8. SUMMARY

The proper functioning of the endothelial barrier is one of the conditions for maintaining homeostasis of the cardiovascular system. In the course of endothelial dysfunction, changes in cell-cell and cell-extracellular matrix adhesion, cytoskeleton organization, cell migration and death, and thus also in endothelial permeability are observed. One of the factors leading to endothelial dysfunction is oxidative stress. The result of redox imbalance in the cell is damage to lipids, proteins and DNA molecules. Increased activity of reactive oxygen species also leads to an increase in the level of Ca^{2+} in the cytoplasm. These ions are an important intracellular messenger, hence their concentration is strictly controlled by numerous channels and ion pumps located in the cell membrane and endoplasmic reticulum. One of the membrane calcium channels, which activation is observed in the course of oxidative stress is the transient receptor potential melastatin 2 (TRPM2) channel. The best described TRPM2 activator is ADP-ribose. The increase in the level of ADP-ribose occurs with the participation of PARP and PARG enzymes in response to single-strand breaks within the DNA, which may be the result of oxidative stress. Thus, the TRPM2 channel is the connector between oxidative stress, Ca^{2+} ions and endothelial dysfunction.

The study aimed to assess the role of the TRPM2 calcium channel in the context of the functioning and response of vascular endothelial cells to oxidative stress conditions, as well as to assess the potential use of TRPM2 channel or the PARP enzyme inhibitors in the prevention and treatment of cardiovascular diseases associated with endothelial dysfunction.

The material used in the study were model endothelial cells of the EA.hy926 cell line. To induce oxidative stress, the cells were cultured in the presence of hydrogen peroxide for 24 h. Downregulation of TRPM2 channel was performed using TRPM2 siRNA introduced into the cells using nucleofection and electroporation technique. Both non-transfected and transfected cells with siRNA sequences lacking human mRNA recognition sites were used as controls. Differences in the response of cells to hydrogen peroxide to TRPM2 expression were examined in terms of: cell viability, type of induced death, intracellular level of Ca^{2+} ions, migration potential, level and localization of selected junctional proteins and organization of the actin cytoskeleton. In order to reduce the activity of the TRPM2 channel, cells were pre-incubated with channel inhibitors (N-(p-Amylcinnamoyl)anthranilic acid – ACA and flufenamic acid – FFA) or the PARP enzyme (3-amonobenzamide – 3-AB and 3,4-dihydro-5 [4-(1-

piperidinyl)butoxy]-1(2H)-isoquinolinone – DPQ) 30 min before exposing the cells to hydrogen peroxide. Cells cultured in growth medium without the addition of hydrogen peroxide served as a control. The response of cells to selected TRPM2 or PARP inhibitors, hydrogen peroxide and a combination of a given inhibitor and hydrogen peroxide was assessed using: the MTT assay, in terms of changes in the distribution of cell cycle phases, type of induced cell death, migration potential and fluorescent labeling of selected junctional proteins and the F-actin cytoskeleton. The concentrations of the TRPM2 or PARP inhibitors and hydrogen peroxide used were selected on the basis of available literature data and the results of the MTT assay.

The assessment of cell viability using the MTT assay showed that in the group of non-transfected cells, after treatment with hydrogen peroxide, their metabolic activity decreases in a concentration-dependent manner. The response of cells transfected with control siRNA responded to selected concentrations of hydrogen peroxide was similar to non-transfected cells. In turn, cells transfected with TRPM2 siRNA were characterized by higher viability compared to the group of non-transfected or control siRNA-transfected cells treated with hydrogen peroxide. Reducing the activity of the TRPM2 channel by pre-incubation of cells with selected inhibitors of the TRPM2 channel or the PARP enzyme also inhibited the negative effect of hydrogen peroxide on EA.hy926 cells. The exception was 3-AB, which induced a significant decrease in cell viability of the EA.hy926 cells and did not show a protective effect against the presence of hydrogen peroxide in the growth medium, which was why it was eliminated from further research. Based on the results of the MTT assay, the concentration of 100 µM hydrogen peroxide was selected for subsequent experiments as the lowest concentration for which a statistically significant difference was observed in the response of non-transfected cells and cells with reduced TRPM2 activity or level. Double staining of cells with annexin V and propidium iodide showed that culture of EA.hy926 cells in the presence of hydrogen peroxide induces apoptotic cell death, which was visible in the group of non-transfected and control siRNA-transfected cells. For cells transfected with TRPM2 siRNA, there was no statistically significant difference in the percentage of apoptotic cells between untreated control and cells cultured in the presence of hydrogen peroxide. Hydrogen peroxide-induced apoptotic cell death was also effectively limited by the use of selected inhibitors of the TRPM2 channel or the PARP enzyme. In addition, hydrogen peroxide resulted in a rapid increase in fluorescence of the Ca2+ ion indicator in non-transfected cells. This effect was significantly reduced in cells transfected with TRPM2 siRNA. Cytometric evaluation of PI

fluorescence intensity in the presence of RNAse showed a change in cycle distribution of cells treated with hydrogen peroxide for 24 h compared to control cells. Incubation of cells with ACA and FFA resulted in no apparent changes in cell cycle distribution relative to controls. In contrast, in the case of cells treated with DPQ, a slight increase in the number of cells with DNA content specific to those between the G1 and G2 phase of the cycle was observed. No changes were confirmed in relation to control cells in terms of cell cycle distribution in groups of cells treated with ACA, FFA and DPQ and then cultured in the presence of hydrogen peroxide for 24 h. Wound-healing and transwell migration assays showed that the culture of EA.hy926 cells in the presence of 100 µM hydrogen peroxide for 24 h results in a reduction in the migratory capacity of the cells. In the group of non-transfected cells, after treating them with 100 µM hydrogen peroxide, statistically significant differences were observed in relation to the control in the wound area at selected time points as well as a decrease in the number of cells able to migrate through the membrane of inserts with pores of 3 µm. In the case of cells transfected with TRPM2 siRNA there was also a slight decrease in the migratory potential of the cells, but these differences were not significant comparing to control cells. Cell culture in the presence of selected TRPM2 or PARP inhibitors did not significantly affect the motility of the cells. In the case of cells pretreated with inhibitors for 30 min and then cultured in the presence of hydrogen peroxide, the negative effect of hydrogen peroxide on cell migration was limited. The inhibitor of the PARP enzyme – DPQ almost blocked the reduction of cell migration caused by hydrogen peroxide. Fluorescent labeling of junctional proteins and the actin cytoskeleton showed that treatment of cells with hydrogen peroxide resulted in the conversion of the intercellular junction pattern into a punctuate distribution. Moreover, the loss of cells through apoptosis and the reduction of the continuity of intercellular connections resulted in the appearance of additional spaces between cells. Downregulation of TRPM2 showed a protective effect on the cell monolayer. Even under the influence of hydrogen peroxide, the continuous nature of intercellular connections was preserved and no significantly larger spaces between adjacent cells were observed. There was also no increase in the number of cells with features of apoptosis. However, in the case of cells treated with selected TRPM2 or PARP inhibitors 30 min before treatment with hydrogen peroxide for 24 h, features of polyploidy were observed, expressed by the presence of additional micronuclei, but these changes were not as intense as in the case of cells treated only with hydrogen peroxide. Moreover, significantly fewer cells with features suggestive of apoptosis were

observed, while the density of the monolayer and the continuity of the structure of intercellular junctions were similar to those observed in the control. These results were confirmed by assessing post-translational protein expression by Western blot. In non-transfected cells, after treating them with 100 µM hydrogen peroxide for 24 h, a significant decrease in the expression of intercellular junctions proteins was confirmed, which was not observed for any of the other study group.

The obtained results indicate that oxidative stress in EA.hy926 cells leads to changes characteristic of endothelial dysfunction. Downregulation of TRPM2 channel by transfection with TRPM2 siRNA and reduction of its activity using TRPM2 or PARP inhibitors show a protective effect on endothelial cells of the EA.hy926 cell line treated with hydrogen peroxide. The TRPM2 channel may therefore be considered as a potential therapeutic target in the prevention and therapy of cardiovascular diseases caused by oxidative stress. In addition, the inhibitors of the TRPM2 channel (ACA and FFA) or the PARP enzyme (DPQ) used in the research effectively inhibit the response of EA.hy926 cells to hydrogen peroxide, but the possibility of their clinical application requires further research.