The role of phospholipids, GTPase activating proteins and extracellular vesicles in superoxide production of neutrophilic granulocytes

Ph.D. thesis

Márton Ákos Lőrincz, M.D.

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor: Erzsébet Ligeti, M.D., D.Sc., member of the HAS

Reviewers: László Cervenák, Ph.D., senior research fellow
József Prechl, Ph.D., senior research fellow

Chairman of the comprehensive examination board:
András Falus, D.Sc., member of the HAS

Members of the comprehensive examination board:
Gabriella Sármay, D.Sc., doctor of the HAS
Sára Tóth, Ph.D., associate professor

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Introduction

Neutrophilic granulocytes are short living and abundant leukocytes in the peripheral blood. As a key player of the innate immunity they fight in the first line against pathogens. The activated neutrophil recognizes, engulfs and kills them with granule proteins and reactive oxygen species (ROS). ROS are produced in the phagosome by NADPH oxidase that transports electrons onto molecular oxygen. The produced superoxide anion and other derivates can harm pathogens and the own tissue as well. The inadequate spatiotemporal regulation of ROS production leads to epidemiologically significant diseases (e.g. autoimmune diseases, neurodegenerative diseases, cardiovascular diseases). In physiological circumstances the NADPH oxidase is under a sophisticated control that prevents excessive production of ROS.

The function of NADPH oxidase is controlled by several factors, inter alia lipid mediators and Rac interacting regulators. Among lipid mediators arachidonic acid (AA) increases, glucocerebrosides and oxidized phospholipids (oxPL) decrease the superoxide production. Direct effect on NADPH oxidase of AA and glucocerebrosides is already known, however there are only assumptions about the mechanism of oxPL effect.

GTPase activating proteins (GAP) are other negative regulators of superoxide production. A few GAPs were examined

previously in context with NADPH oxidase but no comprehensive study was made to test the effect of different GAPs under the same conditions or evaluate the effect of membrane bound GAPs.

Neutrophils kill pathogens not only intracellularly, but they also neutralize them extracellularly. Besides the formation of neutrophil extracellular trap (NET), they are able to secrete antibacterial extracellular vesicles (EV). NET formation and antibacterial effect of NET requires active NADPH oxidase, however there are no data about connection between NADPH oxidase and EVs.

The aim of my work was to investigate new aspects of regulation of phagocyte NADPH oxidase.

Objectives

The major questions of my Ph.D. work were the following:

1. Do oxidized phospholipids affect the assembly and activity of phagocyte NADPH oxidase?

2. Does ARHGAP25 regulate the superoxide production of NADPH oxidase in a cell free system?

3. Which membrane-bound GAPs can regulate the superoxide production of NADPH oxidase?

4. Do neutrophil derived EVs, especially antibacterial aEVs produce superoxide? Have ROS any importance in the antibacterial effect of aEVs?

Methods

Isolation of neutrophils. Venous blood was drawn from healthy adult volunteers according to procedures approved by the Institutional Review Board of the Semmelweis University. Polymorphonuclear cells (PMN) were obtained by dextran sedimentation followed by Ficoll-Paque gradient centrifugation.

Preparation of neutrophil subcellular fractions. Isolated PMN cells were suspended in protease inhibitor containing buffer thereafter cells were broken by ultrasonic treatment. Membrane and cytosolic fractions were prepared on a discontinuous sucrose gradient (17%/40%) by ultracentrifugation (160 000 *g*, 30 min). Cytosol fraction was collected from the top of gradient, membrane fraction from the border of the 17% and 40% sucrose layers.

Recombinant protein expressing bacteria. The GST fusion protein Rac1, Bcr and ARHGAP1 expressing *E. coli* species were generous gifts from Alan Hall. The GST fusion protein $p67^{phox}$ and $p47^{phox}$ expressing *E. coli* species were generous gifts from Frans Wientjes. The GST fusion protein ARHGAP25 expressing *E. coli* was made in our workgroup by Roland Csépányi-Kömi. The GST fusion protein ARHGAP35 expressing *E. coli* was made in our workgroup by Magdolna Lévay.

Immunoprecipitation experiments. Membranes from human PMN were solubilized for 20 min in PBS containing 40 mM octyl-β-D-glucopyranoside. Immunodepletion was performed in three successive steps, incubating the solubilized material each time for 15 mins with antibody-loaded Sepharose beads. For the separation of the beads Spin-X Centrifuge Tube Filter with 450nm pore was used. The immunodepleted probes were tested in Western blots. Densitometry was performed using „ImageJ" software (National Institutes of Health, USA).

Measurement of $O2^{\bullet-}$ production in semi-recombinant cell-free system. The rate of $O2^{\bullet-}$ production was determined as the superoxide-dismutase-sensitive portion of ferricytochrome c reduction measured at 550 nm in a Labsystem iEMS microplate reader. A two-step activation system was used. Membrane fraction and recombinant $p67^{phox}$ and $p47^{phox}$, were pre-incubated for 10 min in the presence of arachidonic acid (AA), and cytochrome c. Superoxide production was initiated by addition of NADPH and followed for 20 min. For calculation of the $O2^{\bullet-}$ production, the absorption coefficient of ferricytochrome c ($21000 M^{-1} \cdot cm^{-1}$) was amended to the properties of the plate reader.

Isolation of extracellular vesicles. PMNs (typically 10^7 cell/ml) were incubated with or without activating agent for 30 minutes at 37° C on a linear shaker. After incubation, PMNs were sedimented (500 *g*, 5 minutes, 4° C) and the supernatant was filtered through a 5 µm pore sterile filter. The filtered fraction was sedimented again (15 700 *g*, 10 minutes, 4° C). The sediment contained the EV fraction. EVs produced during spontaneous cell death were isolated similarly, except the cells were in DMEM medium for 1, 2 and 3 days at 37° C in the presence of 5% CO_2.

EV detection by flow cytometry. EVs were labelled with a monoclonal RPE conjugated Ab against CD11b. Flow cytometric measurements were performed on a FACSCalibur flow cytometer. In our EV gating strategy, first an EV-size gate was set with the help of calibrating GFP-expressing bacteria (*S. aureus*, diameter 0.8 µm) and 3.8 µm SPHERO rainbow alignment particles. The fluorescent gate was set above the signal of the control isotype antibody labelled EVs. To confirm the vesicular nature of detected events TritonX-100 detergent was used to solubilize vesicles. To avoid swarm detection the flow rate was held below 1 000 events/s.

Measurement of $O2^{\bullet-}$ production of EVs. The rate of $O2^{\bullet-}$ production was determined as the superoxide-dismutase-sensitive portion of ferricytochrome c reduction measured at 550 nm in a Labsystem

iEMS microplate reader. Intravesicular $O2^{\bullet -}$ production was determined with lucigenin-based chemiluminescence. Superoxide production was initiated by adding PMA or opsonized *S. aureus.*

Measurement of bacterial survival. EV samples in HBSS were incubated with opsonized *S. aureus* for 30 minutes at 37°C. Samples were taken at starting point and 30 min later. Samples were lysed in ice-cold HBSS containing saponin and frozen at -80°C for 20 minutes to inactivate EVs. Lysed samples were diluted in LB medium, then bacteria were grown in a shaking plate reader (Labsystems iEMS) at 37°C for 10 hours, and the OD was followed continuously at 650 nm.

Statistics. Statistical analysis was performed with STATISTICA 7.0 software (Statsoft Inc. Tulsa, OK, USA) with two-sample *t*-test. Results were considered significant at a (two sided) p value less than or equal to 0.05.

Results

Effect of phospholipids on NADPH oxidase in a cell free system

We demonstrated that oxidized phosphatidylcholine inhibits the assembly of NADPH oxidase subunits in cell-free system without affecting the enzymatic activity. This result gives molecular explanation for the previously observed effect of oxidized phospholipids in a whole-cell system. It also supports the negative feedback effect of oxidized phospholipids on ROS production.

Effect of recombinant GAPs on NADPH oxidase in a cell free system

It was also demonstrated in subsequent experiments that the recombinant forms of some Rac GTPase activating proteins (GAP), such as ARHGAP1, ARHGAP25, ARHGAP35 and Bcr are equally effective in accessing the membrane-bound Rac and in inhibiting the assembly of NADPH oxidase. In contrast, these soluble GAPs are not able to regulate superoxide production of the already assembled enzyme complex.

Effect of membrane-bound GAPs on NADPH oxidase in a cell free system

In accordance with previous data from our workgroup, it was demonstrated that NaF increases the superoxide production if added before or after the assembly of the NADPH oxidase enzyme complex. As NaF is an efficient inhibitor of GAPs, the effect of

fluoride on the assembled oxidase means that membrane-bound GAPs are indeed able to regulate the enzyme complex.

Immunodepletion of GAPs

In order to evaluate the effect of membrane-bound GAPs on NADPH oxidase we depleted each GAP in the membrane fraction. On the average we succeeded in depleting 70%-65% of ARHGAP1, ARHGAP25 and ARHGAP35 from the solubilized membranes. Bcr depletions were not successful.

NADPH oxidase activity in immunodepleted membranes

Depletion of ARHGAP1 or ARHGAP25 alone resulted in a greater than two-fold increase in the rate of $O2^{\bullet-}$ generation. Moreover, the effect of the two RacGAPs was additive: in double-depleted membranes $O2^{\bullet-}$ production was more than 3 times higher than in the isotype-treated membranes. In contrast, depletion of ARHGAP35 from the membranes did not affect $O2^{\bullet-}$ production at all. Apparently, some but not all of the membrane-localized RacGAPs do interact with Rac in the Nox2 complex and constitutively diminish $O2^{\bullet-}$ production. Based on structural studies we propose that the interaction of GAP with the Rac effector protein $p67^{phox}$ underlies the discrimination between different GAPs.

Effect of storage on physical and functional properties of extracellular vesicles derived from neutrophilic granulocytes

A systematic study was carried out on different storage conditions for EVs to define an appropriate storing method. PMN-derived EVs could not be stored at room temperature or at +4° C without loss of vesicle number and function. Storage at -20° C induces structural changes may be due to swelling of vesicles. Parallel to structural changes the antibacterial capacity of vesicles was decreased. Storage at -80° C for 1 month resulted in a slight decrease of the antibacterial function. Snap freezing/ thawing or presence of albumin during storage did not improve the results. Well-known cryoprotectants destroyed the vesicular structure.

Characterization of spontaneous death derived EVs

Neutrophils are short living cells. They produce huge amount of EVs during cell death. These EVs contain less proteins per vesicles than activation induced in a short period secreted EVs. This was confirmed by electron microscopy. Cell death induced vesicles were empty compared to the electron dens appearance of activation induced EVs.

Superoxide production of neutrophil derived EVs

Neither spontaneous cell death-induced nor spontaneously formed or phagocytosis-induced EVs produced superoxide after PMA

activation. Intravesicular superoxide production was also not detected by chemoluminescent method.

ROS do not play a role in the antibacterial effect of EVs

In bacterial survival test, activation-induced EVs decreased the bacteria number to 61% during a 30 minutes incubation period. Spontaneously formed or spontaneous cell death-induced EV had no antibacterial effect, indeed *S. aureus* could grow on these samples. The antibacterial effect of activation induced EVs weas tested in the presence of DPI. The non-specific inhibitor of NADPH oxidase did not change the antibacterial capacity of EVs. Therefore enzymatic function of NADPH oxidase does not have a role in the antibacterial effect of activation induced EVs.

Conclusions

According to our aims, we sum up the following conclusions:

1. OxPAPC inhibits the assembly of NADPH oxidase in a cell free system. The non-oxidized form of PAPC has no effect on it.

2. Soluble forms of GAPs, such as ARHGAP1, ARHGAP25, ARHGAP35 and Bcr are equally effective in accessing the membrane-bound Rac and in inhibiting the assembly of NADPH oxidase. In contrast, these soluble GAPs are not able to regulate superoxide production of the already assembled enzyme complex.

3. Membrane-bound ARHGAP1 and ARHGAP25 inhibit the superoxide production of assembled NADPH oxidase, however ARHGAP35 does not.

4. Neutrophilic granulocytes derived EVs are not able to produce superoxide. Therefore enzymatic function of NADPH oxidase does not have a role in the antibacterial effect of activation induced EVs.

List of publications

The PhD thesis is based on the following publications (in a chronological order):

Bluml S, Rosc B, Lorincz A, Seyerl M, Kirchberger S, Oskolkova O, Bochkov VN, Majdic O, Ligeti E, Stockl J. (2008) The oxidation state of phospholipids controls the oxidative burst in neutrophil granulocytes. J Immunol, 181: 4347-53.

Timar CI*, Lorincz AM*, Csepanyi-Komi R, Valyi-Nagy A, Nagy G, Buzas EI, Ivanyi Z, Kittel A, Powell DW, McLeish KR, Ligeti E. (2013) Antibacterial effect of microvesicles released from human neutrophilic granulocytes. Blood, 121: 510-8. *(*These authors contributed equally)*

Lorincz AM, Szarvas G, Smith SM, Ligeti E. (2014) Role of Rac GTPase activating proteins in regulation of NADPH oxidase in human neutrophils. Free Radic Biol Med, 68: 65-71.

Lorincz AM, Timar CI, Marosvari KA, Veres DS, Otrokocsi L, Kittel A, Ligeti E. (2014) Effect of storage on physical and functional properties of extracellular vesicles derived from neutrophilic granulocytes. J Extracell Vesicles, 3: 25465.

Other publications:

Timar CI, Lorincz AM, Ligeti E. (2013) Changing world of neutrophils. Pflugers Arch, 465: 1521-33.

Wuertz CM*, Lorincz A*, Vettel C, Thomas MA, Wieland T, Lutz S. (2010) p63RhoGEF--a key mediator of angiotensin II-dependent signaling and processes in vascular smooth muscle cells. FASEB J, 24: 4865-76. *(*These authors contributed equally)*