ROLE OF DUAL OXIDASE 1 IN SQUAMOUS CELLS

Ph.D. thesis

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Introduction

Mammalian NADPH oxidases, namely NADPH oxidase (NOX) 1-5 and Dual oxidase (DUOX) 1 and 2, are the primary source for regulated production of reactive oxygen species (ROS) in mammalian cells. These membrane-integrated enzymes transport an electron across the membrane to reduce molecular oxygen into superoxide. Superoxide is then rapidly, spontaneously, or enzymatically converted to hydrogen peroxide. Most isoforms require subunits or auxiliary proteins, such as $P22^{PHOX}$ or DUOXA1, or DUOXA2, for proper function. The presence of at least one of the NADPH oxidases has been described in almost all organs, most isoforms show characteristic tissue distribution, and in some cases, their physiological role is known, such as the importance of NOX2 in host defense or the crucial role of DUOX2 in thyroid hormone synthesis. However, several unanswered questions remain about the function of other isoforms.

DUOX enzymes contain seven transmembrane α-helical domains, including two hemes that transfer electrons from NADPH-FAD through the membrane. Additionally, DUOX enzymes possess an N-terminal, extracellular peroxidase-homology domain and two calcium-binding EF-hand motifs. Notably, DUOX1 can be stimulated by calcium through these EF hands. Without DUOXA1 maturation factor, DUOX1 is not

properly glycosylated and remains in the endoplasmic reticulum.

DUOX1 was first described in the thyroid gland but is expressed in airway epithelial cells, gastrointestinal tract, and bladder. The role of DUOX1 in thyroid hormone synthesis seems insignificant compared to DUOX2. Its function in host defense has been studied, especially in the airways and gastrointestinal tract, where it seems to be associated with lactoperoxidase activity. DUOX1 has been extensively studied in the context of wound healing, particularly in zebrafish. Besides its presence in other tissues, DUOX1 is highly expressed in skin, but its physiological role there is poorly understood.

Objectives

The aim of my work was to gain a better understanding of the role of the hydrogen peroxide-producing NADPH oxidase, DUOX1 in squamous cells. Therefore, the following objectives were set:

1. to investigate the source of epidermal growth factor (EGF)-induced hydrogen peroxide production in squamous cells.
2. to characterize the molecular mechanism of hydrogen peroxide production induced by increased intracellular calcium concentration.
3. to identify the molecular targets of DUOX1-produced hydrogen peroxide.
4. to confirm the presence of DUOX1 in mouse skin.
5. to develop an antibody against mouse and human DUOX1 and establish a genetically controlled system to verify the specificity of the antibodies.
6. to investigate the selectively altered nociceptive behavior of DUOX1-deficient mice.

Methods

Cell culture

A431, HEK293, HEK293T, HEK293A, and HaCaT human cell lines and primary mouse keratinocytes prepared from the back skin of wild-type and DUOX1-deficient mice were used.

Animals

Duox1 knockout mice (Model #TF1226, Taconic) and C57BL/6N wild-type mice were utilized. Experiments were approved by the Ethics Committee on Animal Research of the University of Pécs.

Cutaneus nociceptive tests

Mustard oil-induced thermal hyperalgesia on the tail and formalin test were conducted. For the first experiment, an increasing temperature water bath was used and thermal hyperalgesia was evoked by immersing the tail into TRPA1 agonist allyl isothiocyanate (AITC) containing mustard oil for 30 sec. After the pretreatment, their tail was hung into the water bath. The bath temperature was considered a noxious heat threshold when the mouse removed its tail.

For formalin test, paw lickings and paw flinches after intraplantar formalin injection were measured, which resulted in a composite pain score (composite pain score=(2x paw licking time + 1x paw flinchings)/observation time). The

swelling induced by formalin was determined 3 h after injection by measuring the thickness of the paw with a digital caliper.

Amplex Red assay

We detected the amount of extracellular hydrogen peroxide with Amplex Red fluorometric assay. In the presence of horseradish peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin.

Calcium imaging

Cells were loaded with calcium-sensitive, fluorescence dye, Fura-2-AM. Ratiometric fluorescence intensity was measured by an inverted microscope.

siRNA transfection

Stealth siRNAs were transfected in 20 nM final concentration. Measurements were carried out 48 h after transfection.

Western blot experiments

Proteins were detected by Western blot. Samples were never boiled when processed for DUOX1 detection.

Non-commercial DUOX1 antibodies

A rabbit polyclonal anti-DUOX antibody, which was raised against the Arg618-His1044 fragment of human DUOX1 was applied. Later our own rabbit polyclonal antibody was developed against a 411-amino-acids-long sequence of recombinant human DUOX1 (amino acids 622-1032). This

antigen was produced and purified from BL21 Competent Cells and injected intracutaneously with Freund's adjuvant into New Zealand white rabbits. For polyclonal antibodies, rabbits were sacrificed, and antibodies were affinity purified from the sera using Affigel 10 beads.

DUOX1 CRISPR in HaCaT

HaCaT cells were genetically mutated for DUOX1, using a pSpCas9(BB)−2A-GFP vector, following Target Sequence Cloning Protocol by ZhangLab.

Biotinylation of reduced thiols

HEK293T cells were transfected with FLAG-tagged KCNQ4. The next day the transfected cells were treated with H_2O_2 and collected in lysis buffer with biotin polyethyleneoxide iodoacetamide (BIAM). FLAG-tagged KCNQ4 proteins were immunoprecipitated with monoclonal anti-FLAG M2 antibody.

Biotinylation of reversibly oxidized thiols

The experiment started like the biotinylation of reduced thiols. After H_2O_2 treatment, biotin labeling of reversibly oxidized thiols was conducted. KCNQ4 was immunoprecipitated, and signals were detected by Western blot.

Biotinyl tyramide assay

HaCaT and HaCaT *DUOX1* knockout cells were treated with thapsigargin in the presence or absence of horseradish peroxidase (HRP) and biotinyl tyramide. In the presence of

H_2O_2, HRP converts a tyramide substrate into a highly reactive form that can covalently bind to tyrosine residues on proteins in the immediate proximity of H_2O_2 production. The biotinylated tyramide reagent is visualized through incubation with fluorescently labeled streptavidin. Cells were fixed and cell nuclei were stained with To-Pro-3. The fluorescent streptavidin signal was detected by fluorescent microscope.

Quantitative PCR

Mouse tissue RNA was isolated from dorsal root ganglions, hind paw, and tail skin. Human keratinocyte RNA was also purified. TaqMan Gene Expression Assays were applied. For each cDNA sample, the expression of the target was divided by the expression of the endogenous control.

Measurement of ATP secretion

Fluorescence ATP sensor, $GRAB_{ATP}$ expressing HEK293A cells were treated with GSK 1016790A, H_2O_2 or ATP and fluorescence changes were detected.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism 7.0 and Origin Pro 8 software programs.

Results

EGF stimulates H_2O_2 production in A431 and HaCaT cells

We applied Amplex Red fluorometric assay to assess H_2O_2 production in A431 and HaCaT cells after EGF stimulation. Both cell lines showed increased H_2O_2 production compared to non-treated control cells. The stimulated cells produced approximately 3 nmol/h/10^6 cells H_2O_2.

Intracellular Ca^{2+}-induced H_2O_2 production in keratinocyte cell lines

Using Fura-2-AM, we confirmed the increase in intracellular calcium levels after EGF stimulation in both A431 and HaCaT cells. To elicit the maximal Ca^{2+} response, cells were treated with thapsigargin, a non-competitive inhibitor of endoplasmic reticulum Ca^{2+}-ATP-ase (SERCA).

We also found that calcium plays an essential role in EGF-induced H_2O_2 production. Treatment with BAPTA-AM, a calcium chelator, prior to EGF stimulation prevented the previously observed growth factor-evoked increase in H_2O_2 production. Furthermore, we used various stimuli to elicit intracellular calcium signals and assessed H_2O_2 production, which increased in response to thapsigargin, ATPγS, niacin, and TRPV4 agonist GSK 1016790A in both cell lines.

Expression of NADPH oxidases in A431 and HaCaT cells

NADPH oxidases are the primary source of regulated ROS production in mammalian cells. We investigated their involvement in EGF-induced H_2O_2 production and found that *DUOX1* was the most abundantly expressed NADPH oxidase isoform, and its maturation factor *DUOXA1* was also highly expressed in both A431 and HaCaT cells. We also confirmed the presence of DUOX1 at the protein level.

Role of DUOX1 in intracellular Ca^{2+}-induced H_2O_2 production

Intracellular calcium signal stimulates DUOX1 activity via the calcium-binding EF hand of the enzyme. We investigated the role of DUOX1 in the H_2O_2 response to intracellular Ca^{2+} levels increase. Silencing of *DUOX1* by siRNA treatment reduced the basal production of H_2O_2, and there was no significant increase in ROS generation in response to either EGF or thapsigargin stimuli. Silencing of DUOX1 maturation factor, *DUOXA1* had a similar effect on EGF-induced H_2O_2 production.

We wanted to investigate whether DUOX1-derived H_2O_2 modulates the intracellular calcium level in A431 or HaCaT cells. After siRNA treatment, cells were loaded with FURA-2-AM, then stimulated with EGF and thapsigargin. SiRNA-mediated downregulation of *DUOX1* did not affect the stimuli-induced increase in intracellular calcium.

Pattern of tyrosine phosphorylation in the absence of DUOX1 activity

We investigated the effect of DUOX1 silencing on tyrosine phosphorylation pattern in response to EGF, but no visible difference was observed. We used Human Phospho-Kinase Array Kit to analyze phosphorylation patterns of 43 kinase phosphorylation sites and two related total proteins, but no significant differences were found between control and silenced samples.

Distinct behavioral responses to nociceptive stimuli in *Duox1* knockout and wild-type mice

We studied DUOX1-deficient mice and subjected them to different tests. Collaborating with Zsuzsa Helyes' laboratory in Pécs, we used the animals for nociception experiments. Although I was not involved in these measurements, I would like to present two important results because, during my Ph.D., part of my work was looking for an explanation of these phenomena.

Duox1 knockout and wild-type animals displayed the first significant difference in the AITC containing mustard oil-induced thermal hyperalgesia test. AITC stimulates capsaicin-sensitive sensory nerve terminals, and they release proinflammatory neuropeptides, causing acute neurogenic inflammation and consequently increased sensitivity toward

thermal stimuli. In every timepoint after the pretreatment with AITC *Duox1* knockout animals thermonociceptive threshold was significantly lower than wild-types. The decrease of the thermonociceptive threshold of the pretreated DUOX1-deficient and control mice was also significantly different.

DUOX1-deficient mice displayed increased paw licking and flinching after intraplantar formalin injection, resulting in a higher composite pain score. However, swelling induced by formalin was not significantly different from control mice. The formalin-induced behavioral response also involves TRPA1 activation, as the above-detailed AITC treatment.

Our team has investigated if there are any observable changes in the structure and differentiation of mouse skin. We used paraffin-embedded tissue sections of tail and paw skin from both wild-type and *Duox1* knockout mice, which were then stained with hematoxylin and eosin or labeled with specific antibodies. However, we were unable to detect any observable difference between the wild-type and DUOX1-deficient samples.

Establishment of Duox1 knockout HaCaT cell line via CRISPR-Cas9

DUOX1 knockout HaCaT cell line was established by CRISPR-Cas9 technique. The success of the gene modification was first verified by Surveyor assay and then by sequencing. Loss of a

guanine and a cytosine nucleotide led to a frame-shift mutation and an early stop codon.

To confirm the lack of DUOX1 activity, H_2O_2 production was measured after GSK 1016790A, ATPγS, and thapsigargin. H_2O_2 generation of HaCaT wild-type cells was significantly increased after the treatments, while stimulated *DUOX1* KO cells did not show any significant difference compared with the knockout control.

To visualize the presence or lack of DUOX1 function, we applied tyramide signal amplification-based assay. Using this technique, we can detect the DUOX1-dependent generation of H_2O_2 on a cultured monolayer of keratinocytes. Biotinylated tyramide and HRP were added into the medium of thapsigargin-stimulated or non-stimulated wild-type or DUOX1-deficient HaCaT cells. The biotinylated tyramide reagent was visualized by fluorescence streptavidin. At cell-cell borders of thapsigargin-stimulated wild-type cells, intense tyramide labeling was observed. Despite the treatment, no signal was detected in the absence of DUOX1.

Antibody development against human DUOX1

At the same time the *DUOX1* knockout HaCaT cell line was established, we started developing antibodies against human DUOX1. We immunized rabbits with a human DUOX1 sequence to produce an antibody. The antibody works in

Western blot, and recognizes endogenous DUOX1 in HaCaT wild-type cells and wild-type tissue lysate from mouse skin and bladder, but not in immunohistochemistry or immunocytochemistry.

Expression and activity of DUOX1 in mouse skin

After successfully developing an antibody, we returned to the original question and started investigating the role of DUOX1 in mouse skin. We confirmed the presence of DUOX1 in mouse skin both at RNA and protein level. It was abundantly expressed in the tail and paw skin, and in primary mouse keratinocytes. Finally, we also wanted to investigate protein activity. Therefore we applied Amplex Red reagent to detect H_2O_2 production by primary mouse keratinocytes. Wild-type and *Duox1* KO cells were stimulated with GSK 1016790, and thapsigargin. While in the presence of DUOX1, there was a significant increase in H_2O_2 production, in the absence of the enzyme, the cells did not respond to the stimuli.

ATP release from stimulated keratinocytes

Keratinocytes release adenosine-5′-triphosphate (ATP) through temperature increase and respond to mechanical stimulation by increasing intracellular calcium concentrations. We assumed that stimuli that cause an increase in intracellular calcium concentrations would simultaneously activate DUOX1 and stimulate the secretion of ATP. To investigate the role of

DUOX1 in ATP release, we used a genetically encoded, fluorescence ATP sensor, $GRAB_{ATP}$. The binding of ATP enhances the fluorescence of cpEGFP. Wild-type or DUOX1 deficient HaCaT cells were cocultured with $GRAB_{ATP}$ expressing HEK293A cells and stimulated with GSK 1016790A. We observed transient local oscillations followed by sustained high signals of extracellular ATP in GRABATP expressing cells. However, no obvious difference was detected between activation of sensor cells cocultured with wild-type or DUOX1-deficient HaCaT cells.

Expression of transient receptor potential receptors and redox-sensitive ion channels in dorsal root ganglia and skin

We analyzed the transient receptor potential (TRP) receptor family in wild-type and *Duox1* knockout skin but found no significant differences in the expression of the channels involved in thermosensory function. We turned our interest to DUOX1-produced H_2O_2. It is known that H_2O_2 can also act as a signaling molecule. Therefore, we browsed the literature to find a possible target for DUOX1-produced H_2O_2. We hypothesized that the keratinocyte-derived H_2O_2 might also act on the primary sensory neuron. Ion channels and regulators of ion channels were analyzed by qPCR, which have been previously described as redox-sensitive proteins.

The expression pattern showed no difference between wild-type and *Duox1* knockout dorsal root ganglia. In the following, we have investigated two redox-sensitive proteins in more detail.

H_2O_2-mediated redox changes of TRPA1-dependent intracellular calcium signal

To examine the redox sensitivity of the TRPA1, we expressed recombinant TRPA1 channels in HEK293T cells, loaded them with Fura-2-AM, and followed their intracellular calcium signals upon treatment with H_2O_2 and a TRPA1 agonist, AITC. Following the addition of H_2O_2, the TRPA1-expressing HEK cells - in contrast to the non-transfected cells - displayed an increase in intracellular calcium level. Additionally, after pretreatment with H_2O_2, the AITC-evoked relative response was much smaller than in the non-pretreated cells. Consequently, H_2O_2 significantly affects the response of cells to allyl isothiocyanate.

Expression and H_2O_2 mediated redox changes of KCNQ4 potassium channel

The activity of the voltage-gated M-type potassium channel, KCNQ4 is enhanced by oxidative modification. Prolonged maintenance of the open state results in hyperpolarization and a decrease in the frequency of action potential firing. Using qPCR, we showed that *Kcnq4* is present in the DRG and in

negligible amounts in the skin. Next, we confirmed the redox sensitivity of KCNQ4 through experiments using biotinylated iodoacetamide (BIAM) to alkylate cysteine thiol groups. If KCNQ4 bound BIAM, it could be detected by streptavidin-HRP on Western blot. However, if the thiol reacted with H_2O_2, it could no longer bind BIAM and no signal was detected. The higher concentration of H_2O_2 was used, the weaker the biotin signal was, because oxidized protein can not bind BIAM and streptavidin-HRP. To confirm these results, and examine the reversibility of oxidation we also carried out a reverse BIAM labeling. In this case, after the H_2O_2 treatment, we first alkylated the non-oxidized KCNQ4 with N-ethyl-maleimide, then we reduced all the reversibly oxidized molecules with dithiothreitol, and finally, we labeled these reduced molecules with BIAM, then proceeded as in the previous experiment. This way the biotin signal is linearly proportional to the amount of oxidation.

Conclusions

Based on our objectives and the results described above, the following conclusions can be drawn:

1. Dual Oxidase 1 is the source of the epidermal growth factor-induced H_2O_2 production in squamous cells.
2. Other mediators (niacin, ATPγS, TRPV4-agonist) can also activate DUOX1.
3. DUOX1 is the most abundant NADPH oxidase in A431, HaCaT cells and mouse keratinocytes.
4. The amount of PGE2 and ATP released from keratinocytes was found to be similar in both wild-type and DUOX1-deficient keratinocytes.
5. We identified TRPA1 and KCNQ4 as potential targets of DUOX1-produced H_2O_2, which might explain the altered nociceptive behavior of DUOX1-deficient mice.

Bibliography of the candidate's publications

Candidate's publications related to the thesis:

1. **Pató A**, Bölcskei K, Donkó Á, Kaszás D, Boros M, Bodrogi L, Várady G, Pape VFS, Roux BT, Enyedi B, Helyes Z, Watt FM, Sirokmány G, Geiszt M. *Hydrogen peroxide production by epidermal dual oxidase 1 regulates nociceptive sensory signals*. Redox Biol. 2023
2. Sirokmány G, **Pató A**, Zana M, Donkó Á, Bíró A, Nagy P, Geiszt M. *Epidermal growth factor-induced hydrogen peroxide production is mediated by dual oxidase 1*. Free Radic Biol Med. 2016

Candidate's publications unrelated to the thesis:

1. Scherr AL, Jassowicz A, **Pató A**, Elssner C, Ismail L, Schmitt N, Hoffmeister P, Neukirch L, Gdynia G, Goeppert B, Schulze-Bergkamen H, Jäger D, Köhler BC. *Knockdown of ATG7 Induces Nuclear-LC3 Dependent Apoptosis and Augments* Chemotherapy in Colorectal Cancer Cells. Int J Mol Sci. 2020
2. Birtalan E, Danos K, Gurbi B, Brauswetter D, Halasz J, Kalocsane Piurko V, Acs B, Antal B, Mihalyi R, **Pato A**, Fent Z, Polony G, Timar J, Tamas L. *Expression of PD-L1 on Immune Cells Shows Better Prognosis in*

Laryngeal, Oropharygeal, and Hypopharyngeal Cancer. Appl Immunohistochem Mol Morphol. 2018

3. Dános K, Brauswetter D, Birtalan E, **Pató A**, Bencsik G, Krenács T, Peták I, Tamás L. *The Potential Prognostic Value of Connexin 43 Expression in Head and Neck Squamous Cell Carcinomas.* Appl Immunohistochem Mol Morphol. 2016