Examination of the function of peroxidases and NADPH-oxidases

Ph.D. thesis

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1. Introduction

Reactive oxygen species (ROS), including oxygen-derived free radicals have been long considered as damaging molecules of our body, which are responsible among others for ageing and the development of different tumors or neurodegenerative diseases. Researches over the last few decades lead to the recognition that ROS are involved in several physiological functions in the body, for instance host defense, hormone biosynthesis or different signal transduction pathways. Alteration of physiological ROS production can result in immunodeficiency disorders, hypothyreosis or coronary heart diseases. In the human body ROS can have several sources, among them the most known are the mitochondria, where superoxide ($O_2^{\bullet -}$) is produced as a byproduct of the respiratory electron transport chain. In mammalian cells the first example of controlled ROS production was the oxidative burst of phagocytic cells, where the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) is expressed. In recent years more homologues of the phagocytic oxidase were discovered and they are now called as the Nox family of NADPH-oxidases.

The function of NADPH-oxidases is the regulated production of ROS.

The mammalian peroxidases are hem-containing enzymes, using the ROS produced by the NADPH-oxidases to oxidize different substrates. In the human body peroxidases and NADPH-oxidases co-operate in different processes like host defense or thyroid hormone synthesis.

A common feature of mammalian peroxidases is that they can produce dityrosine bridges through the crosslinking of tyrosine amino acids, which function in invertebrate species to stabilize the extracellular matrix (ECM). One example for the ECM stabilization is the cuticle synthesis of the nematode *C. elegans* (Ce), in which the certain layers of cuticle are crosslinked through dityrosine bridges. The formation of dityrosine bridges requires both the Ce-Duox (NADPH-oxidase) and MLT7 Ce-peroxidase. In absence of these enzymes the cuticle biosynthesis becomes insufficient and consequently blisters develop on the worms. The function of the dityrosine-forming activity of peroxidases hasn't been examined in vertebrates yet.

The lactoperoxidase (LPO) is a peroxidase expressed in milk, tears, saliva and different mucosal surfaces. Its major function is the defense of mucosa against adhering pathogens.

On the mucosal surfaces LPO utilizes H_2O_2 synthesized by Duox enzymes to produce hypothiocyanate ($OSCN^-$), which is an effective antimicrobial agent. The antimicrobial effect of LPO-catalyzed dityrosine formation hasn't been characterized yet.

Peroxidasin (PXDN) is an additional member of the family of mammalian peroxidases. PXDN is distinct from other peroxidases, because in addition to its peroxidase-homology domain it contains immunoglobulin-C2 type domains, leucine-rich repeats and a von Willenbrand C-type domain. These domains were described as protein-protein interacting regions. Based on its structure PXDN incorporates the features of peroxidases and of extracellular matrix proteins. PXDN was first described in *Drosophila*, where its mRNA is expressed in the early phase of embryogenesis. The *Drosophila* PXDN is an active peroxidase, it has dityrosine-forming activity, that presumably contributes to the consolidation of ECM, for instance during the wing development.

We have little information about the human PXDN (hPXDN) and its function is not known. In the literature there are contradicting data about the expression of hPXDN, once it's regarded as being melanoma-specific, other times as ubiquitous protein. It is not known whether the hPXDN has

peroxidase or dityrosine-forming activity like other peroxidases.

Duox enzymes are members of the NADPH-oxidase enzyme family. Besides the NADPH-oxidase homology domain Duox enzymes also contain an extracellular peroxidase homology domain. Unlike Ce-Duox, the heterologously expressed peroxidase domain of hDuox1 doesn't have dityrosine-forming activity.

Duox1 and Duox2 enzymes were first described in the apical membrane of thyrocites, where they produce H_2O_2, which is then utilized for the synthesis of thyroid hormones by thyroid peroxidase.

Nonsense mutation of the *Duox2* gene causes hypothyreosis even in a heterozygote form. There is no known mutation in *Duox1* causing hypothyreosis and Duox1 is not able to compensate the loss of function mutations of *Duox2* in the thyroid gland. These findings imply that Duox1 and Duox2 have distinct functions.

Another function of Duoxes is the protection of mucosal surfaces against invading microorganisms. Between the two homological domains of Duox, there is an intracellular loop, containing two Ca^{2+}-binding EF hand motifs, which make possible the activation of the enzymes through Ca^{2+} ions.

Epithelial cells of the thyroid gland and respiratory tract have been described to produce H_2O_2 in a calcium-dependent manner. It is not known whether epithelial cells of the urinary bladder can produce H_2O_2 or they have an antimicrobial system similar to the Duox-LPO system of airway mucosa.

2. Objectives

The main aims of our experiments were the following:

1. *In vitro* characterization of lactoperoxidase-catalyzed dityrosine-formation and determination if the reaction is suitable to the quantification of H_2O_2, also investigation of the function of dytirosine-formation in mammalian organism.

2. Determination of the peroxidase activity, tissue expression and intracellular localization of the human peroxidasine enzyme.

3. Investigation of the H_2O_2-producing ability of urinary bladder epithelial cells and identification of the enzymatic source of H_2O_2. Furthermore determination if the urothelium possess a similar Duox-LPO antibacterial system as does have the airway epithelia.

3. Methods

Plasmid constructions: for stable and transient transfection the coding regions of human Nox4 and PXDN enzymes were cloned into pCDNA3.1 plasmid.

Cell culture and transfection: for preparation of Nox4-expressing clones the FreeStyle 293F human embryonic kidney cells were transfected by Fugene®6 then clones were selected in Geneticin-supplemented media. Simian renal COS-7 cells were transfected by Fugene®6 and Lipofectamin®2000 with the plasmid containing the coding region of PXDN.

Northern blot: total RNA's were isolated from Geneticin-surviving clones with Trizol reagent. RNAs were blotted onto Whatman Nytran™ nylon membrane and were hybridized with P^{32}-labelled isotope in PerfectHyb™ Plus hybridization buffer. We used the whole coding region of hNox4 as a probe. The radioactive signals were detected on FUJI Super RX film. For the human PXDN mRNA detection, human multiple tissue (2 μg of poli $[A]^+$ RNA) Northern blot membranes from Clontech were probed with the cDNA fragment corresponding the 3'- untranslated regions of PXDN mRNA.

Measurement of peroxidase activity: after 2 days of transfection COS-7 cells expressing PXDN were lysed in PBS

containing 1% hexadecyltrimethylammonium bromide. Peroxidase activity of the lysates was determined by the Amplex Red peroxidase assay KIT.

Measurement of H_2O_2 by LPO-catalyzed dityrosine formation: Nox4-expressing stable Freestyle 293F cells were suspended in H-medium, then cells were incubated in 96-well black plate with 1 mM tyrosine and 0.5 μg/ml LPO at 37 ºC for 30 min. The dityrosine production was detected in a fluorescence plate reader at excitation and emission wavelengths of 320 and 405 nm, respectively.

Measurement of H_2O_2 by Amplex Red: Nox4-expressing and freshly isolated urothelial cells were suspended in H-medium, then cells were incubated in the presence of 50 μM Amplex Red and 0.1 U/ml horseradish peroxidase at 37 ºC for 30–60 min. Resofurin fluorescence was measured at 590 nm. The H_2O_2-production of urothelial cells were stimulated by thapsigargin, ATP or GSK 1016790A, which is a selective agonist of the TRPV4 channels.

Measurements with human blood plasma: human blood samples were collected and mixed with heparin. Cells were separated from the plasma by centrifugation. Plasma samples were supplemented with 12.5 mg/ml LPO and 2 mM H_2O_2 and

incubated at 37ºC for 30 min. Dityrosine production was detected in a fluorescence plate reader.

Preparation of polyclonal anti-PXDN antibody: rabbits were immunized with intracutaneous injections of glutathione S-transferase-PXDN fusion protein suspended in Freund adjuvant. The antibody was affinity purified using Affigel 10 beads.

Western blot: plasma proteins and lysates of COS-7 and urothelial cells were separated on polyacrylamide gels and blotted onto nitrocellulose membranes. LPO, PXDN and Duox proteins were detected with the corresponding primary and HRP-labeled secondary antibodies with the ECL method.

Backcrossing of Duox1 knockout mice to C57BL/6 background: Duox1 knockout mice were purchased from Lexicon Genetics with mixed 129/SvEv^{Brd} and C57BL/6J genetic background, hence we backcrossed mice to C57BL/6 background.

Genotyping of Duox1 mice: purified tail genomic DNA was used as a template for PCR, which is based on the detection of genetic insertion site of the trapping cassette. Amplified PCR products were separated and evaluated on 1.5 % agarose gels.

RT-PCR: urothelium RNA from urinary bladders were isolated using Trifast reagent. cDNAs were synthesized from total RNA

using oligo$(dT)_{18}$ and RevertAid M-MuLV Reverse Transcriptase. Quantitative PCR experiments were carried out in a LightCycler 1.5 instrument and amplicons were detected with SYBR Green. Crossing point was determined by the second derivative method. The target gene expression was normalized to β-actin and relative expression levels were presented.

Immunohistochemistry on frozen sections: mouse urinary bladders were embedded and frozen in TissueTek OCT. Sections of 8 μm were fixed in aceton. After blocking in goat serum Duox was detected with anti-Duox primary and Alexa-conjugated secondary antibodies.

Confocal laser microscopy: images were taken using a Zeiss LSM 510 confocal laser microscope, excited by argon and helium/neon lasers. Emission was detected using a 500–530 nm band pass filter for A488, and a 560 nm long pass filter for A568.

Preparation of primary urothelial cells: urinary bladders were cut open and urothelium was removed from smooth muscle with the use of forceps. Urothelium was treated with trypsin, then cells were harvested by centrifugation and finally suspended in H-medium. Animal experiments were authorized

by the National Animal Experiment Committee under permission No. 22.1/1100/003/2008.

4. Results and conclusions

During my PhD work we characterized the LPO-catalyzed dityrosine formation *in vitro*. We concluded that LPO effectively catalyze the formation of dityrosine from free tyrosine amino acids of the reaction solution. Dityrosine is a fluorescent product, which can be detected at excitation and emission wavelengths of 320 and 405 nm, respectively. Adding catalase to the reaction mix we observed a decrease in fluorescence, which proves that LPO catalyze dityrosine formation only in the presence of H_2O_2.

Based on our experiments we propose that the formation of dityrosine can be suitable for the measurement of H_2O_2 concentration. This H_2O_2-detecting reaction would be an alternative and more economic method to the commercially available Amplex Red. In our experiments we showed that besides L-tyrosine LPO can also produce dityrosine from D-tyrosine, therefore the reaction can be suitable to measure H_2O_2 in such systems, where the L-tyrosine concentration is decreasing for example due to amino acid transport. Moreover we defined the required and sufficient concentrations of tyrosine and LPO with which the reaction is suitable for the quantification of H_2O_2 with a known reaction time and volume.

With LPO-catalyzed dityrosine formation as little as 0.5 μM H_2O_2 can be detected, and up to 20 μM, dityrosine fluorescence changed linearly with the H_2O_2 concentration. Using LPO-catalyzed dityrosine formation we detected successfully the H_2O_2 produced by glucose oxidase or by FreeStyle 293F cells, which stably expressed Nox4 enzyme.

We wanted to examine the role of the LPO-catalyzed dityrosine formation in mammalian organisms. To explore this question we carried out experiments on human blood plasma. We chose blood plasma, because like LPO-containing secretions it has high protein concentration. Furthermore it doesn't contain any peroxidase, hence the tyrosine amino acids presumably aren't crosslinked. In our experiments we demonstrated that using H_2O_2 LPO crosslinks plasma proteins trough dityrosine bridges along with itself. We hypothesize that this process *in vivo* can contribute to boost the antimicrobial activity of LPO, because it could inactivate bacterial proteins or it would enhance the antimicrobial effect of LPO through anchoring it to bacterial surfaces.

To examine the dityrosine-forming activity of human PXDN (hPXDN) we expressed it in COS-7 cells. Using Amplex Red Peroxidase Assay KIT we demonstrated that like

other peroxidases, hPXDN has peroxidase activity. We planned to examine the dityrosine-forming activity of hPXDN on purified peroxidase domain. Therefore we expressed the peroxidase domain of hPXDN in Sf9 insect cells, but we couldn't purify the peroxidase domain from the insoluble protein fraction of the cell lysate.

To clarify the contradicting expression data, we examined the tissue expression of hPXDN with Northern blot and we concluded that it is expressed in several tissues except brain, thyroid gland and peripheral blood leukocytes.

Because there was no commercially available antibody against PXDN, we prepared polyclonal anti-PXDN antibody with the immunization of rabbits. We detected PXDN in COS-7 cells in the endoplasmatic reticule. In further experiments our lab determined that in human dermal and lung fibroblast PXDN is also localized in the ER. During fibroblast to myofibroblast transition in response to TGFβ1 treatment, PXDN is secreted into the media, where it forms fibril-like structures with ECM proteins.

We were interested whether the urothelium has a similar Duox-LPO antimicrobial system like airway mucosa. In our experiments we showed that like airway and thyroid

epithelial cells, urothelial cells also produce H_2O_2. With the help of Duox1 knockout mice we proved that the source of H_2O_2 in the urothelium is Duox1. In immunohystochemical analysis on frozen sections we determined that Duox1 is expressed in the urothelial layers of urinary bladder.

In real-time quantitative PCR experiments we concluded that LPO is not expressed in the urothelium, hence in the urothelium the presence of a Duox-LPO antimicrobial system like in the airways is not likely.

In freshly isolated mouse urothelial cells Duox1 produced H_2O_2 in response to intracellular Ca^{2+}-concentration elevation evoked by thapsigargin, ATP or TRPV4 agonist. TRPV4 is the most abundantly expressed transient receptor potential cation channel of the urothelium, which opens to mechanical stimuli and generate intracellular Ca^{2+}-signal. Activation of Duox1 trough TRPV4 raises the possibility that Duox1-produced ROS contribute to the mechanotransduction of the urothelium, which hypothesis needs further investigation.

5. List of publications

Related to the thesis

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Philos Trans R Soc Lond B Biol Sci 2005; 360: 2301-2308.
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Free Radic Biol Med 2010; (under revision)

Other publication

Orient A, **Donkó Á**, Szabó A, Leto TL, Geiszt M.
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