Intracellular calcium ions kinetics and chemiluminescent activity in human neutrophils

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Abstract

Chemiluminescence of granulocytes is a renowned method for the estimation their overall activity as measured by the production of reactive oxygen species. The aim of this study was to compare chemiluminescent activity of human granulocytes to kinetics of intracellular calcium ions concentration in children with recurrent infections of respiratory tract (RRTI) and in healthy children. The studies was performed with the use of isolated peripheral blood neutrophils from 41 RRTI children (21 girls, 20 boys) 3 to 10 years old with more than five episodes of RRTI per year and from control group of 30 healthy children age and sex matched free from allergic, immune and hematological disorders. Neutrophils were activated by bacterial peptide fMLP, opsonised zymosan (OZ) and phorbol myristat acetate (PMA). Intracellular Ca concentration kinetics was assessed by flow cytometry (Coulter Epics XL) with the use of Fluo3 and Fura Red fluorescent dyes. fMLP and OZ induced Ca mobilization lasted shorter in RRTI group ($p<0.05$).The peak influx of free Ca and its concentration in resting state after stimulation with fMLP were lower in patients ($p<0.05$). In RRTI group stimulation with OZ was delayed comparing to control ($p<0.01$). In response to PMA free Ca concentration decreased faster. Chemiluminescent response to all the examined stimuli in children with recurrent infections was significantly lower compared to the control. Significant negative correlation was observed between intracellular calcium level and emission of light measured by chemiluminescence assay ($r=–0.9643$, $p<0.001$) after stimulation with fMLP. Intracellular calcium concentration did not correlated with chemiluminescent activity induced by OZ or PMA.Increased sensitivity to infections in RRTI children may be related to the disturbances in neutrophil activation via intracellular Ca concentration and subsequent production of free oxygen radicals. Chemiluminescent activity after stimulation with fMLP seems to be dependent on intracellular calcium ions concentration. PMA and OZ induce chemiluminescence in Ca^{2+} independent manner.

Key words: *granulocyte, chemiluminescence, calcium concentration, recurrent infections of respiratory tract.*

(Centr Eur J Immunol 2007; 32 (2): 72-76)

Introduction

Chemiluminescence test is commonly used to determine neutrophils function, which play an important role in nonspecific immunological response [1, 2]. Due to various stimuli granulocytes are producing free oxygen radicals (respiratory burst), resulting in light emission (chemiluminescence). It could be induced by chemotactic agonist (fMLP), antibodies and complement Fc-fragment receptor (opsonized zymosan – OZ) or in non-receptor way (PMA – phorbol 12-myristate 13-acetate) [3, 4]. When stimulated, granulocytes produce large amounts of superoxide that dismutates to hydrogen peroxide (H_2O_2). The enzyme myeloperoxidase utilizes H_2O_2 and chloride to produce hypochlorous acid (HOCl), a potent oxidant that reacts with a wide range of biological targets and is implicated as a cause

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of inflammatory tissue damage. Correct granulocytes response to extrinsic stimuli depends on proper function of cell signalling pathway [5, 6]. Cytosolic free calcium (Ca^{2+}) is believed to play a key role in the regulation of cellular function. Intracellular calcium ions changes are essential for some enzymes in signal pathway activation, therefore monitoring of its concentration could help in assessment of stimuli-dependent granulocytes reactivity [7-12]. Neutrophils recruitment and activation in response to inflammatory stimuli is regulated through complex parallel and sequential events [9]. The process begins after coupling of specific ligand to the surface receptor or by tight cell-cell contacts and triggering a signaling cascade inside the cell. The molecular signals initiate directional cell movement, endocytosis, degranulation, superoxide generation and chemiluminescence. Chemiluminescence initiated by chemotactic agonists such as fMLP or by binding of opsonised particles to FcRγ, may be Ca^{2+} dependent, as decrease of extracellular Ca^{2+} level significantly reduces emission of light.

The aim of this study was to compare effects of fMLP, OZ and PMA on respiratory burst and intracellular calcium ions level changes in human neutrophils.

Materials and methods

Patients

Based on physical examination and disease history 71 children were divided into two groups. Forty-one children (21 girls and 20 boys) of age ranging from 1 to 15 years (mean 7,0±4,19) who suffered from more than eight episodes of recurrent respiratory tract infections (RRTI) per year referred to the Children Hospital. A control group consisted of thirty children, 11 girls and 19 boys, aged from 1 to 15 years (mean 6,9±4,03 SD). There were no significant differences in the age and sex between the children with or without RRTI. Control children were free from allergic diseases, immune and hematological disorders and had experienced fewer than five episodes of URTI per year. Healthy children were referred to plastic surgery department and were qualified for minor plastic operations. Blood collection was performed at least three weeks after last episode of infection, vaccination and any medication and at least 3 months after last episode of viral disease as mononucleosis, smallpox. Parents of the investigated children gave informed consent during enrollment visit, having been fully informed of the nature, risk and potential benefits of the study. In cases of elder children we also obtained their permission. The Research and Ethical Committee of Medical University of Warsaw approved the study protocol.

Neutrophils

Three milliliters of venous blood were taken from the ulnar vein to a tube containing heparin (10 U/ml). The blood count was determined using a Coulter HMX analyser. Number of neutrophiles was identified microscopically in the blood smear after haematological staining. Routine laboratory tests, including C-reactive protein, erythrocyte sedimentation rate, and leukocyte count, were performed for each patient. Neutrophils were isolated by 25 min centrifugation at 1200 g on Gradisol G with d=1.115±0.002 g/cm^3 (POLFA, Łódz, Poland) mixed with Histopaque in proportion (3+2). After isolation and final washing cells were pelleted and resuspended in RPMI-1610 medium (Sigma Chemicals St/Luis, MO, USA) and concentration was adjusted to 1-2 mln/ml. 95% of the cells had morphology of neutrophils. To the neutrophils suspension 5 μM of Fluo3 and Fura Red (Molecular Probes, Eugene, OR, and USA) was added and the aliquots were incubated in dark at 37°C. Then, the cells were washed and resuspended in RPMI-1610 at concentration 2 mln/ml and divided into three aliquots.

Flow cytometry

Analysis was performed on Coulter Epics XL flow cytometer (Becman Coulter Hialeh, FL, USA) according the method described earlier [6]. Neutrophils were discriminated by flow cytometric measurements of cellular forward angle and right angle scatter. Fluo 3 and Fura Red were exited at 488 nm with Fluo 3 emission detected at 515-535 nm and Fura Red emission detected at with 665-685 nm. Data were collected in histograms displaying Fluo3 fluorescence vs time and Fura Red fluorescence vs. time and mean channels of fluorescence intensity were used for calculation. The first 40 sec of the analysis was considered as initial, resting state. Then, the measurement was interrupted to add stimuli and the measurement for next 60 sec was continued. As stimulants fMLP (Sigma) at concentration of 10^{-5}M, PMA (Sigma) at concentration of 1 ug/ml and opsonised zymosan (OZ) (20 mg/ml) were used. Intracellular changes of calcium after stimulation are expressed as a percent of initial, resting value. Ratio intensity of Fluo3/Fura Red vs. time was also calculated.

Chemiluminscence

Neturophils luminol – dependent chemiluminescence was assessed before and after stimulation with fMLP (10^{-3}M), OZ (20 μg/ml) and PMA (1 μg/ml). Chemiluminescence was measured with scintillation counter (LKB Wallac 1409, Finland). Results were shown as a chemiluminescence index (spontaneous vs. stimulated).

Results

Changes of intracellular concentration of free Ca in the cytosol of neutrophils isolated from peripheral blood after stimulation with fMLP, OZ or PMA were measured in time dependent manner and expressed in relation to chemiluminescent activity of examined cells (figures 1-3). OZ and fMLP evoked intracellular increase of free Ca concentration in neutrophils of patients and controls. Around

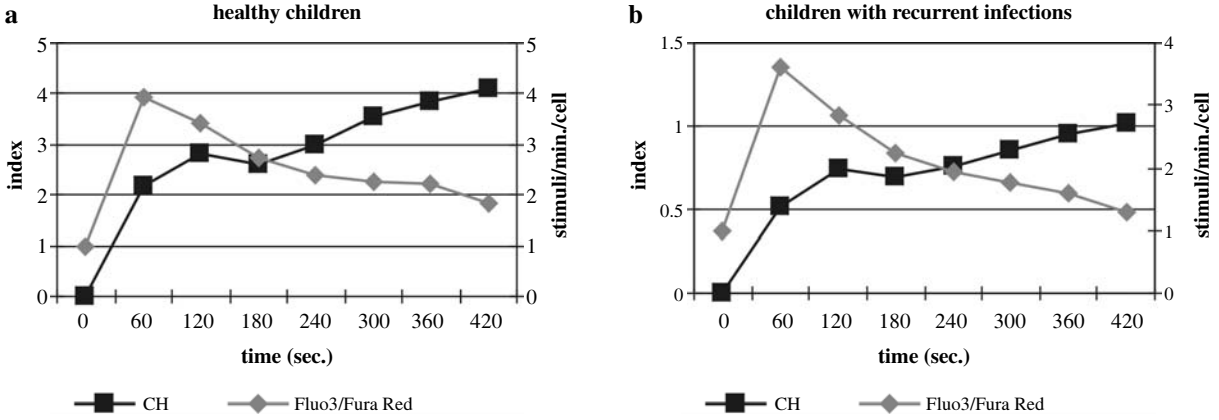

Fig. 1. Effect of fMLP on chemiluminescence and calcium level in granulocytes

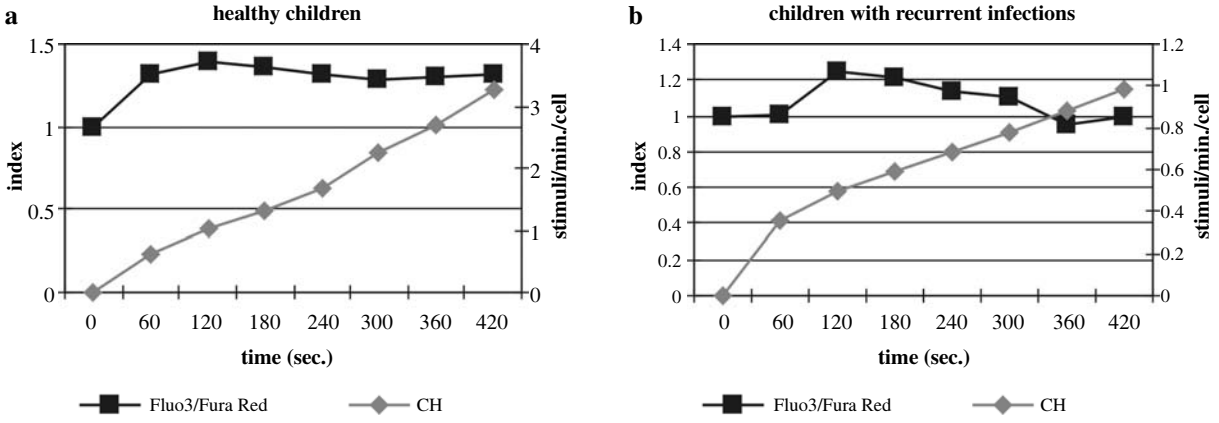

Fig. 2. Effects of OZ on chemiluminescence and calcium level in granulocytes

six minutes after stimulation in RRTI group fluorescence ratio of Fluo 3 to Fura Red returned to the level of resting state. After stimulation with OZ, in both studied groups, non-significant increase of intracellular Ca concentration comparing to the resting state was noticed. fMLP and OZ induced Ca mobilization lasted shorter in RRTI group ($p<0.05$).The peak influx of free Ca and its concentration in resting state after stimulation with fMLP were lower in patients ($p<0.05$). In RRTI group stimulation with OZ was delayed comparing to control ($p<0.01$). In response to PMA free Ca concentration decreased. The kinetic slopes of Ca concentration in both examined groups differed statistically in all measured points. Decrease on intracellular Ca concentration after PMA stimulation was higher ($p<0.01$) and lasted longer in RRTI group.Chemiluminescent response to all the examined stimuli in children with recurrent infections was significantly lower compared to control ($p<0.001$). Kinetic slopes differed depending on the stimulator used (figures 1-3).

After fMLP stimulation in both examined groups peak of calcium ions concentration was present in 60th second after stimulation and was followed by the decrease of this concentration accompanied with light emission (chemiluminescence) (figure 1).

Stimulation with OZ both in healthy children was followed by small increase of intracellular calcium concentration accompanied with slow increase of light emission (CL) (figure 2).

Stimulation with PMA both in healthy children was followed by slow decrease of intracellular calcium concentration accompanied with slow increase of light emission (chemiluminescence) (figure 3).

Significant negative correlation was observed between intracellular calcium level and emission of light measured by chemiluminescence assay ($r=–0.9643$, $p<0.001$) after stimulation with fMLP. Intracellular calcium concentration did not correlate with chemiluminescent activity induced by OZ or PMA.

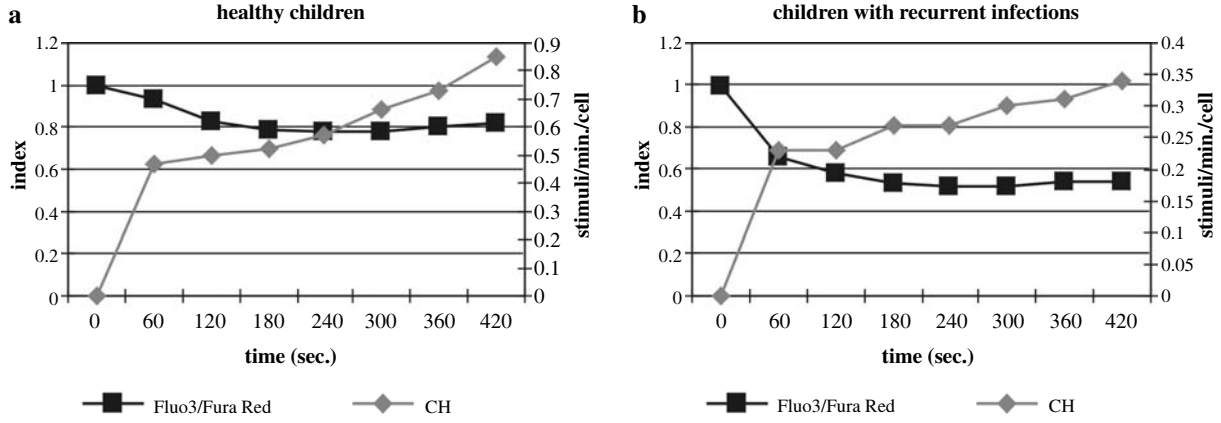

Fig. 3. Effect of PMA on chemiluminescence and calcium level in granulocytes

Discussion

Blood neutrophils play important role in the innate immunity and in inflammatory reaction. Its proper functions are important for effective local and systemic host defense. Neutrophils are major sources of biological oxidants. In polymorphonuclear leukocytes changes in intracellular calcium are associated with multiple cellular events, including activation of cellular kinases and phosphatases, degranulation, phagosome-lysosome fusion, regulation of cytoskeleton binding proteins, transcriptional control and modulation of surface receptors [7, 8]. In the present paper changes in Ca free and Ca stored in stimulated granulocytes in relation to their chemiluminescent activity were evaluated. We have shown opposite relationship between production of free oxygen radicals and intracellular calcium concentration after stimulation with fMLP. Negative trend was also observed after OZ stimulation however obtained results were not significant. PMA induced CL did not correlated with calcium concentration changes. Opposite to our observations Oommen et al. suggest that there is no interaction between respiratory burst and intracellular calcium concentration after fMLP stimulation [13]. On the other hand Xun Shen et al. found the relation between calcium concentration in intra- and extracellular space and chemiluminescent activity of fMLP stimulated granulocytes [14]. These authors show that in calcium free medium production of oxygen radicals by fMLP activated neutrophils is significantly weaker then in the presence of calcium ions in extracellular space [14]. It could suggest that extracellular calcium regulate respiratory burst in neutrophils after receptor-dependent stimulation. The authors investigated the relation between intracellular calcium pool and respiratory burst and showed enhanced chemiluminescent activity of granulocytes in high calcium concentration environment [14]. Furthermore Foyouzi--Youseffi et al. also postulate that elevation of Ca^{2+} critically determine activation of respiratory burst [9]. fMLP induced a rapid and sustained elevation of cytoplasmic Ca^{2+}. Mobilization of Ca^{2+} is one of the early events triggered by binding of a chemoattractant to its receptor. fMLP-stimulated neutrophils phosphorylation is dependent on phosphoinositide 3-kinase, PLD (phospholipase D) and PKC (protein kinase C) activity. Two fMLP receptor subtypes were identified in neutrophils, characterized by a distinct sensitivity to fMLP and antagonistic peptides [15]. Both fMLP receptors involves an action of phosphoinositide 3-kinase, PLD and PKC isotypes. After stimulation with OZ, in both studied groups, non-significant increase of intracellular Ca concentration comparing to the resting state was noticed. In RRTI group the excitation induced by this stimulus was delayed and shorter in comparison to the healthy group. Significant increase of free Ca concentration in neutrophils from controls durated much longer after stimulation then in RRTI patient's cells. OZ induced activation of calcium channels and CL activity has different kinetics. OZ induced CL is a slow process whereas calcium concentration changes fast. High calcium level is toxic for cells so the system of pumps quickly decrease its concentration [1, 10]. The reaction of neutrophils to PMA was clearly different. Concentration of free Ca fell down in neutrophils of the examined both groups. In neutrophils of healthy control significant decrease lasted 120 sec after stimulation. The kinetic slopes of Ca concentration in both groups differed statistically in all measured points. Decrease of intracellular Ca concentration was significantly lower and lasted longer in RRTI group comparing to the control. In both study groups weaker respiratory burst activity after PMA stimulation was noticed. No correlation between CL and calcium ions kinetics was observed. Xun Shen et al. showed that PMA induced respiratory burst is calcium independent [14]. Phorbol myristate acetate as a direct stimulator of protein kinase C (PKC) induces chemiluminescence in Ca^{2+} independent manner. Tianhui Hu et al. showed that

PMA-stimulated respiratory burst is not only due to the changes of free calcium level, but is also due to the higher efficiency of diacylglycerol in activating protein kinase C [16]. Decrease of Ca^{2+} combined with very moderate increase of Ca^{2+} bound might indicate that Ca^{2+} efflux is stimulated by cyclic AMP. Formation of ROS in granulocytes may be induced by different mechanisms, of which activation of the NADPH oxidase is the most important [17]. In resting neutrophils this complex consists of unassembled cytosolic and membrane components. Following activation, certain cytosolic components translocate to the plasma membrane where they associate with flavocytochrome b558 and Rap1A to form the active oxidase [17]. Phosphorylation of the $p47^{PHOX}$ subunit plays a major role in activation of the NADPH oxidase complex [15] and is responsible for transporting the cytosolic NADPH oxidase complex to the membrane during activation [17]. Various protein kinases have been involved in the regulation of NADPH oxidase activity, including phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), and mitogen activated protein kinases (MAPK) [15, 17]. PLC induces formation of DAG and IP3, which is followed by calcium release from intracellular stores and PKC activation. Generally our observation suggests that intracellular Ca^{2+} kinetics in the group with RRTI is different in comparison to healthy control. On the basis of presented results is difficult to explain this observation. It may be the consequence of RRTI or subsequent treatment. We may also hypothesize that children highly susceptible to infections may present inherited disturbances in intracellular mechanisms of calcium distribution. Our investigation indicates that the fMLP induced respiratory burst was at least partly due changes intracellular calcium concentration. The possible mechanism may involve tyrosine kinase mediated activation of PI3K what result in enhanced activation of calcium-dependent PKC by enhanced PLC activity, followed by intracellular calcium release [18, 19].

Conclusions

1. Peripheral blood neutrophils activation followed by respiratory burst causes changes of intracellular calcium ions concentration. Kinetics these changes strictly depend on stimulant used.
2. Calcium ions concentration increase due to stimuli which have membrane receptors like fMLP.
3. fMLP induced respiratory burst is at least partly calcium dependend whereas PMA stimulated chemiluminescence is calcium unrelated.

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