ANALYSIS OF HYDROGEN PEROXIDE SIGNALING IN MAMMALIAN CELLS USING NOVEL MOLECULAR TOOLS

Aim of the project

The aim of this research project was to characterize the subcellular organization and function of intracellular H_2O_2 signals

1. Analysis of EGF-induced H_2O_2 production using genetically encoded FRET-based H_2O_2 sensors

Duox1 is involved in the EGF-induced ROS response of epithelial cells. Although this response was described to have an important role in signaling, the source of the ROS was unknown. In our experiments, we worked with two epidermal cell lines (A431 and HaCaT), which have been previously reported to show calcium-dependent ROS production. Quantitative PCR experiment found that Duox1 was the only significant NADPH oxidase isoform in the two cell types and we also detected DuoxA1, which is an activator protein indispensable for Duox activity. In both cell lines, H_2O_2 production in the extracellular space was detected, which was reduced by BAPTA (intracellular Ca^{2+}-chelator). Furthermore, when we inhibited Duox1 expression, the ROS production induced by EGF was inhibited. In further experiments, we determined that the presence of DuoxA1 protein is also important in the EGF-induced H_2O_2 response. We also investigated, whether the EGF-stimulated Duox1-dependent ROS production affects tyrosine phosphorylation and calcium signaling pathways. In cells treated with Duox1 siRNA, there was no change in the tyrosine phosphorylation cascade activated by EGF and no significant difference was observed in the EGF-induced Ca^{2+} signal. According to our results, the EGF-induced ROS response in A431 and HaCaT cells is mediated by the Duox1 enzyme, which is activated through the formation of a Ca^{2+} signal. Interestingly, intracellularly expressed OxyFRET and PerFRET fluorescence ROS sensor proteins failed to detect intracellular ROS production suggesting that ROS produced by Duox1 would only appear in the extracellular space. However, it is also possible that the increase of intracellular H_2O_2 concentration is too small to be captured by the sensors. Since we could not detect a change in intracellular H_2O_2 by the OxyFRET and PerFRET probes expressed in intracellular space, we tried to test other ways whether H_2O_2 produced by Duox1 influenced the intracellular redox milieu. We found that EGF-stimulated HaCaT cells show oxidation of thioredoxin-1 and peroxiredoxin proteins and this effect can be reduced by suppressing Duox1 expression by the siRNA technique. Since we have previously shown that EGF stimulation induced Duox1 activation is the consequence of the agonist-induced Ca^{2+} signal, our results suggest that the calcium signal in Duox1-expressing cells is transformed into a redox signal. These observations also indicate that H_2O_2

produced by Duox1 appears in the cytosol, but the fluorescence sensors are probably not sensitive enough to detect it. A report describing our results was published in *Free Radical Biology and Medicine* (Sirokmany et al. FRBM, 2016).

2. Analysis of TGF-β-induced Nox4-dependent H_2O_2 production in primary fibroblasts

The intracellular localization of the Nox4 enzyme is currently unknown. The protein has already been described in the plasma membrane, nucleus, endoplasmic reticulum and mitochondria. These results, however, have to be treated with reservation because there are currently no antibodies available which reliably detect Nox4. It is known that primary fibroblast cells stimulated by TGF-β produce significant amounts of H_2O_2 which can be detected in the extracellular space. In our experiments, we wanted to see if H_2O_2-sensitive sensors expressed at different intracellular sites could also detect Nox4-derived H_2O_2 within the cell. First, the transfection of primary fibroblast cells had to be adjusted. In our experiments, two different primary human fibroblast cells were used, lung (HPF) and skin (BJ) fibroblasts. The transfection of the cells with conventional lipid-based transfection reagents was not very effective, therefore, the Invitrogen Neon nucleofection system was applied. Using this method, we achieved an efficiency that has enabled microfluorimetric measurements. First, we used Hyper H_2O_2 sensors with different targeting sequences, which localize in the cytosol, ,mitochondria, endoplasmic reticulum, nucleus, or plasma membrane, respectively. Interestingly, the oxidation of the probes at the various intracellular sites was not increased by TGF-β, while H_2O_2 appeared in the extracellular space. The FRET-based H_2O_2 sensors (OxyFREt and PerFRET) were similarly ineffective in capturing the TGF-β-elicited H_2O_2 signals at intracellular sites. Studying the intracellular localization of the $p22^{phox}$ protein that is a component of the Nox4 complex, the protein was primarily found in the endoplasmic reticulum of differentiating cells. Based on this observation, we started to set up and study a cell model where the oxidation state of the endoplasmic reticulum (ER) can be investigated in permeabilized cells using our fluorescent sensors. In our experiments, we found that the inner space of the endoplasmic reticulum is also highly oxidized in permeabilized cells and the oxidation state is surprisingly stable.

Our results obtained in experiments on fibroblasts suggested that the Nox4-$p22^{phox}$ complex is present in the membrane of the endoplasmic reticulum (ER). We were interested in determining the orientation of the enzyme complex in the ER membrane. In order to achieve that we applied a protein dimerization technique. The essence is of this techniques is that a protein (FRB) is attached to the C-terminal portion of $p22^{phox}$, which is able to bind to the cytosolic expressed FKBP12 protein in the presence of rapamycin. To determine the localization of proteins, two different fluorescence proteins were coupled to them. We found

that rapamycin caused the translocation of FKBP-YFP protein to the ER, suggesting that the FRB-CFP labeled C-terminal portion of $p22^{phox}$ looks toward the cytosol. Based on this observation and previous literature data we suggest that the Nox4-$p22^{phox}$ complex produces ROS in the direction of the ER lumen.

The $p22^{phox}$ protein is a partner of several different Nox proteins (Nox1, Nox2, Nox3, Nox4) and it forms a complex with Nox2 in phagocytic cells such as neutrophils or macrophages. In phagocytic cells, the protein expression of Nox2 and $p22^{phox}$ is mutually dependent: no $p22^{phox}$ is expressed in the absence of Nox2, and mutation of the $p22^{phox}$ gene leads to decreased Nox2 expression. The genetic deficiency of Nox2 or $p22^{phox}$ always results in severe immunodeficiency, chronic granulomatosis disease. It is known that in the primary fibroblast cells TGF-β significantly increases the expression of Nox4. Interestingly, in our experiments studying the relationship between Nox4 and $p22^{phox}$, we found that $p22^{phox}$ expression in primary fibroblast cells is independent of the presence of Nox4 and the expression of $p22^{phox}$ does not increase when fibroblast cells are treated with TGF-β. The presence of $p22^{phox}$, however, is absolutely necessary for the expression of Nox4, since the TGF-β-induced ROS-production of $p22^{phox}$-mutant fibroblasts is significantly reduced. This "asymmetric" relationship between the Nox4 and $p22^{phox}$ proteins raises the possibility that the two proteins form a complex with a previously unknown mechanism that differs from the Nox2-$p22^{phox}$ interaction. This result is also interesting because it raises the possibility that the $p22^{phox}$ protein may be involved in other, previously unknown protein-protein interactions in primary fibroblast cells. A revised version of a manuscript describing the above-described results is currently under review at *Free Radical Biology and Medicine*. I have also co-authored a review on the redox environment of the endoplasmic reticulum (Margittai E. et al., FRBM, 2015).

3. Identification of ROS-dependent bacterial killing in B lymphocytes

Regulated production of ROS is now mainly attributed to members of the Nox family of NADPH oxidases. In phagocytic cells, Nox2 has a crucial role in bacterial killing, and the absence of phagocytic ROS production leads to the development of a serious immunodeficiency, chronic granulomatous disease (CGD). Expression of Nox2 was also detected in B lymphocytes, where the role of Nox2 is still poorly understood and mainly signaling functions were attributed to the enzyme. We showed that peritoneal B cells, which were shown previously to possess phagocytic activity, have a high capacity to produce ROS in a Nox2-dependent manner. In phagocytosing B cells, we detected intense intraphagosomal ROS production. Furthermore, by studying two animal models of CGD, we demonstrate that phagocyte oxidase-deficient B cells have a decreased capacity to kill bacteria. These observations extended the number of

immune cell types that produce ROS to kill pathogens. We published these results in *Journal of Leukocyte Biology* (Kovacs I. et al, JLB, 2015). We also published a review paper on Nox/Duox knockout models in *Trends in Biochemical Sciences* (Sirokmany et al., TIBS, 2016).