ANALYSIS OF THE PHYSIOLOGICAL FUNCTIONS OF NOX5 USING A KNOCKOUT RABBIT MODEL SYSTEM

Members of the NOX/DUOX family of NADPH oxidases are primarily responsible for the regulated production of reactive oxygen species (ROS) in living organisms. In humans, there are seven members of the NOX/DUOX family (NOX1-5, DUOX1&2). These enzymes have a wide variety of biological functions and play an important role in immune defense, hormone biosynthesis, and regulation of vascular function. Most members of the NOX/DUOX family of enzymes are also found in mice, so different gene-deficient mice models play an important role in identifying NOX/DUOX functions and analyzing the role of enzymes in disease development. However, NOX5 is absent in rodents, so the function of the enzyme cannot be tested in KO mice. Because NOX5 is found in rabbits, we decided to create a NOX5-deficient rabbit model and use it to study the function of NOX5. The NOX5-deficient rabbit model was generated in collaboration with the rabbit genomics team of the NAIK in Gödöllő, using the CRISPR technique. The CRISPR guide RNA was targeted to exon 3 of the rabbit NOX5 gene. We chose this exon because it is found in all NOX5 splice variants described so far. Targeting NOX5 has resulted in a wide variety of genetic modifications, which are likely to have led to loss of gene function. We performed the vast majority of our experiments on rabbits with a 10 bp deletion in exon 3 of the NOX5 gene (line 700). When applying the CRISPR technique, mutations in genes other than the target sequence sometimes occur (off-target effects). Off-target analysis and sequencing of other NOX cDNAs (NOX1, NOX2, NOX4) showed that the CRISPR-induced mutation was specific for the NOX5 gene. (The NOX3 cDNA was not included in the analysis because it is expressed only in the inner ear). The expression pattern of the rabbit NOX5 gene was studied by QPCR technique and we found NOX5 to be expressed in higher amounts in the testis and lymph node, but NOX5 expression was also detected in the

epididymis and ovary. This expression pattern is similar to that of human NOX5, which is important because it suggests that studying NOX5-deficient rabbits may indeed be relevant in exploring the function of NOX5 in humans. NOX5-deficient animals show no obvious phenotype and they are fertile. Although the latter observation is a "negative" result, it is important because it suggests that NOX5 does not play an essential role in the fertilization process. Although sequencing of exon 3 of the rabbit NOX5 gene suggested the KO phenotype, we confirmed the absence of NOX5 protein by Western blot analysis of lymph node protein lysate. It has been suggested in several publications that NOX5 plays a role in the regulation of vascular function. However, in the absence of a KO animal model no genetic evidence support this notion. We prepared aortic rings from the aortas of wild-type and KO animals and the contraction responses of the rings were examined by wire myography. We found no difference in the responses of wild-type and NOX5 KO vessels, suggesting that in rabbits NOX5 does not play a role in the development of acute vascular responses to vasoactive agonists. We also examined whether NOX5 deficiency affected the blood pressure of the animals and found no significant difference in blood pressure between wild-type and KO animals. Next, we examined whether the development of cholesterol-induced atherosclerosis is altered in the absence of NOX5. We were interested in this because several NOX enzymes have been described to be expressed in different vascular cell types and to affect the development of atherosclerosis. In the case of NOX1 and NOX2, decreased atherosclerosis was observed in KO mice, while the development of more severe atherosclerosis was described in NOX4 KO animals. These experiments were performed in mice that were deficient for both the LDL receptor and the particular NOX gene. Rabbits are very sensitive to cholesterol feeding and are therefore often used as model animals in atherosclerosis research. In our experiments, 12-week-old male rabbits were fed a high-cholesterol (120 g / day) diet for 8 weeks. There was no difference in the amount of food consumed and weight gain between wild-type and KO animals. The

cholesterol-rich diet significantly increased plasma cholesterol levels in both wild-type and NOX5 KO rabbits. At the end of the cholesterol feeding period, the animals were sacrificed and plaque formation in the aorta was detected by Oil Red O staining. In the aortic arch area, intense plaque formation was observed in both wild-type and KO animals, whereas more severe plaque formation was observed in the thoracic section of the aorta in NOX5 KO animals. The composition of atherosclerotic plaques was examined by smooth muscle actin and macrophage staining and no difference was detected between wild-type and KO animals.

The deleterious effects of NOX/DUOX-derived reactive oxygen species (ROS) have been reported previously in several disease models. For example, a pathogenic role of the NOX5 has been described in renal and vascular diseases. However, our experiments were the first to investigate NOX5 function by using KO animals, and in these experiments, we found that NOX5-derived ROS protect against the development of atherosclerosis. The explanation for this protective is currently unknown. We don't think that vascular NOX5 expression explains the protective effect, as only a minimal amount of NOX5 was detected in the rabbit aorta, and NOX5 expression was not significantly altered by cholesterol feeding. The protective effect of NOX5 may be related to its expression in immune cells. The results described above were published in Circulation Research.

Results from other NOX5-related studies

Relatively little is known about the physiological function of the NOX5 enzyme and its role in the development of diseases. Our understanding of the NOX5 function is incomplete because NOX5 is the only NOX isoform that is not expressed in rodents and therefore the most commonly used animal models cannot be used in NOX5 research. Experiments on KO animals are also of great importance in investigating fundamental issues such as the exact identity of NOX5-expressing cells. At the beginning of our work, we hypothesized that NOX5 expressed in spermatozoa has a role in the fertilization process. During the project, however, we had

several “negative” results that fundamentally call into question our knowledge of NOX5 to date. The NOX5 gene was known to be expressed in the testis and the protein was also detected in human spermatozoa. NOX5-deficient male rabbits, on the other hand, are fertile, suggesting that NOX5 function is not essential during fertilization. At the beginning of the project, in a biallelic mutant (F0) animal, we found that the volume of the ejaculate increased and the viability of the spermatozoa was decreased significantly. However, we could not reproduce this finding in additional KO animals.

A serious problem in NOX/DUOX research is the lack of good-quality antibodies. Since we did not have an anti-NOX5 antibody at the beginning of the project, we developed guinea pig polyclonal and mouse monoclonal anti-NOX5 antibodies. We tested these antibodies on cells expressing NOX5 in a heterologous manner and showed that the antibodies specifically recognized NOX5. We could also detect NOX5 in human testis lysate by Western blot, but no protein could be detected in either human or rabbit spermatozoa. NOX5 may be expressed in spermatozoa at such a low level that our antibodies are not sensitive enough to detect it, but we cannot rule out the possibility that NOX5 is not present in spermatozoa, contrary to the results published so far. We are currently testing whether our antibodies are suitable for immunoprecipitation because, if so, then we will try to enrich the protein from larger amounts of spermatozoa lysate.

Using the *in situ* hybridization-based RNAscope technique, we were able to detect NOX5 mRNA in the seminiferous tubules of the testis. Because NOX5 mRNA is located primarily in the periphery of tubes, we think that early precursors of spermatozoa might express NOX5 in the testis. The exact identity of these cells has yet to be determined.

As I mentioned earlier, only low levels of NOX5 mRNA expression were detected in the aorta and we were unable to detect NOX5 by Western blot and the more sensitive RNAscope technique. Because there are reports about the expression of NOX5 in vascular cells, we

examined whether the antibodies we have developed can detect the protein in different primary human vascular cells. In these experiments, human coronary endothelial and smooth muscle cells as well as human aortic smooth muscle cells were studied. We found that NOX5 was not present in the cells we examined, which calls into question the role of NOX5 in vascular ROS production. In these experiments, a human melanoma cell line (UACC-257) that endogenously expresses NOX5 was used as a positive control, and the specificity of the NOX5 signal was confirmed by the siRNA technique. Our results on vascular cells suggest that reliable data on the expression pattern of each NOX/DUOX isoform can only be obtained using properly tested, specific antibodies. Our results on NOX5 expression are planned to be presented in the form of a poster at the 2022 NOX Gordon Conference.

During the project, we developed a HEK 293-based cell line that expresses NOX5 in a heterologous manner. We used these cells in the antibody development, however, we also made several new observations by examining the ROS production of the cells. We have shown that the calcium-induced production of superoxide and hydrogen peroxide is significantly increased in NOX5-expressing cells. These responses could be inhibited by DPI, a non-specific inhibitor of NOX enzymes. In the heterologous expression system, Nox5 was localized to the endoplasmic reticulum (ER) and also showed enrichment in the ER-associated nuclear membrane. Interestingly, the protein was not detected in the plasma membrane, suggesting that ROS produced by the enzyme first appears in the intracellular space. We think that the proximity of ER and plasma membrane may explain that NOX5-derived ROS is detected in the extracellular space.

In plants, NOX-derived ROS affect calcium signaling through the activation of calcium channels. Since NOX5 shows similarity to plant NOX isoforms we wanted to study whether NOX5 has any effect on the calcium signal induced by different agonists. No such effect was observed in cells heterologously expressing NOX5, as inhibition of the enzyme activity by DPI

did not modify the calcium signals elicited by different agonists. However, we can not rule out the possibility that HEK293 cells do not contain the calcium channels or other signaling proteins which are potential targets of NOX5-derived ROS.

Based on our observations in the heterologous expression system, NOX5 protein localizes to the endoplasmic reticulum (ER). From a redox perspective, the ER is a very exciting organelle as the depletion of its calcium content reduces the oxidation state of the ER. Another important observation is that other forms of NOXes (e.g., NOX4) have been previously described in the ER. In our experiments, we examined how NOX5 activation affects the redox state of the ER. We expressed a fluorescent protein-based redox sensor (Hyper) in the ER and we could detect the oxidative effect of exogenously added H_2O_2. However, NOX5 activation did not alter the redox state of the ER, suggesting that the ROS produced by NOX5 do not enter the lumen of the ER. However, we cannot rule out the possibility that the ROS produced by NOX5 is buffered so efficiently that they cannot be detected by the ROS-targeted ROS sensor. In our further experiments, we investigated that ER stress induced by depletion of internal calcium stores is modified in the presence of NOX5. In these experiments, we observed a change in the expression of well-known ER-stress markers GADD34 and IRE1. However, no difference was observed for other ER stressors. We are currently investigating if an altered ER-stress response is also present in tissues isolated from NOX5-deficient rabbits.