Investigation of NADPH-oxidase and proton channel function in leukocytes

PhD thesis

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Introduction

The phagocyte oxidase (phox) is a multicomponent enzyme, which is responsible for the regulated production of reactive oxygen species (ROS) in leukocytes. The complex consists of membrane bound and cytosolic components. Nox2 and $p22^{phox}$ are the two membrane bound components, the former is responsible for the enzimatic activity of the complex. The cytosolic subunits - $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac - regulate the activity of the oxidase. In its active form, Nox2 transports electrons across the membrane, which react with molecular oxygen and form superoxide anions, a precursor of further reactive oxigen species. ROS play an important role in the elimination of microorganisms, furthermore they influence other functions of the immune system (antigen presentation, signal transduction). The transport of electrons depolarise the plasma membrane, moreover the oxidation of NADPH leads to intracellular acidification. Without compensatory mechanisms these

factors hamper phox-activity. To our current knowledge, voltage-gated proton channel (Hv1) is the most effective way of compensation, as it is able to alleviate both problems jointly, supporting intense and sustained ROS-production.

Despite the plethora of data on the structure and funcion of phox and Hv1, several questions are still unanswered. At the beginning of our experiments mainly circumstancial evidence was available on the expression, dimerisation, and intracellular distribution of Hv1 in human granulocytes. Furthermore, direct evidence was lacking that Hv1 funcions as a proton channel in human white blood cells as well. We did not have any information about the interdependence of Nox2 and Hv1 expression. The relationship of phox subunit and Hv1 expression and respiratory burst intensity in eosinophil and neutrophil granulocytes of healthy individuals was poorly explored.

The role phox in B-lymphocytes is not well understood. Investigation of phox deficient conventional B-cells revealed only minor discrepancies. No data is

available on the importance of phox in B-1 cells, which are abundant in the murine peritoneal cavity. Recently several authors reported, that B-1 cells are able to engulf and kill pathogens, but the role of the oxidase in the process was not adressed.

Objectives

The main aims of our work were as follows.

- Investigation of Hv1 expression, dimerisation and intracellular distribution in human granulocytes. Investigation of proton channel-function of Hv1 in human franulocytes.
- Investigation of the interdependence of Nox2 and Hv1 expression.
- Systematic comparison on the phox subunit and Hv1 expression and respiratory burst intensity of eosinophil and neutrophil granulocytes isolated from healthy individuals.

- Investigation of oxidase activity in bacterial elimination of peritoneal B-cells.

Methods

Solutions and antibodies

Most of the applied materials were purchased from Sigma-Aldrich. Experiments were performed at 21-26°C, unless otherwise stated. All reagents were used as specified by the manufactuter.

Hv1 specific antibody (aHv1-N) was produced by immunisation of rabbits with a protein-construct containing the 99 N-terminal amino-acids of Hv1 together with glutation-S-transferase. The Affigel-binded version of the same construct was used to purify the anti-glutathion-S-transferase depleted rabbit sera. The same procedure was applied to produce antibodies against the mouse homologue of Hv1.

Mice

Mouse strain carrying the mutation in $p22^{phox}$ gene (nmf333) was purchased from Jackson laboratory. Nox2 deficient mice ($Cybb^{tm1Din}$) were generous gift from Professor Ralf P. Brandes. Age matched male C57Bl/6J mice were used as controls.

Cell lisolation and culture

Primer human eosinophil and neutrophil granulocytes, monocytes, T- and B-lymphocytes were isolated from peripherial venous blood of healthy individuals after obtaining their informed consent. Murine cells applied: peritoneal and splenic B-lymphocytes and peritoneal cells.

Human leukemia cell line PLB-985 was from ATCC LCG, PLB-985 X CGD cells were donated by Dr. Mary C. Dinauer. Cells were cultured in accordance with the manufacturer's protocol, in some cases however, medium serum content was decreased from 10% to 0.5% in order to increase the drive for differentation. PLB-985 cells were transfected by electroporation.

Western blot

Cells were lysed in 2x Laemmli buffer. Sapmles were run on 8-10% polyacrylamide gel, then blotted to nitrocellulose membrane. Afrter blocking, membranes were incubated with the primary antibody, followed by thorough washing in PBS supplemented with Tween20. Horseraddish (HRP) conjugated secondary antibodies were applied, then washed once more in PBS-Tween. Signals were detected using enhaced chemiluminscence on Fuji Super RX films.

Densitometric analysis was performed with ImageJ software.

Immunocytochemistry, confocal microscopy and colocalisation-analysis

Cells were fixated with 4% paraformaldehyde, then washed 5 times with PBS. After incubation with 100 mM glycinein in PBS, cells were washed two times in PBS. Samples were permeabilised by Triton X-100-containing solution. Non-specific binding sites (eg. Fc-receptors)

were blocked, then cells were incubated in primary antibody solution for one hour. Cells were washed, and incubated with the secondary antibody. Samles were washed and finally mounted in Mowiol 4-88 antifade reagent.

Confocal images were acquired using LSM 510 or LSM 710 laser confocal unit. Pearson's coefficient was used to describe the extent of colocalisation.

Phagocytosis and intracellular killing ability

Phagocytosis of human granulocytes and PLB-985 cells was induced by serum treated zymosan (STZ). Mouse peritoneal B-cells were incubated with non-viable Alexa Fluor 594 labeled *S. aureus.* Bacterium-to-cell ratio was 10:1. The intracellular killing ability of peritoneal B-cells was investigated with gentamycin protection assay.

All procedures were performed at 37°C.

Measurement of ROS-production and oxygen consumption

ROS-production was measured using Diogenes reagent or luminol + HRP. Superoxide production of peritoneal B-cells was also measured with nitroblue-tetrazolium (NBT)-test.

Oxygen consumption and extracellular pH were measured using Seahorse XF Analyser System.

All measurements were carried out at 37°C.

Molecular biology

Hv1 was cloned from RNA isolated from mature dendritic cells, cDNA was sythetized using revese transcription. The open reading frame of Hv1 was cloned into CDNA3.1/V5-His-TOPO vector. The base sequence was conroled with sequence analysis.

Patch-clamp measurements

Voltage-clamp measrements were performed on Axon Axopatch-1D amplifier equipped with CV-4-

1/100U headstage. Data were collected with pClamp6, analysis was performed with pClamp8 (Axon) software.

Data analysis

All data are mean±SEM unless otherwise specified. Statistical analysis was performed with Satistica 8 software. Student's t-test or Mann-Whitney U-test was applied. Level of significance was $p<0,05$, unless otherwise stated.

Results

Expession of voltage-gated proton channel (Hv1) is detectable in eosinophil and neutrophil granulocytes, monocytes and B-lymphocytes. The protein was not detected in T-cells. The dimer of Hv1 can be stabilized by thiol- and aminoreactive cross-binding agents, by increasing the non ionic detergent content of the sample buffer and by omitting β-mercaptoethanol from the solution. Under non-reducing conditions, N-

ethylmaleimide (which inhibits novel disufide bond formation, but leaves already exsisting bonds unaffected) did not alter dimer formation significantly.

The intracellular distribution of Hv1 and its colocalisation with Nox2 was investigated with immunocytochemistry. Eosinophils showed intense Hv1-labeling and good colocalisation with Nox2 (Pearson's coefficient 0,84±0,03). Neutrophils stained fainter with aHv1-N, Pearson's coefficient was 0,78±0,02. Human leukemia PLB-985 cells showed marked Hv1-labeling and had a Pearson's coefficient of 0,92±0,03. During zymosan-phagocytosis Hv1 and Nox2 accumulated in the wall of the phagosome.

The correlation between Hv1 expression and proton current density was investigated using Hv1 knock down PLB-985 cells. Based on patch-clamp and Western blot data, the two factors are directly proportional.

The interdependence of Hv1 and Nox2 expression was investigated in PLB-985 cell line. Dimethyl-formamide was used to induce granulocyte-like differentiation of PLB-985 cells. The expression of Hv1

and Nox2 was measured on different days of develeopment. The expression of both protein raises constantly during the 6 day long period of differentation. The expression of both proteins was highest after 7 days of differentation under inceased differentaion pressure. In Nox2 deficient cells the pattern and the arcievable highest Hv1 expression was not different of that observed in wild type cells. Similarly, in Hv1 knock down cells decrease in Nox2 expression was not detected.

Comparing the expression of respiratory burst associated proteins in neutrophil and eosinophil granulocytes no major difference was found in the phox subunit content: Nox2, $p22^{phox}$, $p47^{phox}$ and Rac expression did not differ significantly, while $p67^{phox}$ expression showed minor difference (eosinophil/neutrophil ratio 1,6±0,2). On the contrary, voltage-gated proton channelexpression was found to be one order of magnitude higher in eosinophil granulocytes (eosinophil/neutrophil ratio 10,6±2,5).

The expression of Hv1 and Nox2 showed correlation in eosinophil, but not in neutrophil granulocytes.

Neutrophils produced more ROS regardless of detecion method used, when respiratory burst was induced with serum treated zymosan (STZ). ROS-production of neutrophils was also higher if the cells were stimulated with phorbol ester phorbol-myristate-acetate (PMA) and the mostly intracellulatly sensitive luminol was the detecting agent. Extracellular superoxide-release of PMA-stimulated eosinophils was more intense, than of neutrophils. To measure the total Nox2 activity, oxygen consumption measurements were performed. PMA-treated eosinophils consumed 1.4-times more oxigen, than neutrophil granulocytes.

Based on our expreiments, peritoneal B-cells produce more superoxide and express more Nox2 and $p22^{phox}$, than splenic B-cells. In contrast to wild type cells, Nox2 and $p22^{phox}$ KO peritoneal B-cells neither show detectable extracellular superoxide-release after BCR crosslinking, nor produce phoagosomal ROS upon

S.aureus engulfment. Intracellular survival of ingested *S.aureus* was found to be higher in Nox2 and $p22^{phox}$ KO cells, than in wild type cells, as measured with gentamycin protection assay.

Conclusions

Based on our expreimental data Hv1 is indispensible to voltage-gated proton current in human leukocytes.

Hv1 is present in all type of human phagocytes and B-cells, and forms stabile dimers, based on experiments on granulocytes.

Expression of Hv1 and Nox2 is spatially and temporally overlapping in human granulocytes, especially in eosinophils. Nevertheless, in PLB-985 cells, we did not find any evidence that the expression of the two proteins is coupled to each other.

Eosinophils and neutrophils from healthy individuals contain essentially the same amount of phox

subunits, whereas eosinophils express ten times more Hv1

Mouse peritoneal cells produce superoxide around engulfed *S. aureus.* The lack of oxidase activity results in attenuated bacterial elimination of peritoneal B-cells.

List of publications

The PhD thesis is based on the following publications:

Petheő GL, Orient A, Baráth M, **Kovács I**, Réthi B, Lányi Á, Rajki A, Rajnavölgyi É, Geiszt M. (2010) Molecular and functional characterization of Hv1 proton channel in human granulocytes. PLoS One 5: e14081.
IF: 4,411

K**ovács I**, Horváth M, Kovács T, Somogyi K, Tretter L, Geiszt M, Petheő GL (2014) Comparison of proton channel, phagocyte oxidase, and respiratory burst levels between human eosinophil and neutrophil granulocytes. Free Radical Res. 48: 1190-9.
IF (2013): 2,989

Kovács I, Petheő GL, Horváth M, Lányi Á, Geiszt M. (2015) Reactive oxygen species mediated bacterial killing by B-lymphocytes. J Leukocyte Biol. 97: 1133-7.
IF (2013): 4,304.