanism involving the CD40-CD40 ligand.6 In response to acute myocardial ischaemia, neutrophils infiltrate the infarcted myocardium, reaching a maximum number one day after the onset of the disease. Inflammatory Ly6Chi monocytes migrate to the myocardium later than the neutrophils and reach their maximum by the third day of the disease.7

Inflammatory leukocytes are considered the cellular protagonists of vascular inflammation, in triggering disease progression, and the destabilisation that causes ACS.3 Neutrophil accumulation at the coronary culprit lesion site is a strong and independent predictor of mortality in patients with ACS.1 In patients with repeat coronary events within a year, more pronounced activation of oxygen-dependent metabolism of neutrophils and lipid peroxidation was revealed by Kratnov.10 The extensive inflammatory response that occurs during acute cardiac events is considered the process responsible, in part, for the development of depressive symptoms.11 Therefore, treatment of inflammation might be useful for postinfarction myocardial repair.12,13

The neutrophil is an extremely active cell with pleiotropic functions involved in inflammation [phagocytosis, synthesis of reactive oxygen species (ROS) and chemotaxis]. Activated neutrophils and monocytes/macrophages produce ROS and, consequently, result in oxidative stress in the myocardium and blood vessels, which aggravates the inflammation, forming a vicious circle.14 Undoubtedly, oxidative stress is an important pathogenetic event in ACS, along with inflammation.15

A rise in 8-iso-PGF2a during ischaemia indicates the activation of lipid peroxidation.16 In one study, advanced oxidation protein...
products were significantly increased in patients with ACS with ST-segment elevation myocardial infarction and tended to increase in patients with non-ST-segment elevation myocardial infarction.\(^{25}\) Oxidative stress markers (total thiol groups, catalase, superoxide dismutase and glutathione reductase) predict early left ventricular systolic dysfunction after acute myocardial infarction, treated with primary percutaneous coronary intervention.\(^{11}\)

ACS patients had significantly higher levels of thiobarbituric acid reactive substances (TBARS), while levels of nitric oxide, hydrogen peroxide, superoxide dismutase and catalase activity were lowered.\(^{18}\) Therefore, the development of various biomarkers of oxidative stress is important for the prevention and diagnosis of ACS, and patient stratification and treatment.\(^{26}\)

In laboratory practice, the number of neutrophils and the ratio between neutrophils and lymphocytes or platelets are predominantly used. The ratio between neutrophil and lymphocyte counts (NLR) has been proposed as a new low-cost predictor for cardiovascular risk.\(^{25}\) Higher neutrophil count, monocyte count and NLR, and lower lymphocyte count on admission were associated with the presence of coronary collateral vessels in patients with non-ST-segment elevation ACS.\(^{27}\) In patients with a recent ACS, an increased pretreatment NLR value was effective in predicting the risk of mortality.\(^{27}\) Awan et al. demonstrated a strong correlation between rising NLR and cardiac troponin-I increase in patients with non-ST-segment elevation ACS.\(^{28}\)

The ratio and number of cells provide a simple and quick means of assessing neutrophil disturbances, but these indices are not sufficient since neutrophil activity varies widely. Therefore, the assessment of activity should be performed along with a clinical blood test. Respiratory metabolism of neutrophils can be assessed by the nitroblue tetrazolium test, but it has many drawbacks and, in general, is not informative. However, we have failed to find such studies in ACS. Myeloperoxidase, an enzyme of polymorphonuclear neutrophils and macrophages, was studied as a possible marker of plaque instability and a useful clinical tool in the evaluation of patients with coronary heart disease.\(^{27}\)

A promising method for assessing oxidative metabolism of neutrophils is luminol-enhanced chemiluminescence, stimulated by stimuli such as zymosan, phorbol-12-myristate-13-acetate (PMA), N-formylmethionyl-leucyl-phenylalanine (fMLP) and others.\(^{26}\) However, neutrophils are activated in two stages, and priming precedes the activation. Therefore, stimulation by one stimulus is incomplete and depends on the previous state of the neutrophils in the body. Therefore, it is impossible to obtain uniform indices of activity, even for healthy donors.

We previously proposed an original approach based on two-stage stimulation, which allows us to quantify the total oxidative resource of the neutrophils.\(^{27}\) We have also determined some patterns of neutrophil response in pathology.\(^{28}\) In this study, we aimed to apply this new protocol to quantitatively study the oxidative metabolism of neutrophils in ACS.

**Methods**

The study included 42 male and female patients, aged 46–91 years (mean age 61.5 ± 10.8 years), hospitalised in the Demikhov State Clinical Hospital, Moscow, Russia. The control group included 25 male and female volunteers, aged 42–55 years (mean age 49.2 ± 4.1 years) without acute or chronic disease. Blood samples for the study were taken on admission after the cardiologist’s examination.

Inclusion criteria were patients over 18 years of age, verified diagnosis of non-ST-segment elevation ACS or ST-segment elevation ACS (the first 24 hours). Non-ST-segment elevation ACS symptoms included the clinical manifestations within a period of not more than 48 hours, plus electrocardiographic features of ischaemia. Exclusion criteria included patients under 18 years of age with acute infectious diseases, malignant neoplasms or manifested autoimmune disorders.

The patients were divided into three subgroups: ST-segment elevation ACS (\(n = 10\); mean age 57.8 ± 7.9 years); non-ST-segment elevation ACS (\(n = 12\); mean age 61.9 ± 13.7 years); and patients with verified ischaemic heart disease (IHD): stable angina of New York Heart Association functional class II–III (\(n = 20\); mean age 62.7 ± 8.1 years). Information about the patients is presented in Table 1.

The study was performed in accordance with the Helsinki Declaration (adopted at the 64th General Assembly of the WMA in 2013), and approved by the Ethics Committee of Demikhov City Clinical Hospital. All subjects signed informed consent.

Chemiluminescence measurements were performed with a Lum-1200 chemiluminometer (DISoft, Russia) at 37°C. ROS production by neutrophils was assessed with the previously developed protocol based on the two-step sequential stimulation of neutrophils with PMA and fMLP.\(^{27}\) Briefly, in all the experiments, 5 ml of blood was taken from the cubital vein into vacutainers with heparin (the final concentration of heparin was 5 U/ml). Blood samples were stored for up to two hours before analysis.

Aliquots of 450 µl of Hank’s buffer with Hepes (HHBS), 25 µl of 1 mM luminol solution (5-amin-1,2,3,4-tetrahydro-1,4-phthalazinedione; Fluka, Germany) (final concentration = 45 µM), and 25 µl of whole blood were placed into the chemiluminometer cuvette and spontaneous chemiluminescence was recorded for 10 minutes. Next, 50 µl of 0.5 mg/ml PMA (final concentration = 0.081 nM) was added. After 20 minutes of incubation, 55 µl of 100 mg/ml fMLP (final concentration = 10 nM) was added and the chemiluminescence response was recorded for at least 60 minutes.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Subgroup 1 + ST-segment elevation ACS ((n = 22))</th>
<th>Subgroup 2 (ST-segment elevation ACS) ((n = 20))</th>
<th>Subgroup 3 (IHD: stable angina class II–III) ((n = 20))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (mean ± SD)</td>
<td>60.8 ± 12.3</td>
<td>62.7 ± 8.1</td>
<td>62.7 ± 8.1</td>
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<td>Smoking, n (%)</td>
<td>7 (31.8)</td>
<td>2 (10.0)</td>
<td>2 (10.0)</td>
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<tr>
<td>Males/females, n (%)</td>
<td>14/8 (63.6/36.4)</td>
<td>13/7 (63.6/36.4)</td>
<td>13/7 (63.6/36.4)</td>
</tr>
<tr>
<td>PCI during hospitalisation, n (%)</td>
<td>1 (4.5)</td>
<td>8 (40.0)</td>
<td>8 (40.0)</td>
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<tr>
<td>PCI before hospitalisation, n (%)</td>
<td>3 (13.6)</td>
<td>10 (50.0)</td>
<td>10 (50.0)</td>
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<tr>
<td>Post-infarction cardioidrosis, n (%)</td>
<td>3 (15.1)</td>
<td>14 (70.0)</td>
<td>14 (70.0)</td>
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<td>Hypertension, n (%)</td>
<td>16 (72.7)</td>
<td>20 (100)</td>
<td>20 (100)</td>
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<td>Acute heart failure (Killip 1), n (%)</td>
<td>14 (63.6)</td>
<td>2 (10.0)</td>
<td>2 (10.0)</td>
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<td>Type 2 diabetes mellitus, n (%)</td>
<td>2 (9.1)</td>
<td>8 (40.0)</td>
<td>8 (40.0)</td>
</tr>
<tr>
<td>Congestive heart failure, n (%)</td>
<td>9 (40.1)</td>
<td>18 (90.0)</td>
<td>18 (90.0)</td>
</tr>
</tbody>
</table>

ACS, acute coronary syndrome; IHD, ischaemic heart disease; PCI, percutaneous coronary intervention.
A kinetic plot of neutrophil response to double-step stimulation involves three parts (Fig. 1A): a spontaneous (or basal) chemiluminescence ($A_{bas}$); a PMA-stimulated response; and a double-step stimulated response ($A_{bas+MLP}$). $A_{bas}$ values were determined from blank chemilumograms (blood samples with luminol but without stimuli); $A_{PMA+MLP}$ values were determined at peak maximum after double-step sequential stimulation. Specific values (divided by the number of neutrophils) of $A_{bas}'$ and $A_{bas+MLP}'$ were calculated and compared with clinical data and laboratory results.

**Statistical analysis**

The size of the groups was not previously determined. For statistical analysis, Statistica for Windows v.10.0 (StatSoft Inc, USA) was used. The normality was checked according to the Shapiro–Wilk test. Normally distributed variables are presented as the arithmetic mean ± standard deviation (SD). Otherwise, medians and first and third quartiles were calculated. Comparative analysis of two independent groups was carried out using the parametric $t$-test. The relationship was evaluated by the Spearman correlation coefficient ($r$). The differences were considered statistically significant at $p \leq 0.05$.

**Results**

In patients with ischaemic heart disease and non-ST-segment elevation ACS, the neutrophil response was similar to chemilumograms of blood of healthy donors (Fig. 1A), whereas the neutrophil response for patients with ST-segment elevation ACS had qualitative and quantitative differences (Fig. 1B). In ST-segment elevation ACS, the amplitude of two-step stimulated chemiluminescence was several times higher. In several cases of ST-segment elevation ACS, there was a slight ‘slow flash’ on the chemilumogram, which indicates the activation of phagocytosis. Total and specific (divided by neutrophil count) parameters of neutrophil activity are presented in Table 2.

The data show that the specific indices of spontaneous and stimulated chemiluminescence did not differ for the subgroups of healthy donors, and ischaemic heart disease, unstable angina pectoris and acute myocardial infarction patients. Total indices did not differ for healthy donors, and ischaemic heart disease and unstable angina patients, but were significantly higher for acute myocardial infarction patients. This increase was caused, not by increased activation of the neutrophils, but by an increase in their numbers.

The correlation coefficient between $A_{PMA+MLP}$ and the neutrophil number was 0.63 for the total ACS subgroup. However, the correlation between $A_{PMA+MLP}$ and NLR was weak ($r = 0.35$). A significant correlation was found between $A_{PMA+MLP}$ and lactate dehydrogenase (LDH-1) in ACS patients but not in IHD patients. The correlation coefficients were 0.72 (ACS) and 0.09 (IHD).

**Discussion**

The complex and ambiguous role of neutrophils makes them therapeutic targets, but clearly requires the distinction of beneficial effects from harmful side effects. Many strategies are being developed, including enhancing, inhibiting or restoring neutrophil function. For this purpose, adequate methods for assessing the activity of neutrophils are needed.

In addition to the haemogram indices, neutrophils are assessed by the activity of their enzymes, for example, elastase, or by the amount of ROS (NBT test, stimulated chemiluminescence). A distinctive feature of our method is the two-step stimulation,
which allows, in contrast to the one-step one, to assess the full resource of the neutrophil after priming with the first stimulus, to unify the response, and to quantify reference intervals. Unlike the NBT test, two-step chemiluminescence makes it possible to also assess the kinetics of the response.

According to the response kinetics, we did not see any principal differences between neutrophils from patients with IHD or ACS and healthy donors. In some patients with ACS, a slow flash (a delayed ROS release) was expressed. According to our assumptions, the slow flash is due to intracellular ROS. However, the intensity of this slow flash was too low to be clinically significant. In sepsis and trauma, the intensity of a slow flash can be an order of magnitude greater than a fast flash.

Quantitatively, the specific double-step activity of neutrophils did not differ significantly in patients with ACS and IHD, which indicates the absence of mechanical changes in the function of ROS production of neutrophils. Therefore, an increase in their number leads to an increase in the total production of ROS. This is in line with other studies where the number of neutrophils or the NLR was used as a marker of ACS. Since the specific indices of spontaneous and double-step stimulated chemiluminescence did not differ in the subgroups of patients with ACS and IHD, which makes it possible to use this parameter for a differential diagnosis. Since an increase in ROS production is caused precisely by an increase in the number of neutrophils, numerical indices remain important in the diagnosis of ACS. We plan to study the oxidative metabolism of neutrophils in larger groups in the dynamics of ACS.

References


