Functional investigation of the Nox4 NADPH oxidase

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Doctoral theses

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Introduction

Regulated production of reactive oxygen species (ROS) is now considered an essential component of maintaining homeostasis of live organisms. Among ROS, hydrogen peroxide (H_2O_2) has emerged as a particularly important molecule with pleiotropic functions that include roles in host defense, thyroid hormone production, synthesis of the extracellular matrix and signal transduction. H_2O_2 is produced at different intracellular sites as a byproduct of various biochemical pathways, but regulated production of H_2O_2 is mediated by members of the Nox/Duox family of NADPH oxidases (Nicotinamide adenine dinucleotide phosphate oxidase). NADPH oxidase enzymes responsible for controlled ROS production in multicellular organelles, their common feature is an electrontransport cross membranes via preserved, transmembrane catalytic electrontransfer domain, where NADPH is used as an electron donor, molecular oxygen as an acceptor. The reaction results superoxide anion or hydrogen peroxide.

Currently, the most intensively studied, dedicated ROS source is Nox4, which was originally identified in kidney epithelial cells. Subsequent studies revealed that the expression of Nox4 is not restricted to the kidney, but other cells including endothelial and alveolar epithelial cells, osteoclasts, cardiomyocytes and activated fibroblasts also contain the enzyme. The physiological function of Nox4 remains unclear.

The Nox4 protein was cloned in full length by Geiszt et al. in 2000. The human NADPH oxidase 4 is encoded by the NOX4 gene, mapped to the chromosome 11q14.2-q21 location. The human Nox4 cDNA encodes a deduced 578-amino acid protein with an apparent molecular mass of 66.5 kD, which shows 57% homology and 39% match with the Nox2 enzyme. Despite the high degree of homology, it does not really fit into any of the subgroups of the Nox4 NADPH oxidase family.

Nox4 is also $p22^{phox}$-dependent but probably in uncommon manner. Those cofactors, cytosolic regulators that are essential for the activation of other $p22^{phox}$-dependent oxidases (Nox1, Nox2 and Nox3) is not required for Nox4. This is confirmed by the fact that the Nox4's functionality is not altered by disrupting motifs which essential for Nox1 Nox2 and Nox3 in C-terminus of $p22^{phox}$ protein.

Also unique among $p22^{phox}$-dependent Noxs that the Nox4's dehydrogenase domain after activation remains in intrinsic activated conformation, the electrontransfer from the NADPH to FAD then to the hem groups is continuous. Nox4 produced ROS is 90% H_2O_2 in two-step manner by formation of superoxide byproduct.

Based on *in situ* hybridization experiments and RNA seq data the largest tissue expression is in the kidney, but is also present in other tissues and cell types to a lower extent in heart, adipocytes, hepatocytes, endothelial cells, skeletal muscle, brain and airway epithelial cells.

There is no unified position on the exact subcellular localization of Nox4. In terms of structure no signal or ER retention sequences were found, however, in most cases it was detected in the membrane of ER and mitochondria.

It is an open-ended question of what function can the Nox4 perform, when largest amount of it is expressed in the kidney, it generates a signaling messenger H_2O_2 molecule, and its constitutive active enzyme. The Nox4 may be an oxygen sensor in the kidney, the catalytic constant of oxygen binding is in the range where the already known oxygen sensors is. It can convert the tension of oxygen into a redox signal, which capable of modify the protein kinases related signal transduction. It induces proliferation in cardiovascular tissues, transmits survival signals in pancreas and liver cells. Its increased activity and the produced ROS would generate oxidative stress, which activates redox-dependent transcription factors such as NFχB, Keap1, Bach2, which may initiate oncogenesis or cell death.

The role of Nox4 in the fibrotic changes in wound healing of tissues is significant and intensively researched also from a pharmacological point of view. Fibroblasts produce H_2O_2 during myofibroblast differentiation, which enhances extracellular matrix proteins, e.g. fibronectin, proteoglycans, α-smooth muscle actin production, activates the MAPK cascade, further kinase pathways that also initiate differentiation and cell proliferation. Producing ROS itself increases the efficiency of differentiation and maintains feed-forward regulation. Fibroblast-type cells in patients with idiopathic pulmonary fibrosis showed higher Nox4 expression. That results drive us to better understanding the function, exact localization, and functioning mechanism of Nox4, what may propose a new opportunity for a target molecule that could specifically inhibit or reduce the onset of renal or pulmonary fibrosis.

Objectives

During my doctoral studies we have examined several closely related issues focusing on the following main objectives:

1. Characterization of the principle mechanism of the genetically encoded intracellular H_2O_2 PerFRET probe by mutation analysis.

2. Endogenous Nox4 expression and activation of primary human pulmonary and dermal fibroblast cells.

3. Characterization of the relationship between Nox4 and $p22^{phox}$ in Nox4 KO and $p22^{phox}$-deficient mice models.

4. Mapping of the subcellular localization of Nox4 and $p22^{phox}$ and enquiring the intracellular presence of H_2O_2 produced by the activated Nox4 oxidase complex.

Methods

***Plasmid construction*:** for transient transfection of human Nox4 and $p22^{phox}$ Open reading frames were cloned into pCDNA3.1 vector. The V5- and AU1-epitope-encoding sequences were cloned into the N-terminal site of Nox4 and C-terminal region of $p22^{phox}$ by site directed mutagenesis. The sequence of CFP-FRB-HA, part of the rapamycin-based induction system was inserted into the vector by restriction enzymes and T4 ligase at the N-terminus of Nox4 and the C-terminal end of the $p22^{phox}$.

Cell culture and transfection: We have intoduced the plasmids with transient transfection into the cells by using Lipofectamine LTX (Life Technologies) or with Neon Transfection System (Life Technologies) in the stage of 60-70% confluency. We applied standard siRNA treatment for gene silencing: 60-80% confluent adherent cells were transfected with 25 pM specific or scrambled siRNA with Lipofectamine RNAiMAX (Life Technologies), then the cells were incubated for further 2-3 days. The tail tip fibroblasts of $p22^{phox}$-mutant (nmf333) and Nox4 knockout (3FAFyh) mice were generated from 8-weeks-old animals. After 30 min collagenase digestion of the tail tips, cells were cultured for several days. We serum deprived the cells in the presence of 0.05% serum, then Cells were treated with 5 ng/mL TGF-β1 for 24 h in the absence of serum.

Western blot: Cells were washed once with cold PBS, then lysed in RIPA buffer enriched with proteinase inhibitor cocktail. After that, the supernatant was combined with 4×Laemmli sample buffer then loaded without boiling on 12% SDS–polyacrylamide gels. After electrophoresis, the gels were blotted onto nitrocellulose membranes, blocked. The primary antibody was visualized by peroxidase-coupled goat anti-mouse or anti-rabbit IgG using the enhanced chemiluminescence method.

***QPCR*:** Fibroblast cells were isolated complete RNA with Trifast reagent and then transformed to cDNA with oligo (dT)18 primer and M-MuLV reverse transcriptase. QPCR reactions were run on Roche LightCycler 1.5, PCR products were detected with SYBR Green, and Cp values were determined by the 2nd derivative method. Relative expression levels were plotted against resting control fibroblasts for the same gene or normalized for the β-actin gene.

Measurement of extracellular H_2O_2 level with Amplex Red method: The extracellular H_2O_2 levels were measured with Amplex Red method. Adherent confluent cells were incubated in the presence of 50 μM Amplex Red and 0.1 U/ml horseradish peroxidase in H-medium. Resorufin fluorescence was measured at 590 nm on POLARstar Spectrophotometer after 40 min incubation at 37°C.

Immunocytochemistry and confocal microscopy: Immunostained cell images were collected on a Zeiss LSM710 confocal laser scanning microscope equipped with a 63x1.4 oil immersion numerical aperture plan Apochromat objective. Images were acquired from optical slices of 1–2 μm thickness in multitrack mode. Alexa488 immunofluorescence detection involved excitation with a 488 nm argon laser, while in case of Alexa568 the 543 nm helium/neon laser was applied. Emissions were collected using a 500-530 nm band-pass filter. Image analysis was performed using Zen software (Zeiss).

Detection of intracellular H_2O_2 levels with HyPer1 probe: The ratiometric measurements of HyPer1 were performed on an inverted microscope equipped with 40x1.4 oil-immersion objective and a Cascade II. Camera. Excitation wavelengths were set by a random-access monochromator connected to a DeltaRAM xenon arc lamp. The excitation wavelengths of HyPer1 are 490 and 420 nm combined with a 505 nm dichroic filter and a 525/36 nm emission filter set. Data acquisition was handled by Metafluor software.

Rapamycin-based inducible heterodimerization: We applied FKBP12-FRB system as a chemically inducible translocation assay with rapamycin as an

inducer. Within a cell, after administration of 300nM rapamycin, the molecule first binds to FKBP12 and only then the FKBP12–rapamycin complex binds to FBR, which is the FKBP and rapamycin binding domain of TOR26 kinase. Dermal fibroblasts were cotransfected the CFP-FKBP12-Nox4 or $p22^{phox}$-FKBP12-CFP with FRB-YFP together with electroporation. Kinetic measurements were performed in 1 ml H-medium, where stimuli were added in 300 nM final concentration. Images were acquired every 5-10 seconds for a period of 15 min.

Results

We examined which cysteines are responsible in the PerFRET probe for the specific response to H_2O_2, so we have mutated all cysteine amino acids occurring in the Orp1 and Yap1 cCRD domains, and also the major combinations. All mutated PerFRET variants were expressed successfully and showed cytosolic localization in HeLa cells. The mutation of Orp1's 36-position cysteine was sufficient to completely eliminate in response of PerFRET to H_2O_2. Based on C64S and C82S variants, the second and third cysteine in Orp1 is not essential. The 36-position cysteine of Orp1 interacts with C620 in Yap1 cCRD, while the mutation of the other two Yap1 cCRDs did not interfere with H_2O_2 detection.

We investigated the H_2O_2 production of human dermal and pulmonary fibroblasts cells upon TGF-β1 treatment. Fibroblasts were serum depleted and induced with TGF-β1 for 24 hours. The level of produced H_2O_2 was measured in Amplex Red assay for 60 minutes by controls with DPI and Nox4 siRNA-treated cells. The TGF-β1 stimulation significantly increased H_2O_2 production in the extracellular space of primary fibroblasts. The H_2O_2 production could be inhibited by DPI and specific siRNA Nox4 treatment.

Tail tip fibroblast were prepared from 8 weeks old Nox4 KO, $p22^{phox}$ deficient mice, and wild type counterparts. After serum depletion and 24 hours TGF-β1 treatment the produced H_2O_2 amount was measured in the extracellular space of cells by Amplex Red assay. H_2O_2 production was induced by TGF-β1 in wild-type fibroblasts, but in case of Nox4 KO mice it was lagged behind. and was significantly lower in the $p22^{phox}$-deficient cells. An Y121H amino acid exchange of the $p22^{phox}$ gene results in an unstable protein in $p22^{phox}$-deficient mice, that can form with Nox4 also an unstable, but functional complex. These experiments proved that endogenously expressed $p22^{phox}$ supports the activity of Nox4.

We investigated whether TGF-β1 induction has an effect at transcriptional level on the mRNA amount of $p22^{phox}$ and Nox4. For this purpose, dermal and pulmonary fibroblasts were induced with TGF-β1 about serum depletion for 24 hours, then total RNA was isolated, converted to cDNA, and their relative expression levels were measured in QPCR reaction., The amount of Nox4 mRNA was significantly increased by TGF-β1 treatment compared to resting levels in both human fibroblast cells, whereas the amount of $p22^{phox}$ mRNA was not influenced by TGF-β1 treatment. We checked whether human primary fibroblasts also express other Nox systems, which are possible sources of the ROS production. Control and TGF-β1 induced cells were checked in QPCR reaction. Based on our experience, these primary human pulmonary and dermal fibroblasts are unlikely to have other NADPH oxidase systems. We did not detect any other Nox component in significant amount, the $p22^{phox}$ mRNA is present independently of induction, but interestingly the expression of Nox4 can only be detected after induction.

As a next step, we examined whether the amount of $p22^{phox}$ protein is dependent on the expression of Nox4 and on the TGF-β1 treatment. Human primary fibroblasts, tail tip fibroblasts isolated from Nox4 KO, $p22^{phox}$-deficient and wild-type mice, were treated with TGF-β1 for 24 hours, then the amount of $p22^{phox}$ protein was investigated in Western Blot experiments. The level of $p22^{phox}$ protein was found to be unchanged regardless of TGF-β1 treatment and presence of Nox4. Altogether, the results suggested that the expression of $p22^{phox}$ is independent of Nox4, and also of TGF-β1 treatment in transcriptional and translational level.

We prepared V5- and AU1-epitope labeled Nox4 and $p22^{phox}$ expression vectors, which were transfected in dermal fibroblasts then visualized their intracellular localization by immunostaining. Based on our confocal microscopic images, the Nox4 and $p22^{phox}$ are colocalized with the BiP ER resident protein and also with each other. In addition, no nuclear or

mitochondrial staining was observed, nor did the TGF-β1 treatment affect the subcellular localization of the complex.

The HyPer1 H_2O_2 sensor was directed into major intracellular compartments in dermal fibroblasts to map the oxidation state prior to TGF-β1 treatment. There was no significant difference in the oxidation state of intacellular compartments in control and TGF-β1-induced cells. Although HyPer1 expressed in the ER lumen was present already in the maximally oxidized form, so we could have only seen a decrease, but the level was unaltered.

H_2O_2 production by Nox4 in TGF-β1-stimulated fibroblasts is readily detected in the extracellular space, although the enzyme complex localizes to an intracellular compartment. We, therefore, became interested in determining the orientation of the Nox4-$p22^{phox}$ complex in the ER membrane. To study the orientation of $p22^{phox}$, we applied a chemically induced protein dimerization technique, which is based on the rapamycin-induced heterodimerization of the mammalian FRB domain with the FK506 binding protein.

We coupled FRB along with the Cyan Fluorescent Protein to the C-terminus of $p22^{phox}$ and FKB12 was labeled with the Yellow Fluorescent Protein shows that the FRB-CFP labeled $p22^{phox}$ localized to the ER, whereas the FKB12-YFP protein was cytosolic. After the addition of rapamycin the FKB12-YFP protein rapidly relocated to the ER indicating that the dimerization event occurred on the cytosolic surface of the ER. In other experiments, we introduced the FRB-CFP tag to N-terminus of Nox4. This Nox construct also located to ER and following the addition of rapamycin, the YFP-linked FKBP12 showed colocalization with Nox4. Based on these experiments, the orientations of $p22^{phox}$ and Nox4 are compatible with ROS release into the lumen of the endoplasmic reticulum.

Conclusion

It was found by mutation analysis of the new FRET-based real-time hydrogen peroxide level PerFRET sensor, which cysteine residues from the cysteine-rich regions of the assembled Orp1 and Yap1 is essential for the proper functioning of the probe. Both Orp1 and the cCRD of Yap1 contain three cysteines residues, of which upon oxidation by H_2O_2 resulting in disulfide bridges forming conformational changes, the fluorophores fused to them distancing and reducing their emissions. We monitored the reactivity of mutated sensors to addition of external H_2O_2 in HeLa cells. The Orp1 C36S mutant fully aborted the H_2O_2 sensing. Originally in yeast, the C36 cysteine of Orp1 forms also an intermolecular disulfide bridge with the C598 cysteine of Yap1. We experienced that in our probe C620 of Yap1 is the partner of Orp1 C36. The other two cysteines of Orp1 and Yap1 are not essential, however certain mutation combinations reduced the FRET ratio. So these cysteine residues probably support the formation of proper conformation with additional disulfide bridges for the maximal response of the probe.

Currently the most intensively studied dedicated ROS source is the Nox4, which was originally identified as renal oxidase in kidney epithelial cells. Subsequent studies revealed that the expression of Nox4 is not restricted to the kidney; we have characterized the Nox4-$p22^{phox}$ oxidase complex in human primary lung and dermal fibroblasts and also in mice tail-tip fibroblasts. According to our results the Nox4 system is constitutively active during TGF-β1 induced differentiation of fibroblast to myofibroblast. The produced ROS is H_2O_2, which was detected in the extracellular space of the cells. We observed that the H_2O_2 production was failed in absence of Nox4 or $p22^{phox}$. We confirmed this observation in mice tail tip fibroblast based genetic models.

Our transcriptional and translational experiments have shown that $p22^{phox}$ is essential and sufficient interactional partner for stabilizing the Nox4 complex.

We showed for the first time that the relationship between Nox4 and $p22^{phox}$ is asymmetrical. Both mRNA and protein expression of $p22^{phox}$ are independent of neither induction nor Nox4 presence, but the Nox4 is unstable without $p22^{phox}$ or TGF-β1 induction in fibroblast cells.

Conflicting data were published regarding the expression pattern of Nox4 in different intracellular locations within the cell; hence we built a heterologous dimerization system to see where it is located. We concluded that after induction the Nox4 complex was formed in the ER as its H_2O_2 production released into the luminal site of the ER. These observations were verified by immunohistochemical staining; and indirectly by fluorimetrical single-cell measurements, where we could not detect any footprints of extra H_2O_2 production during activation in less oxidative compartments than the ER. Future studies are needed to clarify the possible H_2O_2 channels, or connection among plasma membrane and ER.

Our results suggest that after activation of myofibroblast differentiation the Nox4 and its stabilizing partner, $p22^{phox}$ is formed a constitutively active NADPH oxidase complex in the ER membrane, where its H_2O_2 production may support the increased requirements of oxidative milieu in the luminal site of ER.

Own publication list

Publications on which the dissertation is based:

1. **Zana M.**, Péterfi Z., Kovács H., Tóth E.Zs., Enyedi B., Morel F., Paclet M.H., Donkó Á., Morand S., Leto T.L., Geiszt M.
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Antioxid Redox Signal. 2013 Aug 20;19(6):523-34.
IF:7,667

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1. Sirokmány G., Pató A., **Zana M.**, Donkó Á., Bíró A., Nagy P., Geiszt M.
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2. Donkó Á., Morand S., Korzeniowska A., Boudreau H.E., **Zana M.**, Hunyady L., Geiszt M., Leto T.L.
Hypothyroidism-associated missense mutation impairs NADPH oxidase activity and intracellular trafficking of Duox2

Free Radic Biol Med. 2014 Aug;73:190-200.
IF: 5,736