MOLECULAR AND STRUCTURAL STUDY OF UMBILICAL CORD ENDOTHELIAL DYSFUNCTION ASSOCIATED WITH MATERNAL SMOKING DURING PREGNANCY

PH.D. THESIS

SZABOLCS ZAHORÁN

SUPERVISOR

DR. EDIT HERMESZ

ASSOCIATE PROFESSOR

PH.D. SCHOOL IN BIOLOGY

UNIVERSITY OF SZEGED

FACULTY OF SCIENCE AND INFORMATICS

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

SZEGED

2020.

INTRODUCTION

The functionality of the umbilical cord (UC) blood vessels is crucial for the proper supply of oxygen and nutrients to the developing fetus. The UC is fully embryonic in origin and the vessels within can be considered as an extension of the fetal vascular system. Oxygen-rich blood is transported from the placenta to the fetus by the UC vein, and the two arteries carry oxygen-poor blood back to the placenta. Therefore, these vessels are directly and primarily exposed to harmful substances not retained by the placenta, so changes in their condition may indicate damage to intrauterine development. The UC vessels are almost completely lack innervation, therefore the regulation of their tone is governed by locally acting effects and active communication with their environment (endocrine factors, oxygen concentration, shear force, etc.). One of the most important parameters is the amount of bioavailable nitric oxide (NO), which determines endothelial function and regulates redox balance. NO is a highly diffusible, vascular relaxant molecule, and its main source is the nitric oxide synthase family. In endothelial cells the dominant isoform is the endothelial nitric oxide synthase (NOS3), which is activated by well-coordinated pathways that can be compromised at several points. Activation is regulated at several levels by e.g. dimerization, post-translational modifications, and protein-protein interactions. Thus, analysis of UC vessels, including endothelial cell-related complications, could be a useful tool for understanding pathological pathways leading to vascular complications in neonates. Because endothelial cells play a complex role in maintaining homeostatic conditions, their morphological and functional changes clearly contribute to the development of cardiovascular disease.

Although little is known about the molecular consequences / background of toxic substances on fetal exposure, there is growing evidence that these environmental factors can adversely affect intrauterine development and even mediate long-term health consequences. During pregnancy, transitional organs such as placenta and UC ensure fetal development. Therefore, changes in intrauterine conditions are directly or indirectly associated with placental or UC disorders such as intrauterine hypoxia and / or inadequate fetal blood supply. In addition, a recently published chemo-biological interactome analysis confirmed and provided evidence that components of tobacco smoke have intense effect on gene expression during both embryonic and fetal development. The effects of conventional cardiovascular

risk factors, such as diabetes, smoking, or dyslipidemia, on the cardiovascular system in adults are well-studied and associated with the development of endothelial dysfunction (ED). One of the main starting points for ED is the reduced NO production and / or bioavailability. As a result, oxidative stress in the endothelium is increased, leading to further severe consequences. If NOS3-dependent NO production get compromised, alternative compensation mechanisms can be activated. In addition, vascular endothelium is in constant contact with circulating red blood cells (RBCs) and a close relationship between the two has recently been reported in a number of diseases. Assuming that circulating RBCs sense ED, the bioavailability of NO could possibly be increased by the NOS3 activation pathway of RBCs. Based on our previous studies on maternal and fetal RBCs, we observed significant changes in the morphological parameters, elasticity and plastic properties of RBCs and, in this context, altered composition of their membrane-forming lipids. These alterations are related to an impaired RBC-NOS3 activation, which can be clearly associated with maternal smoking. The results thus suggest that this route cannot give a sufficient compensation. Another obvious solution could be an increase in the expression of inducible nitric oxide synthase (NOS2) in the endothelium due to its high catalytic activity (100-1000 times that of NOS3). Activation of xanthine oxidoreductase (XOR) is also a potential candidate. Although the latter may seem astonishing due to the basic properties of XOR, but recent studies have clearly demonstrated that activation of the XOR pathway contributes beneficially to NO production through reduction of inorganic nitrite.

AIMS

Initially in this study, we hypothesized that improper maternal lifestyle *e.g*. smoking can induce morphological and functional alterations in the UC vessels that could serve as fingerprints of harmful effects on the developing fetus. Accordingly, we searched for parameters that could serve as early markers, indicating, not only the direct damages to the UC vessels, but also the general condition of the vascular system of new-borns. In our work, we have pursued the harmful consequences due to maternal smoking on the UC arteries and veins, which could be a long-term determining factor for theirs later life. The objectives of the project are summarized as follows:

1) Study of the NOS3 endothelial expression and its active state determinant i.e. phosphorylation at SER1177 position in parallel to the stress-inducible ARG1 expression as a key player in L-arginine substrate competition

2) Investigation on the role of NOS2 and XOR in the light on possible compensatory mechanisms

3) Evaluation of the redox status in the blood vessels and analysis of the subsequent macromolecular damages

4) Examination of the endothelial cells ultrastructure in connection with their molecular alterations, and endothelial cell viability

5) Comparative gene expression analysis upon acute stress effects in the smoker and non-smoker derived umbilical veins

APPLIED METHODS

1) Transmission electron microscopy
2) Immunohistochemistry on frozen sections
3) *In situ* detection of $O_2^{\bullet -}$ with dihydroethidium
4) Laser scanning confocal microscopy
5) Evaluation of confocal images with an objective semiquantitative approach developed by us
6) Isolation of humane umbilical vein endothelial cells (HUVEC)
7) Viability assay with annexin V and propidium iodide
8) Flow cytometry
9) Development and application of an acute stress response induction model
10) Isolation, pulverizing and homogenization of UC vessels
11) Determination of the total nitric oxide derivatives (tNOx) concentration with Griess reaction
12) RNA extraction, reverse transcription
13) Quantitative real-time PCR

RESULTS AND CONCLUSIONS

Using the umbilical cord system in our study is advantageous over any general practices, such as studying the placenta, the umbilical cord blood, or *in vitro* cell cultures. The umbilical cord blood vessels can be considered as a direct elongation of the vascular system of the developing fetus. Thereby, any alteration in the vessel's functionality reflects the *in-vivo* circumstances, experienced by the fetus. Our results may contribute to a better understanding in the molecular changes occurring during intrauterine toxic exposure, and in-depth comprehensive knowledge of the pathways involved in these alterations. These may identify additional therapeutic targets for clinical outcomes and applied research in the future.

1) NO production is impaired in the directly exposed veins, which can be attributed to the altered phosphorylation of the NOS3 at SER1177 position. In the arteries, apart from the decrease in NOS3 levels, the NO supply is considered to be more or less satisfactory. Evidence of this can be traced back to the retained phosphorylation, therefore the arterial NOS3 population can be considered as active. The competitive ARG1 enzyme showed significantly higher expression in smoker-derived arteries, compared to the veins. The detailed effect of this result on the NOS3-NO pathway is not clear, since in the arteries the dimeric form of NOS3 appears to be intact based on their phosphorylation. In parallel, in the case of veins, it can be assumed that NOS3 loses the dimer structure, which is ultimately manifested to a decrease in activating phosphorylation.

2) NOS2 and XOR expression showed significant induction in veins. Based on the Janus-faced properties of NOS2, NOS2-NO pathway may promote vasodilation to a limited extent. Furthermore, it contributes with higher extent in the increase of passive, non-directly available tNOx pool. Elevated tNOx levels in a hypoxic environment may help to reveal the active role of the tNOx-XOR-NO pathway. In addition to NOS2, XOR expression was also drastically increased in the veins. In contrast, we found lack of compensatory pathways induction in the arteries.

3) Significant macromolecular damages were observed in veins with primary exposure. This may be greatly contributed to the large amount of NO, synthesized by increased NOS2 over a short period of time. This pathway, in a highly oxidative environment, shifts the role of NO, toward nitrosative stress rather than in a biologically active and beneficial direction. This is based on the high concentration of $O_2^{\bullet -}$ observed in the veins, where most likely $ONOO^-$ is formed in a spontaneous reaction. Traces of nitration and / or oxidation of the macromolecules, potentially associated with $ONOO^-$, which were significantly detectable in the veins. The presence of 4-HNE adducts as the marker of membrane damage and 3-NT-type modification of proteins also showed a significant increase. The sign of macromolecular damages in arteries are negligible, presumably due to their secondary, indirect exposure. In this case, the increase in 4-HNE compared to the veins is very small in our smoking samples. In parallel, the 3-NT level also does not reach the value of the control arteries. The latter can be associated with NOS2 expression in both vascular types. Our results in evaluating DNA double strand breaks and the related repair mechanisms revealed a smaller average size of the foci detected in smoker veins. This suggesting, that the proper assembly of the DNA repair pathways was inhibited.

4) The ultrastructural changes observed with transmission electron microscopy also suggest varying degrees of exposure of vessel types, which supports our molecular data. The lesions observed in the vascular endothelial layer indicate increased venous damage due to direct exposure to toxic substances. In the case of veins, progress of degenerations has been observed that lead to increased CASP3-mediated cell death. Examining the MMP-9 – TIMP-1 ratios in the vein, it was demonstrated that MMP-9 expression exceeds TIMP-1 levels, which despite the generally decreasing trend, leading to tissue degradation.

5) Comparing the basal expression levels, we found that all the examined genes (*hsp90*, *mmp-9*, *timp-1*, *mt1e*, *mt2a*) with the exception of *mt3* are lower expressed in smoker-derived veins. Based on this, the direct damaging effects on the smoking vein lead to a general decrease in transcriptional mechanisms.

The stress response of the smoker group to Cd^{2+} treatment was less pronounced compared to the control samples. In parallel, we hypothesize that the expression level of these genes never reached the control level due to the continuous stress effect (lasting for several months) and proves prolonged heavy metal exposure has occurred during pregnancy.

FUNDING

This work was supported by the European Union and the Hungarian Government in the framework of the GINOP-2.3.2-15-2016-00040 project.

LIST OF PUBLICATIONS

MTMT identifier: 10051798

Publications related to the PhD process:

1. Zahorán, S.; Szántó, P.R.; Bódi, N.; Bagyánszki, M.; Maléth, J.; Hegyi, P.; Sári, T.; Hermesz, E. Sustained Maternal Smoking Triggers Endothelial-Mediated Oxidative Stress in the Umbilical Cord Vessels, Resulting in Vascular Dysfunction. Antioxidants 2021, 10, doi:10.3390/antiox10040583.

2. Dugmonits, K.N.; Ferencz, Á.; Zahorán, S.; Juhász, R.; Talapka, P.; Orvos, H.; Hermesz, E. Elevated Levels of Macromolecular Damage Are Correlated with Increased Nitric Oxide Synthase Expression in Erythrocytes Isolated from Twin Neonates. BRIT J HAEMATOL 2016, 174, 932–941, doi:10.1111/bjh.14156.

Publications in refereed journals:

1. Zahorán, S.; Szántó, P.R.; Bódi, N.; Bagyánszki, M.; Maléth, J.; Hegyi, P.; Sári, T.; Hermesz, E. Sustained Maternal Smoking Triggers Endothelial-Mediated Oxidative Stress in the Umbilical Cord Vessels, Resulting in Vascular Dysfunction. Antioxidants 2021, 10, doi:10.3390/antiox10040583. IF: **5.014**

2. Dugmonits, K.N.; Chakraborty, P.; Hollandi, R.; Zahorán, S.; Pankotai-Bodó, G.; Horváth, P.; Orvos, H.; Hermesz, E. Maternal Smoking Highly Affects the Function, Membrane Integrity, and Rheological Properties in Fetal Red Blood Cells. OXID MED CELL LONGEV 2019, doi:10.1155/2019/1509798. IF: **5,067**

3. Zhou, Z.; Mahdi, A.; Tratsiakovich, Y.; Zahorán, S.; Kovamees, O.; Nordin, F.; Uribe, G.A.; Alvarsson, M.; Ostenson, C.; Andersson, D.; et al. Erythrocytes From Patients With Type 2 Diabetes Induce Endothelial Dysfunction Via Arginase I. J AM COLL CARDIOL 2018, 72, 769–780, doi:10.1016/j.jacc.2018.05.052. IF: **17.93**

4. Dugmonits, K.N.; Ferencz, Á.; Zahorán, S.; Juhász, R.; Talapka, P.; Orvos, H.; Hermesz, E. Elevated Levels of Macromolecular Damage Are Correlated with Increased Nitric Oxide Synthase Expression in Erythrocytes Isolated from Twin Neonates. BRIT J HAEMATOL 2016, 174, 932–941, doi:10.1111/bjh.14156. IF: **5.67**

Cumulative impact factor: 33.681

Other publications:

1. Zahorán, S.; Hermesz, E. Az Oxidatív Stressz És Endotél Diszfunkció Összefüggéseinek Vizsgálata Humán Köldökzsinór Modellrendszerben.; Poór, P., Blázovics, A., Eds.; Szegedi Tudományegyetem: Szeged, 2019; p. 18.
2. Chakraborty, P.; Dugmonits, K.N.; Zahorán, S.; Hermesz, E. Mapping the Morphological and Functional Alterations of the Red Blood Cells in Active Maternal Smoking during Pregnancy. FEBS OPEN BIO 2018, 8.
3. Zahorán, S.; Dugmonits, K.N.; Szántó, P.; Bartos, K.; Hermesz, E. A terhesség alatti dohányzás rövid és hosszú távú hatásának molekuláris vizsgálata a köldökzsinór endotél diszfunkciójának és a lehetséges kompenzációs hatások tükrében. In Magyar Szabadgyök-Kutató Társaság IX. Kongresszusa és az MTA ÉKB Mikroelem Munkabizottságának Tudományos Ülése; Mézes, M., Ed.; SzIE: Gödöllő, 2017; pp. 28–28 ISBN 978-963-269-666-9.
4. Kiss, A.A.; Zahorán, S.; Popovics, N.; Márton, K.; Monika, R.; Hermesz, E.; Boldogkői, Z.; Katalin, C.; Mink, M. Chronic Inflammation, Oxidative-Nitrosative Stress, Mitochondrial Dysfunction, Improper Integrin Expression, Loss of Sarcomeres and Proteolysis of Myofibrillar Proteins Allow Diagnosis of Muscular Dystrophy in Drosophila Type IV Collagen Col4a1 Mutants.; Nikos, K., Ed.; Spetses, 2017; pp. 85–85.
5. Zahorán, S.; Dugmonits, K.N.; Bódi, N.; Hermesz, E. Cord Endothelial Dysfunction Can Be Sensed by Circulating Red Blood Cells? FEBS J 2017, 284, 122–122.

6. Chakraborty, P.; Dugmonits, K.N.; Ferencz, Á.; Zahorán, S.; Hermesz, E. P 097 - Is Twin Pregnancy Being a Medical Boon? – Comparative Evaluation on Oxidative Stress in Multiple Pregnancy 2017.

7. Zahorán, S.; Dugmonits, K.N.; Hermesz, E. Novel Insights of Nitric Oxide Signaling: The Importance of Red Blood Cells Activity in Twin Neonates.; Vancouver, 2016; pp. 73–73.

8. Zahorán, S. A dohányzás károsító hatásának molekuláris vizsgálata dohányzó és nem dohányzó édesanyáktól született újszülöttek köldökzsinór mintáiban. In XXXII. Országos Tudományos Diákköri Konferencia; Putnoky, P., Galambos, A., Eds.; Pécsi Tudományegyetem Természettudományi Kar (PTE TTK): Pécs, 2015; pp. 78–78 ISBN 978-963-642-739-9.

9. Zahorán, S.; Dugmonits, K.N.; Hermesz, E. A dohányzással összefüggésbe hozható fémfelhalmozódások hatásainak vizsgálata humán köldökzsinór mintákon.; Lugasi, A., Fébel, H., Eds.; 2015; pp. 33–34.

10. Dugmonits, K.N.; Ferencz, Á.; Zahorán, S.; Hermesz, E. Az antioxidáns vélemi rendszer vizsgálata intrauterin növekedési retardációval született újszülöttekben. In Tavaszi szél : absztraktkötet 2015; Csiszár, I., Ed.; Doktoranduszok Országos Szövetsége (DOSZ): Budapest, 2015; pp. 385–385 ISBN 978-963-397-702-6.

11. Zahorán, S.; Ferencz, Á.; Dugmonits, K.N.; Hermesz, E. Fémfelhalmozódás és oxidatív stressz: a dohányzás hatásainak molekuláris vizsgálata humán köldökzsinór mintákon.; 2015; pp. 44–44.

12. Ferencz, Á.; Dugmonits, K.N.; Zahorán, S.; Hermesz, E. Little babies-big warriors:The antioxidant response in the umbilical cord of neonates with intrauterine growth restrictio. In Molecules of Life. FEBS3+ Meeting. 11th Meeting of the Slovenian Biochemical Society; Janko, K., Nataša, P.U., Eds.; Slovenian Biochemical Society: Ljubljana, 2015; pp. 252–252 ISBN 978-961-93879-1-7.

13. Dugmonits, K.N.; Juhász, R.; Ferencz, Á.; Zahorán, S.; Hajnalka, O.; Hermesz, E. Smoking and Pregnancy: Molecular Study of Oxidative Stress Response in the Human Umbilical Cord. FEBS J 2014, 281, 8–9.