CHARACTERIZATION OF PEROXIDASIN AND PEROXIDASIN-LIKE PROTEINS IN MAMMALIAN CELLS

Ph.D. Thesis

Hajnal A. Kovács M.D.

Doctoral School of Molecular Medicine
Semmelweis University

Supervisor:
Miklós Geiszt M.D., D.Sc.

Official Reviewers:
Beáta Lontay Ph.D.
Nikolett Wohner M.D., Ph.D.

Head of the Final Examination Committee:
Gábor Varga D. Sc.

Members of the Final Examination Committee:
Tamás Kardon M.D., Ph.D.
Katalin Erdélyi Ph.D.

Budapest
2021

1. Introduction

Reactive oxygen species (ROS) are usually considered as harmful chemical agents due to their high reactivity. Examples are superoxide, hydroxyl, hydroperoxyl radicals, hydrogen peroxide (H_2O_2), and organic peroxides. They can be produced spontaneously or enzymatically. Uncontrolled ROS production can cause oxidative damage to DNA, proteins, and lipids. On the other hand, ROS has several physiological functions in the body, such as host defense, extracellular matrix (ECM) modification, hormone synthesis, cell signaling, or even balance sensation. Important regulated ROS sources are the NADPH oxidases (Nox/Duox). The Nox/Duox family often cooperates with heme peroxidases. Peroxidase enzymes oxidize their substrates with the help of H_2O_2. In different kingdoms of life, there are four heme peroxidase superfamilies. These proteins contain heme as a prosthetic group in the catalytic site. One of the superfamilies is the peroxidase-cyclooxygenase superfamily. The unique feature of its members is that the heme group is covalently bound to the protein. Six members of the superfamily are expressed in mammals: myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), thyroid peroxidase (TPO), peroxidasin (PXDN), and peroxidasin-like (PXDNL) proteins. MPO, EPO, and LPO are important in host defense, TPO is essential for thyroid hormone synthesis, PXDN is necessary for the crosslinking of collagen IV molecules in the basement membrane, and the function of PXDNL is currently unknown. This Ph.D. thesis focuses on two of the less-known

members: PXDN and PXDNL. The two proteins have the same domain structure, and besides their peroxidase homology domain, they have domains characteristic of ECM proteins (Leucine-rich repeats, C2 type Ig-like domain, C-type vWf domain). One crucial difference between PXDN and PXDNL is that PXDNL doesn't have peroxidase activity due to changes in amino acids of the catalytic site. PXDN catalyzes the formation of crosslinks between Met93 and hydroxylysine (Hyl)/lysine (Lys) 211 of two neighboring NC1 domains of collagen IV. The reaction requires H_2O_2 and bromide, and the result is the formation of a sulfilimine covalent bond. Collagen IV is the major component of basement membranes. Basement membranes are complex, three-dimensional ECM formations underlying endothelial and epithelial cells, but collagen IV can also be found between adipocytes and different kinds of muscle cells.

The proteolytic processing of PXDN by proprotein convertases was described before *in vitro*. We wanted to characterize this processing of the protein.

The biological function of PXDN in *Caenorhabditis elegans* is related to epidermal elongation and epidermal-muscle interactions. Peroxidasin was first discovered in *Drosophila melanogaster*. In this animal, it is also important for the basement membrane integrity. PXDN mutation in mice and humans causes an ocular developmental defect called anterior segment dysgenesis (ASD). PXDN was also linked to cancer pathology (primary and metastatic brain tumors, melanoma, ovarian and prostate cancer, and oral squamous cell carcinoma).

PXDNL function is currently unknown, the gene is absent from mice and rats, but it is expressed in humans and many other mammalian species. Our group described and characterized the human PXDNL first. According to our observations, PXDNL mRNA is only detectable in the heart from the examined human tissues. Furthermore, the protein was related to several diseases like breast cancer, schizophrenia, bipolar disorder, major depressive disorder, and laterality defects.

2. Objectives

We made the following objectives:

1. Establishment of a PXDN knockout PFHR-9 cell line (KO PFHR-9) with the CRISPR-Cas9 technique.

2. Development of a mouse model with CRISPR-Cas9 technique, which expresses an N-terminally hemagglutinin (HA)-tagged PXDN (HA-PXDN).

3. Identification of the proprotein convertase which processes PXDN.

4. Studying the crosslinking activity, proprotein convertase processing, and localization of various double-tagged PXDN constructs.

5. Investigating the role of peroxidase activity in the proteolytic processing of PXDN and PXDNL.

3. Results

3.1 Establishment of a PXDN knockout PFHR-9 cell line (KO PFHR-9) with the CRISPR-Cas9 technique.

We used a mouse embryonic cancer cell line, PFHR-9, as a model system for our experiments. This cell produces a large amount of ECM rich in collagen IV and expresses PXDN. In wild type (WT) PFHR-9 cell lysates, we could detect the crosslinked NC1 dimers and uncrosslinked NC1 monomers. To study various PXDN constructs, we made a PXDN knockout PFHR-9 cell line with the help of the CRISPR-Cas9 technique. We proved the pxdn gene modification with Surveyor mismatch analysis and sequencing. With western blot experiment, we showed the absence of PXDN protein and the lack of NC1 dimers in the KO PFHR-9 cell lysates compared to the WT PFHR-9 cells.

3.2 Development of a mouse model with CRISPR-Cas9 technique, which expresses an N-terminally hemagglutinin (HA)-tagged PXDN (HA-PXDN).

To study PXDN's *in vivo* proprotein convertase processing and localization in mice, we have made a novel knockin animal model. The mouse expresses an N-terminally hemagglutinin (HA)-tagged PXDN (HA-PXDN). This modification was achieved with the CRISPR-Cas9 technique. We thought if proprotein convertase processing of PXDN occurs *in vivo* in mice, we will detect two HA-specific bands around 170 kD. We confirmed this hypothesis with western blot analysis of the kidney and lung lysates of the HA-PXDN mice. We had the same observation with a PXDN-specific polyclonal antibody in the same

organs of WT and HA-PXDN mice. The tag did not effect on the function of PXDN, as we could not observe any phenotypic changes in the knockin mice. Furthermore, the crosslinking activity of PXDN was unaffected by the HA-tag. Based on these experiments, we concluded that proprotein convertase processing of PXDN can happen *in vivo*. We also examined the localization of PXDN in the newly developed mouse. We immunostained the developing mouse eye prepared from fetuses. Immunostaining of HA-PXDN revealed intense staining in the lens, and we could observe a network-like pattern inside the lens and strong labeling at the borders of the lens.

3.3 Identification of the proprotein convertase which processes PXDN.

We prepared a primary cell culture from HA-PXDN mice embryos (mouse embryonic fibroblast, MEF). In MEF culture, we could study the effect of proprotein convertase inhibitor Decanoyl-Arg-Val-Lys-Arg-Chloromethylketone (CMK), which is used to block furin/proprotein convertases. CMK treatment of the HA-PXDN MEF decreased the lower PXDN-specific band's intensity, representing the proteolytically processed form of the protein. We also studied the processing of PXDN in WT PFHR-9 cells. Treatment of the cells with CMK decreased the lower PXDN-specific band's intensity. In addition, we conducted experiments with furin-specific siRNA as well on the PFHR-9 cells. Furin-specific siRNA treatment could reduce the processing. We confirmed that furin has a role in the cleavage of PXDN.

3.4 Studying the crosslinking activity, proprotein convertase processing, and localization of various double-tagged PXDN constructs.

To study the behavior of various PXDN constructs in a cell culture model, we used the KO PFHR-9 cells. We made a double-tagged PXDN construct with a FLAG-tag at the N-terminus after the signal peptide and a V5-tag at the C-terminus. This double-tagged, full-length PXDN coding plasmid can be detected with a V5 antibody as a single band around the predicted molecular weight of PXDN. Meanwhile, with the FLAG antibody, we can see a double band at that molecular weight of PXDN. The lower band represents the cleaved form of PXDN. The newly developed double-labeled PXDN is functional can catalyze the crosslinking of collagen IV. We immunostained the double-tagged PXDN with anti-FLAG-, and anti-V5-antibodies. We could observe an intracellular, cell-associated V5 signal and the FLAG signal, besides being cell-associated, it was also extracellularly located in a network-like structure.

We examined with our newly developed tools how the proprotein convertase processing of PXDN can influence the crosslinking activity- and localization of PXDN. We mutated the predicted cleavage sites of PXDN, Arg1335 and Arg1336 were changed to alanines in one plasmid (RR/AA 1335-1336), and Arg1354 and Lys1355 were mutated to alanines in another construct (RK/AA 1354-1355). We also made a double mutant construct that carries both mutations. The transfection of RR/AA 1335-1336 construct into KO PFHR-9 cells resulted in reduced proteolytic processing of PXDN, and the collagen IV crosslinking

activity was reduced as well. In addition, the mutant PXDN was deposited extracellularly in a net-like pattern, but the V5 signal was only cell-associated.

We also examined the behavior of a PXDN mutant that is unable to form oligomers. Transfection of KO PFHR-9 cells with the trimerization mutant form of PXDN (C736S, C1315S) resulted in a slightly reduced cleavage of PXDN. Localization of the mutant PXDN showed a more diffuse extracellular staining, and the V5-signal was cell-associated.

We wanted to figure out where the cleavage of PXDN occurs. We used a mutant of PXDN that cannot leave the cell because of an ER-retention signal (KDEL). The mutant PXDN (KDEL) was less processed and had no crosslinking activity. The immunostained ER-retention mutant was located intracellularly.

3.5 Investigating the role of peroxidase activity in the proteolytic processing of PXDN and PXDNL.

We investigated whether the peroxidase activity of PXDN has any effect on proteolytic processing. We used a known peroxidase inhibitor, phloroglucinol (PHG). The PHG treatment reduced the proteolytic processing of the wild type PXDN. We transfected the KO PFHR-9 cells with a peroxidase activity-mutant PXDN construct as well. The mutant PXDN's (Q823W, D826E) proteolytic processing was reduced compared to the wild type PXDN. The localization of this construct had a similar pattern as in the case of the wild type PXDN.

Next, we examined the proteolytic processing of PXDNL. We found a candidate site at Arg1319 for cleavage of PXDNL. The sequence of the site was different from the one in PXDN. We made a double-tagged (FLAG-tag at the N-terminus, V5-tag at the C-terminus) full-length PXDNL coding construct to study the proteolytic processing of PXDNL. We couldn't observe any processing after transfection of the KO PFHR-9 cells with this plasmid (PXDNL). We thought this might result from the inability of the protease enzyme to recognize the potential cleavage site. To test this idea, we made another PXDN mutant in which the endogenous proprotein convertase recognition site is replaced with the one in the PXDNL sequence. This mutant (RGR/QKK1333-1335) was effectively cleaved in the KO PFHR-9 cells. This observation, combined with the one we made with PHG treatment and with the Q823W, D826E mutant PXDN, suggests that the peroxidase activity of these enzymes affects their posttranslational proteolytic processing.

4. Conclusions

Based on our observations, we can make the following conclusions:

1. We created a PXDN knockout PFHR-9 cell line (KO PFHR-9) with the CRISPR-Cas9 technique. The cells don't produce a detectable amount of PXDN, and they have an uncrosslinked collagen IV network.

2. We made a new mouse model with the CRISPR-Cas9 technique, which expresses an N-terminally hemagglutinin (HA)-tagged PXDN (HA-PXDN). In this animal, we proved that proprotein convertase processing of PXDN could occur *in vivo*. We also studied the localization of PXDN in the developing eye of this mouse strain.

3. In a primary cell culture (MEF), which we prepared from the HA-PXDN mice embryos, and in PFHR-9 cells, we could decrease the processing of PXDN with a furin/proprotein convertase inhibitor (CMK). Furthermore, in PFHR-9 cells with furin-specific siRNA treatment, we could identify that furin has a role in the proteolytic processing of PXDN.

4. We studied the proprotein convertase processing, crosslinking activity, and localization of a double-tagged wild type, proprotein convertase site mutated, trimerization-, and ER-retention mutant PXDN in KO PFHR-9 cells. We confirmed that the proteolysis site is at 1336Arg and that the cleavage site mutant PXDN has decreased crosslinking activity. Furthermore, we proved that only the processed form of PXDN could be built into the ECM. The trimerization mutant cleavage was slightly decreased, and the ER-retention mutant was not processed and didn't have crosslinking activity.

5. We found that in the case of the pharmacological inhibition of PXDN's peroxidase activity, the proteolytic processing of PXDN is decreased. We saw reduced processing in the peroxidase activity mutant PXDN as well. We made a double-tagged PXDNL and examined its processing. According to our experiments, PXDNL is not cleaved in KO PFHR-9 cells even though it has a potential cleavage site for proprotein convertase enzymes.

5. Bibliography of the candidate's publications

The Ph.D. thesis is based on the following publications:

1. **Kovács, Hajnal A**.; Lázár, Enikő; Várady, György; Sirokmány, Gábor; Geiszt, Miklós

Characterization of the Proprotein Convertase-Mediated Processing of Peroxidasin and Peroxidasin-like Protein

ANTIOXIDANTS 10 : 10 Paper: 1565 , 17 p. (2021)

IF (2020): 6,312

2. Sirokmány, G; **Kovács, HA**; Lázár, E; Kónya, K; Donkó, Á; Enyedi, B; Grasberger, H; Geiszt, M

Peroxidasin-mediated crosslinking of collagen IV is independent of NADPH oxidases

REDOX BIOLOGY16 pp. 314-321., 8 p. (2018)

IF: 7,793

Other publications which are related to the Ph.D. thesis:

3. Zana, M; Peterfi, Z; **Kovacs, HA**; Toth, ZE; Enyedi, B; Morel, F; Paclet, MH; Donko, A; Morand, S; Leto, TL; Geiszt, M

Interaction between p22(phox) and Nox4 in the endoplasmic reticulum suggests a unique mechanism of NADPH oxidase complex formation.

FREE RADICAL BIOLOGY AND MEDICINE116 pp. 41-49., 9 p. (2018)

IF: 5,657

4. Lázár, E; Péterfi, Z; Sirokmány, G; **Kovács, HA**; Klement, E; Medzihradszky, KF; Geiszt, M

Structure-function analysis of peroxidasin provides insight into the mechanism of collagen IV crosslinking

FREE RADICAL BIOLOGY AND MEDICINE83 pp. 273-282., 10 p. (2015)

IF: 5,784

5. Péterfi, Z; Tóth, ZE; **Kovács, HA**; Lázár, E; Sum, A; Donkó, Á; Sirokmány, G; Shah, AM; Geiszt, M

Peroxidasin-like protein: A novel peroxidase homologue in the human heart

CARDIOVASCULAR RESEARCH 101: 3 pp. 393-399., 7 p. (2014)

IF: 5,940