Free Radical Research, 2014; Early Online: 1–10

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715762.2014.938234

informa
healthcare

ORIGINAL ARTICLE

Comparison of proton channel, phagocyte oxidase, and respiratory burst levels between human eosinophil and neutrophil granulocytes

I. Kovács[1], M. Horváth[1], T. Kovács[2], K. Somogyi[1], L. Tretter[2], M. Geiszt[1,3] & G. L. Petheő[1]

[1]*Department of Physiology, Faculty of Medicine, Semmelweis University, Budapest, Hungary,* [2]*Department of Medical Biochemistry, Faculty of Medicine, Semmelweis University, Budapest, Hungary, and* [3]*"Lendület" Peroxidase Enzyme Research Group of the Semmelweis University and the Hungarian Academy of Sciences, Budapest, Hungary*

Abstract

Robust production of reactive oxygen species (ROS) by phagocyte NADPH oxidase (phox) during the respiratory burst (RB) is a characteristic feature of eosinophil and neutrophil granulocytes. In these cells the voltage-gated proton channel (Hv1) is now considered as an ancillary subunit of the phox needed for intense ROS production. Multiple sources reported that the expression of phox subunits and RB is more intensive in eosinophils, than in neutrophils. In most of these studies the eosinophils were not isolated from healthy individuals, and a comparative analysis of Hv1 expression had never been carried out. We performed a systematic comparison of the levels of essential phox subunits, Hv1 expression, and ROS producing capacity between eosinophils and neutrophils of healthy individuals. The expression of phox components was similar, whereas the amount of Hv1 was ~10-fold greater in eosinophils. Furthermore, Hv1 expression correlated with Nox2 expression only in eosinophils. Additionally, in confocal microscopy experiments co-accumulation of Hv1 and Nox2 at the cell periphery was observed in resting eosinophils but not in neutrophils. While phorbol-12-myristate-13-acetate-induced peak extracellular ROS release was ~1.7-fold greater in eosinophils, oxygen consumption studies indicated that the maximal intensity of the RB is only ~1.4-fold greater in eosinophils. Our data reinforce that eosinophils, unlike neutrophils, generate ROS predominantly extracellularly. In contrast to previous works we have found that the two granulocyte types display very similar phox subunit expression and RB capacity. The large difference in Hv1 expression suggests that its support to intense ROS production is more important at the cell surface.

Keywords: healthy individuals, oxygen consumption, reactive oxygen species, NADPH oxidase, Hv1 channel

Introduction

Eosinophil and neutrophil granulocytes undergo respiratory burst (RB) upon activation by diverse stimuli. The extra oxygen consumed during the RB is converted into reactive oxygen species (ROS). ROS can damage invading microorganisms and eventually surrounding host tissues. ROS production is initiated by the heteromultimeric phagocyte NADPH oxidase enzyme complex (phox), which catalyzes the transport of electrons across the plasma membrane to reduce molecular oxygen, thus producing superoxide, the precursor of further, more aggressive ROS [1]. The active phox comprises at least the following subunits: the cytosolic $p67^{phox}$, $p47^{phox}$, and rac1/2 (mainly rac2 in myeloid cells) attached to the membrane bound cytochrome b_{558}, which is a heterodimer of Nox2 (a.k.a. $gp91^{phox}$) and $p22^{phox}$ [2]. Additionally, $p40^{phox}$ also appears important for supporting phagosomal ROS production [3]. Genetic deficiency for any of the aforementioned six subunits can cause chronic granulomatous disease (CGD), an inherited syndrome characterized by severe fungal and bacterial infections and persistent granulomas [4]. The activity of the phox is accompanied with intracellular acidification and membrane depolarization as a consequence of electron extrusion from the cytoplasm. Without effective compensatory mechanism these changes could rapidly inhibit the activity of the oxidase [5] and damage the granulocyte itself [6]. Currently, proton extrusion through the voltage-gated proton channel (Hv1) is regarded as the most effective way of compensation, since it is able to alleviate both problems jointly and with high capacity [5]. Hv1 proton channel is a "voltage-sensor only protein" [7], the activity of which is promoted by intracellular acidosis, extracellular alkalosis, depolarization, and by the activated phox [8]. Importantly, phagocytes from Hv1 deficient mice produce 30–75% less ROS upon activation [2].

Previous studies reported that eosinophil granulocytes express phox subunits in higher quantity and produce more ROS upon stimulation, than neutrophils. Mostly because of technical difficulties many of the works used eosinophils of individuals with hypereosinophilia [9–12]. Therefore, these results possibly provide a poor estimate for the healthy population. Notably, some investigators compared certain specific functions of eosinophil and neutrophil granulocytes from normal blood [13–16], but these studies concentrated on ROS production, and much less effort was focused on the expression of phox subunits [13,16]. Thus a profound and systematic analysis in healthy human granulocytes is lacking. More importantly, we do

Correspondence: Gábor L. Petheő, Department of Physiology, Faculty of Medicine, Semmelweis University, PO Box 259, H-1444 Budapest, Hungary. Tel: + 36-1-459-1500, Ext: 60436. Fax: + 36-1-266-7480. E-mail: petheo.gabor@med.semmelweis-univ.hu

(Received date: 29 November 2013; Accepted date: 20 June 2014; Published online: xx xxx xxx)

not yet have quantitative comparison of the protein level of the more recently discovered Hv1 proton channel [17] in these cells.

To follow the intensity of the RB, ROS formation or oxygen consumption can be measured. There are numerous methods available to follow ROS production, and each has its own spectra of ROS sensitivity and detects a given ROS in different compartments with different efficiency [18]. For example, Cytochrome c (Cyt c) reduction assay, applied in most of the previous comparisons, detects exclusively extracellular superoxide. In contrast to neutrophils, eosinophils tend to produce ROS at the cell surface [10,13]. Therefore, Cyt c reduction is prone to overestimate the difference in ROS producing capacity, favoring eosinophil granulocytes. One possibility to more correctly assess phox activity (i.e., total ROS production) in granulocytes is to follow their oxygen consumption during RB polarometrically, e.g., with a Clark-type electrode. The high cell-demand of polarographic oxygen consumption measurements, however, made it extremely difficult to determine RB intensity in eosinophils until recently. In the past decades novel isolation techniques were developed making it possible to isolate satisfactory amounts of eosinophils from healthy individuals without severely interfering with the resting state of these cells [19]. Moreover, recent advances in fluorometric oxygen detection technologies have made possible to detect the oxygen consumption of only a few thousand cells [20].

In this study we set out to systematically compare the expression of phox subunits, the expression of Hv1 and the intensity of the RB in eosinophil and neutrophil granulocytes of healthy individuals.

Materials and methods

Reagents

All reagents were purchased from Sigma–Aldrich (www.sigmaaldrich.com) and all manipulations were carried out at ambient temperature (21–26°C), unless otherwise specified. H-medium contained (mM): NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 0.8, HEPES 10, Glucose 5, (pH 7.4). Granulocyte isolation (GI) buffer was RPMI 1640 medium supplemented with 2 mM EDTA and 2% fetal bovine serum (FBS, Cat No. DE-14802-F, Lonza, www.lonza.com). Zymosan was opsonized with pooled human serum (from at least three healthy donors) by 30 min incubation at 37°C. Stocks of phorbol-12-myristate-13-acetate (PMA) and diphenyliodonium (DPI) were prepared in DMSO at 5 and 10 mM, respectively.

Antibodies: to detect the voltage-gated proton channel, our affinity purified polyclonal rabbit antibody (aHv1-N) was applied, as previously described [21]. Nox2 and $p22^{phox}$ immunoreactivity was detected using Santa Cruz Biotechnologies (www.scbt.com) antibodies sc-130548 and sc-20781, respectively. Anti-$p47^{phox}$ was purchased from Cell Signaling Technology (#4312, www.cellsignal.com), and anti-$p67^{phox}$ [22] was a generous gift of Dr. Katalin Német. Rac was detected using BD Transduction Biotechnologies antibody (#610650, www.bdbiosciences.com). For loading control in Western blots anti-protein disulphide isomerase antibody (aPDI) was used (ab2792, Abcam, www.abcam.com). To detect $gp91^{phox}$ in immunofluorescence experiments, supernatant of the mouse monoclonal hybridoma 7D5 [23] was used. Alexa Fluor® 488- (aHv1-N) and Alexa Fluor® 568-labeled (7D5) secondary antibodies (F(ab′)2 fragment only) were from Molecular Probes (probes.invitrogen.com). Horseradish-peroxidase-labeled secondary antibodies were from GE Healthcare (www.gelifesciences.com).

Preparation of granulocytes

The studies conformed to the standards set by the Declaration of Helsinki, and the procedures were approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (license #: TUKEB 38/2007). Blood cells were prepared from venous blood drawn from healthy adults after obtaining their informed and written consent. Red blood cells (RBCs) and leukocytes (WBCs) were separated by gravity-driven dextrane (Cat No. 17–0320, GE healthcare, www.gelifesciences.com) sedimentation for 30–40 min by mixing 5 volumes of whole blood with 3 volumes of 4% w/v dextrane in 0.9% w/v NaCl solution and 1 volume of 3.13% w/v Na-citrate solution in a vertical tube. To separate mononuclear cells (MCs) from granulocytes (PMNs), WBCs were layered onto Ficoll-Paque Plus (GE Healthcare, www.gelifesciences.com) and centrifuged at 400 g for 20 min. The remaining manipulations were carried out at 4°C. Residual RBCs were hemolyzed by 20 s exposure to distilled water, followed by reconstitution of the osmolality with an equal volume of 1.8% w/v NaCl solution. After centrifugation (200 g, 5 min), pellets containing purified PMNs were resuspended in GI buffer and separated further into eosinophil (CD16-) and neutrophil (CD16+) fractions utilizing paramagnetically labeled anti-CD16 antibodies (MicroBeads) and a magnetic separator (CS column-equipped VarioMacs) purchased from Miltenyi Biotec (www.miltenyibiotec.com).

Cell lysate preparation

To minimize protein degradation, cells were resuspended in H-medium supplemented with diisopropyl fluorophosphate (DFP, 1:5000), and were incubated on ice for 30 min before lysis. With this treatment we managed to minimize the proteolysis of Hv1, which is prone to proteolytic cleavage [21]. Cells were lysed using 2x Laemmli buffer containing 5% v/v β-mercaptoethanol and 2 mM phenylmethanesulfonyl fluoride.

Immunoblotting

Samples were run on 8 or 10% polyacrylamide gel and blotted onto nitrocellulose membrane. To block nonspecific binding sites in Western blot experiments (WB), 5%

w/v skimmed milk powder was applied in phosphate buffered saline (PBS, pH 7.4) for 1 h. After incubating the membranes with the first antibody (rabbit polyclonal or mouse monoclonal) for 1 h, membranes were washed five times in PBS with 0.1% v/v Tween20 (PBS-T). Horseradish peroxidase-labeled secondary antibody was added in 1:5000 dilution (in PBS-T with 1% w/v skimmed milk powder) for 40 min, followed by washing five times in PBS-T. Signals were detected on FUJI Super RX films (Fujifilm, www.fujifilm.com) using the enhanced chemiluminescence method (GE Healthcare, ECL™ Western Blotting Analysis System). After photo scanning and digitalization, the images of photographic films were analyzed using ImageJ software (Rasband, W. S., U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/). The integrated density of each protein band was determined and normalized to the loading control (protein disulfide isomerase, PDI). The so-obtained values were used to calculate eosinophil/neutrophil ratio (Eo/Ne) of subunit content.

Determination of ROS production

Cells were suspended in H-medium at a concentration of 10^6/ml. To follow the ROS production predominantly intracellularly [24] (Supplementary Figure 1B to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234), the medium was supplemented with 250 µM luminol and 1 U/ml horseradish peroxidase (HRP, but see Supplementary Figure S2 to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234). After 10 min of incubation 100 µl cell suspension was loaded into wells of a 96-well Greiner Lumitrack 200 plate (www.greinerbioone.com). To measure extracellular ROS release (mainly superoxide, Supplementary Figure 1A to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234), the cell suspension was diluted to 5×10^5 cells/ml with Diogenes™ Cellular Luminescence Enhancement System (a trade mark of National Diagnostics, www.national-diagnostics.com), then 100 µl of this suspension was loaded into each well. To induce RB, either a supramaximal dose of PMA (0.6 µM) or 0.4 mg/ml serum-treated zymosan (STZ) was added to the cells. Luminescence signals were recorded using Thermo Fischer Scientific Fluoroscan Ascent lumino-fluorometer (www.thermoscientific.com) at 37°C.

Oxygen consumption and pH measurements

To measure oxygen consumption and pH changes at 37°C, the Seahorse XF Analyzer system (Seahorse Bioscience, www.seahorsebio.com) was used. Fifty microliter suspension of isolated cells ($2–3 \times 10^5$/ml) was loaded into fibronectin-coated wells of 96-well Seahorse XF Analyzer microplate, and was incubated for 60 min to allow adhesion. The fibronectin surface is in the first line of choices if adhesion-induced granulocyte activation is to be minimized [25]. After the adhesion period, 130 µl H-medium was added to each well. RB was induced as described above. Changes in extracellular partial O_2 tension and pH were measured. From these changes Oxygen Consumption Rate (OCR) and Extra Cellular Acidification Rate (ECAR) were calculated, respectively. To preclude major influence of pH shifts on NADPH oxidase activity [26], we applied strongly buffered solutions (10 mM HEPES at pH 7.4). As a consequence, only tiny pH shifts could be observed, limiting the relevance of pH measurements.

Immunofluorescent labeling

For immunofluorescence experiments (IF) PMNs (>90% neutrophil) or purified eosinophils were resuspended in ice cold 4% w/v paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) and incubated on ice for 30 min. Following centrifugation, cells were washed with 5 ml FBS. Following centrifugation, 3 million cells were suspended in 0.1 ml FBS. Thirty microliter drops of the suspensions were dried fast onto coverslips. A second fixation step was carried out using 4% w/v paraformaldehyde in PBS for 20 min. Following fixation, coverslips were rinsed four times with PBS and incubated for 10 min in PBS containing 100 mM glycine. Coverslips were then washed two times in PBS. Cell permeabilization was carried out in PBS containing 1% w/v bovine serum albumin (BSA) and 0.1% v/v Triton X-100 for 20 min. To block nonspecific binding sites (e.g., Fc-receptors), 5% v/v normal goat serum and 5% v/v human Fc-receptor blocking reagent (Miltenyi Biotec) were applied in PBS for 1 h. Coverslips were then incubated with the primary antibodies overnight at 4°C, then washed six times in PBS and incubated with the secondary antibodies for 1 h, and finally washed six times in PBS again. During the application of all antibodies 5% v/v normal goat serum and 5% v/v human Fc-receptor blocking reagent were present. To label nuclei, 0.2 µM TO-PRO®-3 (Life Technologies, www.lifetechnologies.com) was added to the fixed cells together with the secondary antibodies. At the end of the procedure coverslips were washed three times with distilled water and mounted using Mowiol 4–88 anti-fade medium (prepared from polyvinyl alcohol 4–88, glycerol, H_2O, and TRIS pH 8.5).

Confocal laser scanning microscopy

Confocal images were collected on an LSM 710 laser scanning confocal unit (Carl Zeiss, www.zeiss.com) with a 63X 1.4 numerical aperture plan Apochromat objective (Carl Zeiss). Excitations were carried out with 25-mW argon laser emitting at 488 (AF-488) nm and a 1.0-mW helium/neon laser emitting at 543 (AF-568) and 633 nm (TO-PRO-3). Emissions were collected using monochromators at the appropriate wavelengths. Images from optical slices of 0.5 µm thickness were acquired. ZEN software (Carl Zeiss) was used for image acquisition. For analyzing and processing images the ImageJ software was applied. The two different granulocyte types of any donor were labeled the same way in parallel and parameters of confocal imaging were retained between cell types to enable

[AQ9] quantitative comparison. For the sake of better visibility, linear image enhancement (brightness and contrast) was carried out (Figure 3) off-line, strictly to the same extent in the two cell types. No off-line correction was applied to the pseudocolor, 3D reconstructions (Supplementary movies to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234).

Statistical analysis

Statistical analyses were performed with Statistica 8 software (StatSoft, Inc., www.statsoft.com). Student's t-test or Mann–Whitney U-test was applied as appropriate, and a value of $p < 0.05$ was considered statistically significant, unless otherwise stated. Data are represented as mean ± SEM, unless otherwise specified.

Results

The expression level of Hv1 but not of phox is higher in eosinophils than in neutrophils

A detailed comparison between eosinophil and neutrophil granulocytes on the amounts of phox subunits had not yet been performed in healthy individuals. Therefore, to assess the amounts of phox subunits supplemented with that of the Hv1 protein, total cell lysates were prepared from eosinophils and neutrophils obtained from healthy donors. Samples were subjected to Western blot analyses and densitometry. The eosinophil and neutrophil samples from a given donor were loaded next to each other enabling direct comparisons. As shown in Figure 1, we analyzed the expression of the five essential phox subunits (Nox2, $p67^{phox}$, $p47^{phox}$, $p22^{phox}$, and Rac) and of Hv1. PDI was used as loading control and for normalization. Even rough inspection of Figure 1A reveals that the labeling of NADPH oxidase subunits correlates well with PDI labeling, whereas the intensity of the Hv1 band seems to be dependent on the cell type, being much fainter in neutrophils. This pattern suggests no major cell type specific difference in NADPH oxidase expression while indicating cell type dependence for the Hv1 content. Densitometric analysis of the Western blots (Figure 1B) supports this notion, as the PDI-normalized Eo/Ne ratios were ~1 for all phox subunits: 1 ± 0.2 for Nox2, 1.6 ± 0.2 for $p67^{phox}$, 1.4 ± 0.2 for $p47^{phox}$, 1 ± 0.2 for $p22^{phox}$, and 0.7 ± 0.1 for Rac ($n = 8$, $p < 0.05$ for $p67^{phox}$), while Hv1 signal was much higher in eosinophils (Eo/Ne ratio 10.6 ± 2.5, $n = 8$, $p < 0.01$). Importantly, the average PDI signal was not different in the two granulocyte subsets (Eo/Ne ratio of 1 ± 0.2, $n = 8$). As we always attempted to load 10^6 cells/lane, the densitometry results can be interpreted on a per cell basis as well.

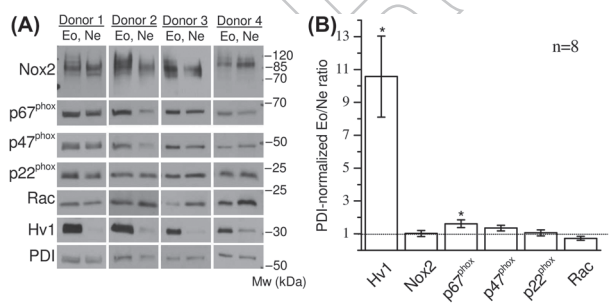


Figure 1. Comparison of the expression of different RB supporting proteins between eosinophil and neutrophil granulocytes. (A) Western blot detection of the essential phagocyte oxidase (phox) components and Hv1. Total cell lysates of 10^6 cells were loaded each lane. PDI was used as loading control. Granulocytes derived from the same donor were loaded next to each other to facilitate visual comparison. (B) Densitometric analysis of the Western blots. Each protein band was normalized to the corresponding PDI band. The normalized values belonging to the same donor and protein were used to calculate eosinophil/neutrophil ratio (Eo/Ne). An Eo/Ne value of 1 (dotted line) would indicate identical protein content (*$p < 0.05$).

Hv1 expression correlates with Nox2 expression in eosinophils

The much higher Hv1 content of eosinophils raises the possibility that their RB depend more on the Hv1 activity than the RB of neutrophils. Furthermore, electrophysiological measurements indicated that the intensity of phox-mediated electron currents was correlated with voltage-gated proton conductance in eosinophils [27] but not in neutrophils [28], also implying a stricter functional coupling between the phox and Hv1 in eosinophils. Supposing that the above conclusions are valid, one would expect that individuals whose eosinophils contain more phox will have higher Hv1 levels as well. To test this hypothesis, we performed linear regression analysis between the levels of Hv1 and Nox2, the core component of the phox, in the two granulocyte types. To establish a spectrum of expected correlation levels, we also performed correlation analyses between essential phox components. Based on literature data, good correlation should be present between $p22^{phox}$ and Nox2 levels, as the stability of each of these proteins in granulocytes depends on the presence of the other one [29]. In granulocytes a correlation between $p47^{phox}$ and $p67^{phox}$ expression would not be surprising, as these are phox specific proteins, which build a complex with each other even in resting cells [29]. In contrast, the small GTPase Rac has diverse cellular functions besides helping phox activity [30], thus a lack of association of its levels with that of other phox components can be anticipated. Linear regression analyses could be carried out in each of these cases, as substantial interpersonal variability (3 to 10-fold difference) in the PDI-normalized expression levels of the phox components was detected with densitometry (Figure 2). As shown in Figure 2, the levels of Nox2 were correlated with $p22^{phox}$, and a similar association was present between $p67^{phox}$ and $p47^{phox}$ levels. Rac content, however, did not correlate with $p67^{phox}$ expression, although their functional and molecular interaction in the active phox had been suggested [31]. All the above correlations were qualitatively the same for both granulocyte types, while an association between Hv1 and Nox2 levels was detected only in eosinophils.

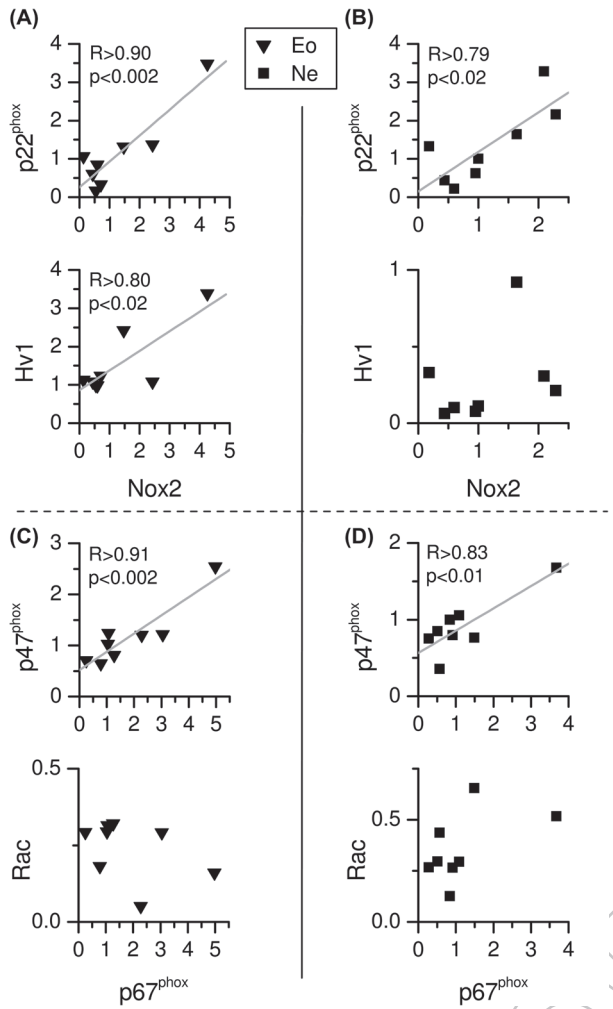


Figure 2. Correlation between the levels of different RB-supporting proteins in eosinophil and neutrophil granulocytes. PDI-normalized expression levels of selected proteins (see text for details) from the eight donors are plotted against each other in granulocyte type specific fashion. Gray lines are results of linear regressions. Only significant correlations are displayed. Different groups of comparisons (A–D) are demarcated by black lines between the scatter plots. As each group contained two comparisons, to accept significance, p was reduced to < 0.0253 for each linear regression analysis.

Hv1 and Nox2 accumulate together at the cell periphery in resting eosinophils

In an earlier study we have shown that the extent of subcellular co-distribution of Hv1 and Nox2 is pronounced in eosinophils [21]. This observation implies that in eosinophils Hv1 has a tendency to accumulate at the cell surface and in small granules as Nox2 does in this cell type [32]. This notion, however, had never been directly addressed. As demonstrated in Figure 3 and in the pseudocolor, 3D reconstruction in the Supplementary movie 1 to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234, Hv1 and Nox2 mainly reside in the same high density foci in eosinophils, most of which localize to the periphery of these cells. On the other hand, in neutrophils the two proteins distribute more evenly throughout the cell, displaying a granular pattern (Figure 3 and

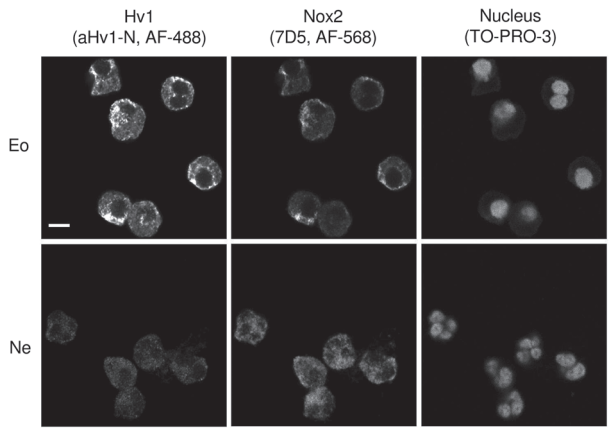


Figure 3. Subcellular distribution of Hv1 and Nox2 in eosinophil and neutrophil granulocytes. Images of resting eosinophil and neutrophil granulocytes, as obtained with confocal laser scanning microscopy. Half micrometer thick slices were taken at the middle of the vertical diameter (i.e., along the Z-axis) of the cells. Cells were labeled for Hv1, Nox2, and dsDNA with aHv1-N, 7D5, and TO-PRO-3, respectively. Note the clustering of Hv1 and Nox2 signals mainly at the perimeter of the eosinophils! Scale bar represents 5 μm and applies to both cell types. AF stands for Alexa Fluor. Pseudocolor, 3D reconstructions of these cells are available as supplemental movies.

Supplementary movie 2 to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234). Furthermore, eosinophils label on average ~3 times stronger for Hv1, while Nox2 labeling is ~1.4 times more pronounced in neutrophils. These results quantitatively differ from those observed with WB but can be in part explained by the co-clustering nature of Hv1 and Nox2 signals in the eosinophils, that may give rise to saturation of the detection system and homo- or heteroquenching of the fluorophores at sites of intense labeling.

The apparent maximal intensity of ROS production is stimulus and detection method dependent

Our results thus far indicate that although the expression level of Nox2 is very similar in the two granulocyte types, its subcellular distribution pattern differs markedly. Furthermore, earlier studies indicate that eosinophils, unlike neutrophils, release ROS mainly to the extracellular space [10,13]. Accordingly, paramagnetically purified eosinophils of healthy individuals released ~3 times more superoxide (as measured with Cyt c) and displayed ~3 times larger plasmalemmal electron currents (i.e., phox activity) upon PMA stimulation than neutrophils [33]. Compiling these data, one would expect that the apparent RB intensity of eosinophils will surpass that of neutrophils even in healthy individuals as long as PMA-induced extracellular superoxide release is measured. Importantly, PMA is a receptor-independent stimulus that induces robust phox activation throughout the cell by activating protein kinase C [34]. On the other hand, this difference should vanish, or even turn over, if intracellular ROS was to be detected, e.g., during phagocytosis-related RB. In a careful study Shult et al. compared RB intensity of the two granulocyte types in six healthy individuals [15]. The data

indicated no difference if phagocytosis-related RB was induced by serum-treated zymosan (STZ). On the contrary, if RB was induced with PMA eosinophils released two times more ROS, as measured with Cyt c. Surprisingly, however, this difference was also present if the ROS-induced chemiluminescence of the membrane permeable luminol was followed. As luminol-enhanced chemiluminescence (LCL) is a peroxidase dependent phenomenon [24], this latter observation could reflect a [AQ10] difference in the capacity of intarcellular peroxidases (myeloperoxidase vs. eosinophil peroxidase) in these cells to support LCL (but see Supplementary Figure 2 to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234). On the other hand, Shult et al. used hypotonic gradient centrifugation to separate eosinophils from neutrophils, which could have affected the two cell types differently. Therefore, we were interested whether these findings are reproducible with our paramagnetically separated cells. In our experiments we used Diogenes reagent to detect extracellular release of ROS (predominantly superoxide, Supplementary Figure 1A to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234), and LCL was used to follow ROS formation predominantly intracellularly [24] (Supplementary Figure 1 and 2 to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234). PMA treatment induced only modest LCL signal in eosinophil granulocytes, as compared to that in neutrophils under these conditions (maximum rate Ne/Eo of 2.2 ± 0.4, $n = 5$, $p < 0.02$, Figure 4A). In marked contrast, PMA-induced maximal ROS release rate was 1.7 ± 0.1 times greater in eosinophils ($n = 5$, $p < 0.01$), as measured with the Diogenes reagent (Figure 4C). STZ-activated neutrophil granulocytes produced on average three times higher maximal LCL signal than their eosinophil counterparts (maximum rate Eo/Ne of 0.3 ± 0.2, $n = 5$, $p < 0.01$, Figure 4B) indicating massive intracellular ROS generation in neutrophils upon STZ-stimulation. On the other hand, if STZ-induced RB was followed with Diogenes, no difference in the maximal ROS release rate was found (Eo/Ne of 0.7 ± 0.2, $n = 3$, Figure 4D). The above results clearly indicate that different ROS detection methods can produce contradictory results if ROS producing capacity of different cell types is compared.

Oxygen consumption of the two granulocyte types during RB is not considerably different

One possible approach to more reliably follow ROS production in granulocytes is to measure their oxygen consumption. During the RB NADPH oxidase is responsible for the vast majority of oxygen consumed, since the mitochondrial respiratory chain is practically inactive in granulocytes [35–38]. As limited number of eosinophils can be obtained from one donor, we performed the

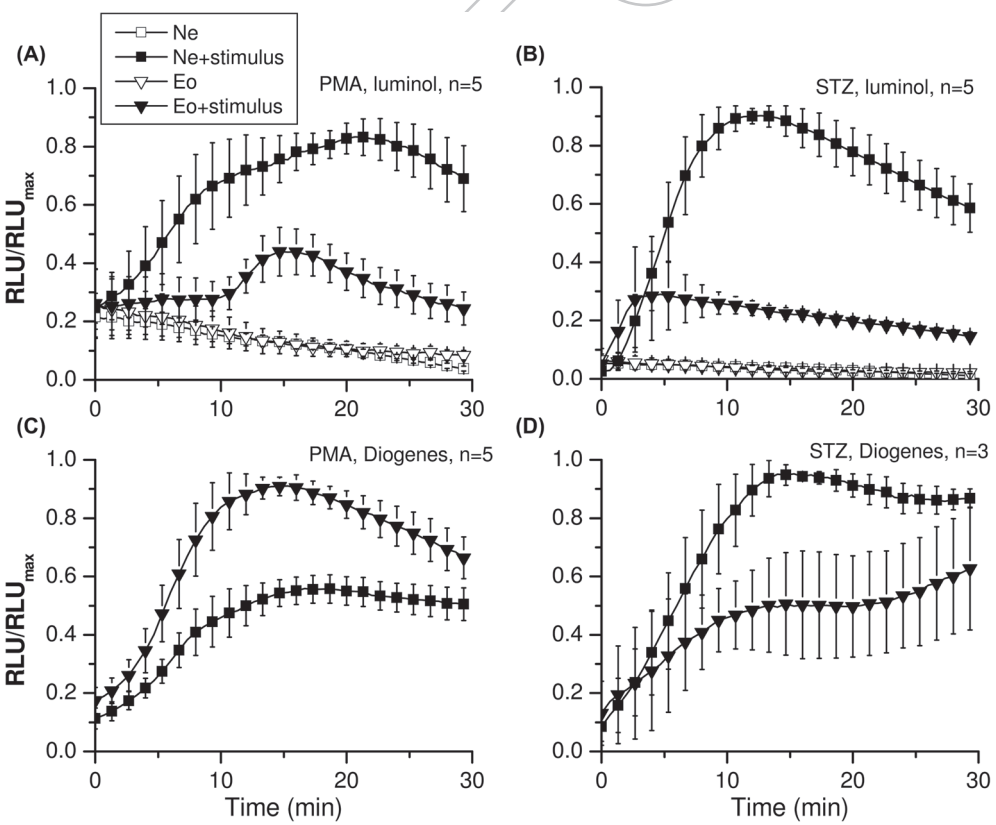


Figure 4. ROS production of neutrophil and eosinophil granulocytes. RB was induced by the addition of phorbol ester (PMA, 0.6 μM) (A), (C) or opsonized zymosan (STZ, 0.4 mg/ml) (B), (D) at 0 min: Extracellular ROS was detected with Diogenes reagent (D and C), while predominantly intracellular ROS formation was followed using luminol chemiluminescence (LCL, A, and B). A separate maximal relative luminescence unit value (RLU_{max}) was calculated for each donor for each stimulus versus detection method combination. In case of LCL the biggest signals were measured with neutrophils and STZ as stimulus, while in the Diogenes experiments PMA-stimulated eosinophils produced the maximal RLU values.

oxygen consumption measurements with the Seahorse XF analyzer system, a novel, fluorometry-based oxygen tension and pH measurement application. To evaluate the intensity of mitochondrial and NADPH oxidase-dependent O_2 consumption, we applied 1 μM antimycin A (respiratory chain inhibitor [39]) or low concentration (1 μM) of DPI (NADPH oxidase blocker [40]), respectively. Granulocytes were activated with either PMA or STZ. As expected for these granulocytes [38], DPI abolished the RB in both cell types, whereas antimycin A exerted no inhibition on the O_2 consumption (Supplementary Figure 3 to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234). The peak of O_2 consumption was on average ~1.4 times higher in PMA-treated eosinophils (4.5 ± 0.7 nmol/min for 10^6 cells) than in neutrophils (3.1 ± 0.3 nmol/min for 10^6 cells, $n = 6$, $p < 0.02$, Figure 5A), indicating that eosinophils possess somewhat bigger ROS producing capacity. STZ-induced RB showed slower activation kinetics in eosinophils (Figure 5B), but the peak value of oxygen consumption did not differ significantly between the two cell types (1.9 ± 0.4 vs. 2.2 ± 0.6 nmol/min for 10^6 Eo vs. Ne, $n = 3$). Although our experimental conditions allowed only minimal extracellular pH change, the average extracellular acidification rate seemed ~1.6-fold higher in eosinophils, but the difference was not significant (Figure 5C).

Discussion and conclusions

Since its first description in 1932 [41] the different aspects of the RB have always been in the forefront of phagocyte research. The most studied model of the RB is human neutrophil granulocyte, as it is relatively easy to obtain in sufficient amounts from donors. Later on the components of the phagocyte NADPH oxidase system were identified and pinpointed as specific proteins needed for mounting intense and regulated ROS formation during the RB [4]. The classical view that the sole aim of phox assembly and activation is the production of toxic ROS to kill pathogens had been recurrently challenged. Today it is accepted that the activity of the phox is also important to promote several other processes needed for efficient pathogen clearing including phagosomal pH regulation, modification of signal transduction pathways [2] or formation of "extracellular traps" [42]. Importantly, most of the above notions are well-established for the best studied neutrophils only and much less so for other phagocytes. It is clear, however, that quantitative and qualitative differences in phox activation are present depending on the phagocyte type or stimulus investigated. During the quest for identifying the nature and aim of phox activation in granulocytes, the RB and sometimes the expression of phox components were compared between neutrophils and eosinophils [9–13,15–17]. In spite of the large amount of data produced during decades of research, controversies exist to what extent are eosinophils capable of mounting a more intense RB than neutrophils (Eo/Ne range from ~1 [10] to ~ 4.7 [12]), and whether differences in the expression of phox components (Eo/Ne range from ~1 [12] to ~3 [11] for cytochrome b558) can account for it. In our view the causes for the controversy are as follows. Comparisons performed earlier on granulocytes mostly had to use different individuals as eosinophil and neutrophil donors. Due to this and to the often very limited number of donors, distortion of data caused by interpersonal differences could not be excluded. Furthermore, eosinophils used in previous studies were often not quiescent, as they stemmed from persons with infectious or proliferative hypereosinophilia [9–12], or because eosinophils were isolated by methods partially activating them [13]. Finally, in most comparisons Cyt c reduction method was used to assess RB intensity (Eo/Ne range from ~2 [14] to ~4.7 [12]), which only measures extracellular superoxide release [23] leaving phox activity in intracellular membranes undetected. Importantly, in the studies in that comparisons were made in normal cells and/or with methods that measure RB more accurately, smaller differences were found between the two cell types (Eo/Ne range from ~1 [10] to ~2.3 [13]).

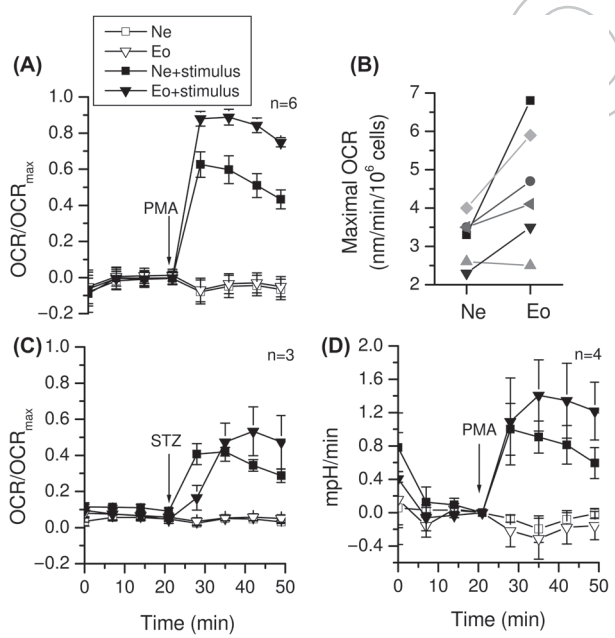


Figure 5. Oxygen consumption and extracellular pH change during the RB of eosinophil and neutrophil granulocytes. Oxygen consumption rate (OCR) was followed during (A) PMA- (0.6 μM) or (C) STZ-induced (0.4 mg/ml) RB. The maximal oxygen consumption rate of PMA-stimulated eosinophils (for both A and C) was defined for each donor and used for all recordings on the given donor as OCR_{max}. (B) Maximal PMA-induced OCR of eosinophils and neutrophils of the donors in (A). (D) pH changes in PMA-activated granulocytes.

This study compares the expression of proteins closely related to ROS production and the intensity of RB in human eosinophil and neutrophil granulocytes derived from healthy individuals. Additionally, we always attempted to minimize the preactivation of granulocytes in our experiments. Granulocytes were used for experiments immediately after isolation. Eosinophils were negatively selected during the separation procedure, while neutrophils were positively labeled with anti-CD16 paramagnetic antibodies, an approach that preserves neutrophils in very good condition [19]. Furthermore, to exclude

the possibility that the labeling had a major influence on neutrophil function, in pilot studies we had compared the RB of neutrophils and unlabeled PMNs (>90% neutrophils) and found no difference (data not shown).

For the assembly of a fully functional phox five subunits are essential, namely Nox2, $p22^{phox}$, $p67^{phox}$, $p47^{phox}$, and Rac [4]. In our experiments the expression of all of these essential subunits was investigated with Western blot and densitometry. The results indicate that the amount of each of these subunits is nearly the same in eosinophils and neutrophils (Figure 1). In contrast, most of the previous studies found that oxidase subunit expression tend to be higher in eosinophils. The cause of this discrepancy most probably lies within the isolation problems mentioned above. As the most important addition to all previous studies we have also compared the amount of the more recently identified voltage-gated proton channel protein [39] in the two granulocyte types. Hv1 proton channel is now considered as a supplementary subunit of the phox, the absence of which can cause a 30–75% reduction in the intensity of the RB in phagocytes of the mouse [2]. In striking contrast with the essential phox subunits, the expression of Hv1 was found to be ~3 (IF) to ~10 (WB) times greater in eosinophils, than in neutrophils. This latter finding is in remarkable accordance with patch-clamp data, as the density of proton current is also ~10 times higher in eosinophils [43,44]. Theoretically proteolysis could have altered the level of Hv1 in our Western blot experiments in a cell type dependent fashion, as Hv1 is reportedly very protease-sensitive [17], and proteolysis is always a special concern when granulocytes, especially neutrophils, are under investigation [45]. To minimize this problem, DFP treatment was routinely applied before cell lysis. DFP is a highly lipid soluble agent that can reach and block granular serine proteases already before the lysis of neutrophils [45]. As we have shown earlier DFP pretreatment is a very effective intervention that dramatically improves the detection of Hv1 in granulocytes, especially in neutrophils, in Western blot and immunofluorescence experiments [21].

Having established that no major difference is present in the expression levels of the essential oxidase components, we were interested whether there was a major difference in the intensity of the RB between the two granulocyte types from healthy individuals. Unfortunately, the experiments with chemiluminescent agents to follow RB yielded contradictory results. If we used Diogenes to follow extracellular superoxide release, the results (Figure 4C,D) were qualitatively in line with all previous studies performed with Cyt c. The differences measured with Diogenes were, however, smaller than in earlier reports with Cyt c (Eo/Ne ratio 1.7 vs. 2–4.7). Two explanations can be given for this quantitative difference: 1) the ROS sensitivity of Diogenes is higher than that of Cyt c, 2) Diogenes was not completely specific for superoxide under our experimental conditions (see Supplementary Figure 1 to be found at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.938234 for details). In striking contrast to extracellular ROS measurements, when we attempted to follow intracellular ROS formation with LCL, we could not reproduce the data obtained by Shult et al. in granulocytes from healthy individuals [15]. In stark contrast to their results, neutrophils produced greater maximal LCL signal than eosinophils in our experiments (Figure 4A,B). Taken together, the observed RB intensity is greatly influenced by the luminescent detection method and possibly by the applied eosinophil purification techniques. Nevertheless, our data reinforce previous findings that eosinophils, unlike neutrophils, produce ROS primarily at the cell surface [13,10]. To approach more accurately the real difference in RB potency between the two cell types, we decided to perform oxygen consumption measurements using the Seahorse XF technology. The advantage of oxygen consumption measurements over ROS detection techniques is that net ROS production will cause net oxygen consumption irrespective of its site. In these experiments PMA induced a somewhat fiercer RB in eosinophils (Figure 5A), while upon STZ stimulation the intensity of oxygen consumption was very similar, albeit with slower activation kinetics in eosinophils (Figure 5B). These results are in good agreement with previous data obtained in polarometric oxygen consumption experiments [16], although the differences detected by us are somewhat smaller.

Taken together, our data indicate that eosinophil and neutrophil granulocytes express very similar amounts of NADPH oxidase subunits and have similar ROS producing potency, whereas eosinophils contain Hv1 in about one order of magnitude greater amounts. So why is this huge difference in Hv1 expression if other fundamental quantitative aspects of the phox system are so similar? As no tool is available yet to specifically modify Hv1 activity in human granulocytes, the answer to this question can only be speculative currently. Nevertheless, in our view the differences in the localization of phox activity between the two cell types may provide a clue. Based on previous studies [10,13] and on our measurements, eosinophils release ROS mainly to the extracellular space, while neutrophils are prone to produce more intracellular ROS. In line with these observations eosinophils tend to co-distribute Nox2 and Hv1 in "hot spots" at the cell's periphery already in the resting state (Figure 3). Indicating that such hot spot like distribution of these proteins is present at the plasmalemma of activated cells as well, disproportionately large electron and proton currents could be measured in small excised patches from PMA-stimulated eosinophils [27], as compared to the whole-cell currents of these cells [8]. Compiling available data, we assume that intense extracellular but not intracellular superoxide release is dependent on very high level Hv1 expression. Indeed, results of a recent study imply that although the suppression of plasmalemmal Hv1 activity reduces extracellular superoxide generation, it also results in augmented intracellular ROS release in neutrophil granulocytes [46]. Furthermore, unlike in eosinophils, the proton channel expression is not correlated with that of the electron transporting Nox2 in neutrophils (Figure 2). Charge compensation, thus limiting depolarization, may much more

be the bottleneck for phox activity at the plasma membrane than in intracellular vesicles, where the transmembrane pH difference [47] or the accumulation of ROS can rather pose a hindrance. Hv1 is an unbeatable and virtually inexhaustible charge compensatory route for massive phox activity at strongly depolarized membrane potentials, like those observed in activated granulocytes at the plasma membrane [48]. Unfortunately, changes in the phagosomal membrane potential during granulocyte phagocytosis have not yet been defined, but robust phagosomal NADPH oxidase activity is likely sustainable in the presence of fewer Hv1 molecule, as probably other ion channels and proton transporters [2] also provide intense support there. Furthermore, very high proton channel density would probably preclude the complex phagosomal pH and ion composition changes of neutrophils [2].

In conclusion, Hv1 can be an important pharmacological target to limit the extracellular superoxide release of granulocytes without severely impairing the RB around engulfed pathogens.

Acknowledgements

The excellent technical assistance of Ms Beáta Molnár, Mrs Eszter Halász and Mrs Anikó Rajki is highly appreciated. We sincerely thank Dr. Katalin Német (Budapest) for the anti-67^{phox} antibody and for the 7D5 supernatant.

[AQ4]

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

This work was supported by grants from the Hungarian Research Fund (OTKA K106138 and F-68023) and from the National Office for Research and Technology (OMFB-01680/2009) and by "Lendület" grant from the Hungarian Academy of Sciences. The authors declare that no competing interests exist.

[AQ11]

References

[1] Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. Arch Biochem Biophys 2002;397:342–344.

[2] El Chemaly A, Demaurex N. Do Hv1 proton channels regulate the ionic and redox homeostasis of phagosomes? Mol Cell Endocrinol 2012;353:82–87.

[3] Tian W, Li XJ, Stull ND, Ming W, Suh CI, Bissonnette SA, et al. Fc gamma R-stimulated activation of the NADPH oxidase: phosphoinositide-binding protein p40phox regulates NADPH oxidase activity after enzyme assembly on the phagosome. Blood 2008;112:3867–3877.

[4] Malech HL, Hickstein DD. Genetics, biology and clinical management of myeloid cell primary immune deficiencies: chronic granulomatous disease and leukocyte adhesion deficiency. Curr Opin Hematol 2007;14:29–36.

[5] DeCoursey TE. Voltage-gated proton channels find their dream job managing the respiratory burst in phagocytes. Physiology (Bethesda) 2010;25:27–40.

[6] Demaurex N, Petheo GL. Electron and proton transport by NADPH oxidases. Philos Trans R Soc Lond B Biol Sci 2005;360:2315–2325.

[7] Okamura Y. Biodiversity of voltage sensor domain proteins. Pflugers Arch 2007;454:361–371.

[8] DeCoursey TE. Voltage-gated proton channels and other proton transfer pathways. Physiol Rev 2003;83:475–579.

[9] DeChatelet LR, Shirley PS, McPhail LC, Huntley CC, Muss HB, Bass DA. Oxidative metabolism of the human eosinophil. Blood 1977;50:525–535.

[10] Lacy P, Abdel-Latif D, Steward M, Musat-Marcu S, Man SF, Moqbel R. Divergence of mechanisms regulating respiratory burst in blood and sputum eosinophils and neutrophils from atopic subjects. J Immunol 2003;170:2670–2679.

[11] Segal W, Garcia R, Goldstone H, Cross AR, Jones OT. Cytochrome b-245 of neutrophils is also present in human monocytes, macrophages and eosinophils. Biochem J 1981;196:363–367.

[12] Yagisawa M, Yuo A, Yonemaru M, Imajoh-Ohmi S, Kanegasaki S, Yazaki Y, Takaku F. Superoxide release and NADPH oxidase components in mature human phagocytes: correlation between functional capacity and amount of functional proteins. Biochem Biophys Res Commun 1996;228:510–516.

[13] Bolscher BG, Koenderman L, Tool AT, Stokman PM, Roos D. NADPH:O2 oxidoreductase of human eosinophils in the cell-free system. FEBS Lett 1990;268:269–273.

[14] Petreccia DC, Nauseef WM, Clark RA. Respiratory burst of normal human eosinophils. J Leukoc Biol 1987;41:283–288.

[15] Shult PA, Graziano FM, Wallow IH, Busse WW. Comparison of superoxide generation and luminol-dependent chemiluminescence with eosinophils and neutrophils from normal individuals. J Lab ClinMed 1985;106:638–645.

[16] Yazdanbakhsh M, Eckmann CM, De Boer M, Roos D. Purification of eosinophils from normal human blood, preparation of eosinoplasts and characterization of their functional response to various stimuli. Immunology 1987;60:123–129.

[17] Ramsey S, Moran MM, Chong JA, Clapham DE. A voltage-gated proton-selective channel lacking the pore domain. Nature 2006;440:1213–1216.

[18] Freitas M, Lima JL, Fernandes E. Optical probes for detection and quantification of neutrophils' oxidative burst. A review. Anal Chim Acta 2009;649:8–23.

[19] Sedgwick B, Shikama Y, Nagata M, Brener K, Busse WW. Effect of isolation protocol on eosinophil function: Percoll gradients versus immunomagnetic beads. J Immunol Methods 1996;198:15–24.

[20] Ferrick DA, Neilson A, Beeson C. Advances in measuring cellular bioenergetics using extracellular flux. Drug Discov Today 2008;13:268–274.

[21] Petheo GL, Orient A, Barath M, Kovacs I, Rethi B, Lanyi A, et al. Molecular and functional characterization of Hv1 proton channel in human granulocytes. PLoS One 2010;5:e14081.

[22] Wientjes FB, Hsuan JJ, Totty NF, Segal AW. p40phox, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. Biochem J 1993;296:557–561.

[23] Nakamura M, Murakami M, Koga T, Tanaka Y, Minakami S. Monoclonal antibody 7D5 raised to cytochrome b558 of human neutrophils: immunocytochemical detection of the antigen in peripheral phagocytes of normal subjects, patients with chronic granulomatous disease, and their carrier mothers. Blood 1987;69:1404–1408.

[24] Dahlgren C, Karlsson A. Respiratory burst in human neutrophils. J Immunol Methods 1999;232:3–14.

[25] Raible DG, Mohanty JG, Jaffe JS, Stella HJ, Sprenkle BE, Glaum MC, Schulman ES. Hydrogen peroxide release from human eosinophils on fibronectin: scopoletin enhances eosinophil activation. Free Radic Biol Med 2000;28:1652–1660.

[26] Morgan D, Cherny VV, Murphy R, Katz BZ, DeCoursey TE. The pH dependence of NADPH oxidase in human eosinophils. J Physiol 2005;569:419–431.

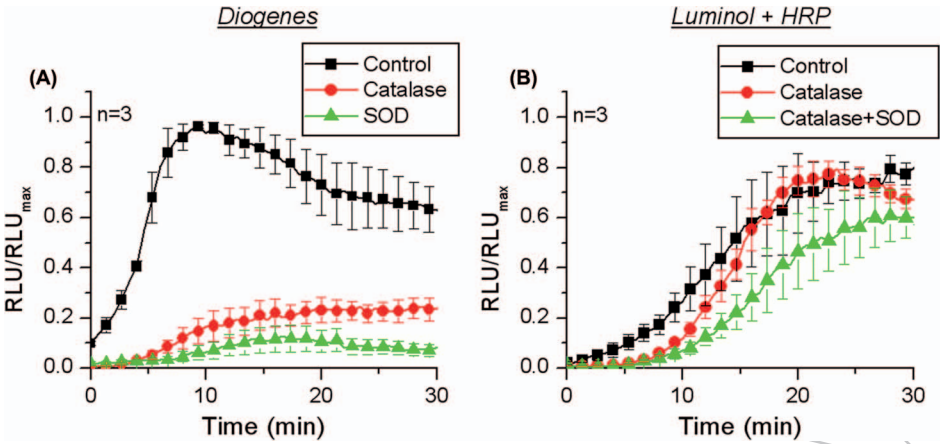
[27] Petheo GL, Maturana A, Spat A, Demaurex N. Interactions between electron and proton currents in excised patches from human eosinophils. J Gen Physiol 2003,122:713–726.
[28] DeCoursey TE, Cherny VV, Zhou W, Thomas LL. Simultaneous activation of NADPH oxidase-related proton and electron currents in human neutrophils. Proc Natl Acad Sci USA 2000;97:6885–6889.
[29] Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 2007;87:245–313.
[30] Dinauer MC. Regulation of neutrophil function by Rac GTPases. Curr Opin Hematol 2003;10:8–15.
[31] Bokoch GM, Diebold BA. Current molecular models for NADPH oxidase regulation by Rac GTPase. Blood 2002; 100:2692–2696.
[32] Ginsel LA, Onderwater JJ, Fransen JA, Verhoeven AJ, Roos D. Localization of the low-Mr subunit of cytochrome b558 in human blood phagocytes by immunoelectron microscopy. Blood 1990;76:2105–2116.
[33] DeCoursey TE, Cherny VV, DeCoursey AG, Xu W, Thomas LL. Interactions between NADPH oxidase-related proton and electron currents in human eosinophils. J Physiol 2001;535:767–781.
[34] Robinson M, Badwey JA, Karnovsky ML, Karnovsky MJ. Release of superoxide and change in morphology by neutrophils in response to phorbol esters: antagonism by inhibitors of calcium-binding proteins. J Cell Biol 1985; 101:1052–1058.
[35] Bainton DF, Ullyot JL, Farquhar MG. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. J Exp Med 1971;134:907–934.
[36] Borregaard N, Herlin T. Energy metabolism of human neutrophils during phagocytosis. J Clin Invest 1982;70:550–557.
[37] Fossati G, Moulding DA, Spiller DG, Moots RJ, White MR, Edwards SW. The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis. J Immunol 2003;170:1964–1972.
[38] Peachman KK, Lyles DS, Bass DA. Mitochondria in eosinophils: functional role in apoptosis but not respiration. Proc Natl Acad Sci USA 2001;98:1717–1722.
[39] Slater EC. The mechanism of action of the respiratory inhibitor, antimycin. Biochim Biophys Acta 1973;301: 129–154.
[40] Doussiere J, Vignais PV. Diphenylene iodonium as an inhibitor of the NADPH oxidase complex of bovine neutrophils. Factors controlling the inhibitory potency of diphenylene iodonium in a cell-free system of oxidase activation. Eur J Biochem 1992;208:61–71.
[41] Baldridge CW, Gerard RW. The extra respiration of phagocytosis. Am J Physiol 1932;103:235–236.
[42] Goldmann O, Medina E. The expanding world of extracellular traps: not only neutrophils but much more. Front Immunol 2012;3:420.
[43] DeCoursey TE, Cherny VV. Potential, pH, and arachidonate gate hydrogen ion currents in human neutrophils. Biophys J 1993;65:1590–1598.
[44] Gordienko DV, Tare M, Parveen S, Fenech CJ, Robinson C, Bolton TB. Voltage-activated proton current in eosinophils from human blood. J Physiol 1996;496:299–316.
[45] Amrein PC, Stossel TP. Prevention of degradation of human polymorphonuclear leukocyte proteins by diisopropylfluorophosphate. Blood 1980;56:442–447.
[46] Decleva E, Menegazzi R, Fasolo A, Defendi F, Sebastianutto M, Dri P. Intracellular shunting of O contributes to charge compensation and preservation of neutrophil respiratory burst in the absence of voltage-gated proton channel activity. Exp Cell Res 2013;319:1875–1888.
[47] Jankowski A, Grinstein S. Modulation of the cytosolic and phagosomal pH by the NADPH oxidase. Antioxid Redox Signal 2002;4:61–68.
[48] Jankowski A, Grinstein S. A noninvasive fluorimetric procedure for measurement of membrane potential. Quantification of the NADPH oxidase-induced depolarization in activated neutrophils. J Biol Chem 1999;274:26098–26104.

[AQ12]

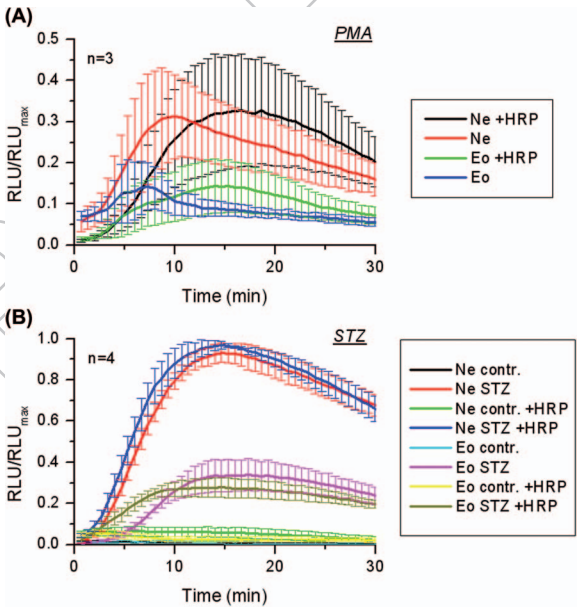
Supplementary material available online

Supplementary Figures 1–3 and Movie 1 and 2.

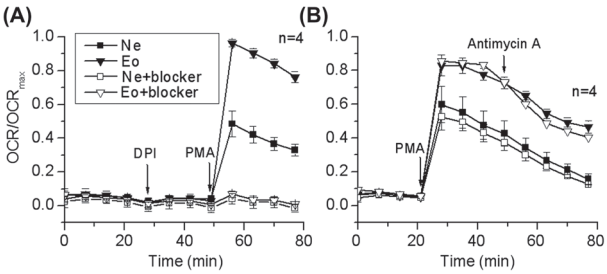
Supplementary material for Kovács I, et al. Comparison of proton channel, phagocyte oxidase, and respiratory burst levels between human eosinophil and neutrophil granulocytes. *Free Radical Research,* 2014*;* DOI: 10.3109/10715762.2014.938234.



Supplementary Figure 1. The effects of extracellularly added superoxide dismutase and catalase on the Diogenes- or luminol-detected ROS production of granulocytes. Polymorphonuclear cells (>90% neutrophil) were stimulated with 0.6 μM PMA at 0 min in the presence or absence of superoxide dismutase (SOD, 12.5 μg/ml) and/or catalase (100 μg/ml). Presence of each of the antioxidant enzymes was capable to substantially reduce the Diogenes signal (A), while an impact on luminol (B) chemiluminescence (LCL) was either absent (catalase alone) or moderate (catalase with SOD). These latter observations support the notion that LCL mainly detects intracellular ROS in granulocytes [1]. The impact of SOD+catalase on LCL may indicate facilitated superoxide secretion at the expense of intracellular ROS release (i.e., more NADPH is consumed near the cell surface). Our results with Diogenes indicate that this compound mainly detects extracellular ROS with a preference for superoxide under the applied experimental conditions (see Methods for details of experimental conditions). The substantial impact of catalase on the Diogenes signal is somewhat surprising, as this reagent had been reported to be specific for superoxide in another experimental system [3]. One explanation that can resolve this apparent contradiction is that at high concentrations of superoxide—with H_2O_2 removed by catalase and with Hv1-extruded protons in the vicinity—the rate of spontaneous dismutation (http://www2.phys.rush.edu/TomD/SuperoxideDisproportionation.GIF) might become comparable to the detection speed of Diogenes.



Supplementary Figure 2. The presence of HRP affects the observed kinetics of LCL-detected respiratory burst but does not influence its maximal intensity in granulocytes. (A) Granulocytes were stimulated with 0.6 μM PMA in the presence or absence of HRP (1 U/ml). (B) Granulocytes were stimulated with or without STZ (0.4 mg/ml) in the presence or absence of HRP. All curves were normalized to the maximal LCL of STZ-stimulated neutrophils on a given plate. Note that although the presence of extracellularly added HRP influences the kinetics of the measured LCL signal, it has no effect on its maximal intensity. Specifically, HRP retards the LCL signal rate if PMA is the stimulus, while HRP accelerates this rate if zymosan is the stimulus. These virtually contradictory observations can be deciphered as follows. HRP will somewhat speed up LCL signal if it has direct access to high concentrations of H_2O_2. This is the case when phagocytosis is induced with zymosan, as it will direct the RB machinery and ROS release toward the zymosan and HRP containing extracellular compartment. On the other hand, in PMA stimulated cells an indirect effect of HRP will dominate, i.e., helping the extracellular removal of H_2O_2 thus facilitating extracellular superoxide release (similarly to extracellular catalase in Figure S1B). Taken together, endogen peroxidase content of the Eo and Ne (eosinophil peroxidase and myeloperoxidase, respectively) appears to be sufficient to support the LCL under PMA-stimulated conditions. The above observations support the notion that LCL mainly detects intracellular ROS in granulocytes [1].



Supplementary Figure 3. Oxygen consumption of activated granulocytes is dependent on NADPH oxidase but not on mitochondrial respiration. To measure oxygen consumption at 37°C, the Seahorse XF Analyzer system was used. To evaluate the intensity of NADPH oxidase-dependent and mitochondrial O_2 consumption, we applied (A) low concentration (1 µM) of DPI (NADPH oxidase blocker [2]) or (B) 1 µM antimycin A (respiratory chain inhibitor [4]), respectively. Granulocytes were stimulated with or without 0.6 µM PMA. The maximal oxygen consumption rate of PMA-stimulated eosinophils in (A) was defined for each of the four donors and used for all recordings on the given donor as OCR_{max}. Note that the longer incubation time in (A) before PMA addition exacerbates the OCR_{max} difference between Eo and Ne, as compared to that in (B). This may be an artificial consequence of the slight mechanical agitation introduced by the periodically lifting and sinking detector of the Seahorse XF Analyzer.

Movie 1

Pseudocolor, 3D reconstruction of the eosinophils in Figure 3. Green: Hv1, red: Nox2, blue: nucleus (see Methods for details on labeling). Bright, yellowish green "hot spots" denote co-clustering of Hv1 and Nox2 labeling.

Movie 2

Pseudocolor, 3D reconstruction of the neutrophils in Figure 3. Green: Hv1, red: Nox2, blue: nucleus (see Methods for the details on labeling).

References

[1] C. Dahlgren and A. Karlsson (1999) Respiratory burst in human neutrophils. J.Immunol.Methods, 232, 3–14.

[2] J. Doussiere and P. V. Vignais (1992) Diphenylene iodonium as an inhibitor of the NADPH oxidase complex of bovine neutrophils. Factors controlling the inhibitory potency of diphenylene iodonium in a cell-free system of oxidase activation. Eur.J.Biochem., 208, 61–71.

[3] P. S. Hole, L. Pearn, A. J. Tonks, P. E. James, A. K. Burnett, R. L. Darley and A. Tonks (2010) Ras-induced reactive oxygen species promote growth factor-independent proliferation in human CD34 + hematopoietic progenitor cells. Blood, 115, 1238–1246.

[4] E. C. Slater (1973) The mechanism of action of the respiratory inhibitor, antimycin. Biochim. Biophys.Acta, 301, 129–154.