Theses of doctoral (Ph.D) dissertation

Effect of maternal smoking on fetal and adult erythrocytes: morphological, rheological and functional studies

Krisztina Nikoletta Dugmonits Hidjapusztayné

Supervisor:
Dr. habil. Edit Hermesz
associate professor
Co-supervisor:
Prof. Dr.habil. Hajnalka Orvos
professor

University of Szeged, Faculty of Science and Informatics

Department of Biochemistry and Molecular Biology

Doctoral School of Environmental Sciences

Szeged
2021

Introduction

Cardiovascular diseases are the major cause of death in the developed countries. There is increasing evidence that environmental agents can exert a marked impact on the outcome of in utero development and even mediate long-lasting health consequences. Many of the compounds present in cigarette smoke are regarded as harmful toxicants playing crucial roles in the pathogenesis of certain severe illnesses. It has been hypothesized that many of the adverse effects may result from oxidative damage to proteins, lipids, and nucleic acids. Such damages could be traced back to direct attack of oxidants present in cigarette smoke and to the activation of the endogenous sources of reactive oxygen species (ROS).

During pregnancy, the physical connection between the mother and the fetus is provided by the placenta and umbilical cord. Smoking during pregnancy the harmful pollutants in the vapour and tar phases might diffuse into the placenta and pass down to the fetal circulatory system through the umbilical cord. With the exception of its most proximal segment, the human umbilical cord lacks innervation and thereby the main regulator of their vascular tone and blood flow is the nitric oxide (NO), a signalling molecule produced by the endothelial nitric oxide synthase (NOS3) from L-arginine. The appropriate fetal development is coordinated by the umbilical cord vessels and the erythrocyte population circulating in the vessels. Chronic smoking jeopardizes proper endothelial functioning by decreasing the formation and increasing the degradation of NO, via generation of ROS or other strong oxidants like peroxynitrite anion ($ONOO^-$). Recently published experimental evidences make it highly supposable that adult red blood cells (RBCs) not only are passive regulators of the endothelium-derived NO level but also actively control systemic NO bioavailability by synthesizing, transporting, and releasing it (1,2). This new “erythrocrine function” helps to maintain the vascular tone and blood flow by releasing bioavailable NO synthetized by the post-translationally modified RBC-NOS3 (3).

Aims

Our goal was to determine how maternal smoking affects the oxygenation of the fetus based on morphological and molecular changes in the circulating RBCs. We looked for possible links between alterations in morphology, endothelial nitric oxide synthase expression and activation, membrane damages and deformability of fetal RBCs. Furthermore, we looked for a potential rescue mechanism/compensatory role of fetal RBCs, based on the newly described "erythrocrine" function in case of any endothelial dysfunction.

In total blood and isolated RBCs with maternal and fetal origins we examined the

- phenotypic changes and stress adaptation
- molecular factors influencing NOS3 activation
- accumulation of free radicals/strong oxidants,
- changes in membrane lipid composition and integrity
- and rheological parameters

as a consecuence of maternal smoking.

Materials and methods

1. Sample collection and preparation of erythrocyte fraction
   Blood was taken from the umbilical cord artery of neonates born to non-smoking mothers (RBC-NS) and mothers who continued smoking during pregnancy (at least 10 cigarettes per day) (RBC-S).
2. RNA preparation, reverse transcription (RT)/ Quantitative polymerase chain reaction (qPCR)
3. Morphological studies and data analysis with Advanced Cell Classifier program on eosin stained blood smears
4. Investigation of rheological properties of erythrocytes with atomic force microscopy (AFM)
5. Lipidome analysis of erythrocytes' plasma membrane
6. Fluorescence activated cell sorting and analysis (FACS) on erythrocytes
7. Spectrophotometric measurements
   a. Determination of protein concentration
   b. Peroxynitrite (ONOO-) measurement
8. *Ex vivo* treatments
   a. Heavy metal treatment
   b. *Candida parapsilosis* infection

Results and Discussion

Our results indicated that RBCs of the developing fetus born to smoking mother (RBC-S) undergo morphological and molecular alterations/aberration. We demonstrated for the first time, that fetal RBCs carry functional NOS3 and during long term *in vivo* exposure to harmful stimuli, the NOS3 level and its activation pathways are altered in a morphology-dependent manner. The impaired activity of NOS3 is well paralleled with an increased ARG1 level. Moreover, both ARG1 protein and *arg1* mRNA expression levels are elevated in the fetal RBC-S populations and high ARG1 expressing cells show basal NOS3 level. We also demonstrated that cells in RBC-S population become a source of oxidizing agents, with the possibility to further inactivate the NOS3 pathway by the ARG1 induction. The importance of this result was underlined by recent findings on adult RBCs, that RBC can intensively contribute to vascular functioning and integrity. NOS3-derived NO export from RBCs mediate a rescue mechanism in case of a vascular dysfunction, but the pathway gets stringently regulated by ARG1. Elevated level of ARG1 inhibits NO export thus reducing NO bioavailability or increasing oxidative stress conditions by the ROS generation, causing serious endothelial dysfunction. This induced dysfunctionality can be prevented by inhibition of ARG1 activity.

In addition, we found that due to maternal smoking, the physico-chemical properties of fetal RBCs underwent significant alterations, with an elevated plasticity and altered lipid composition of the plasma membrane. Additionally, we were able to characterize the viscoelastic response of RBCs to mechanical stimulus and found a remarkable decrease in the recovery ability of the cells after indentation.

We demonstrated a significantly elevated level of $ONOO^-$ in RBCs with smoking origin, which initiates free radical mediated processes that mark an array of macromolecular damages. In agreement with the elevated ROS production, we could also detect a significant increase in the lipid oxidation, based on the formation of 4-hydroxy-2-nonenal, a known product of lipid peroxidation. The increased frequency of lipid peroxidation indirectly signifies a loss of membrane integrity and function and impaired rheological parameters in the RBC-S population.

Taken together - under the influence of cigarette smoke - an imbalance in the redox homeostasis in RBCs enhances the rate of macromolecular damage with membrane stiffness and loss in their intrinsic functional and elastic activities. As a consequence of it, in case of endothelial dysfunction with low bioavailable level of NO, the RBC NOS3-NO production is unavailable as a compensatory mechanism. Moreover, because of the wide increase in the

RBC mass during pregnancy, the elevated ARG1 level might even augments and synergizes the development of vascular dysfunction/comorbidities. And lastly, we believe that the alterations in NOS3 activation pathway and ARG1 expression might serve as an early prognostic marker for not only RBC-linked anomalies but also for endothelial dysfunction and several vascular comorbidities.

References

1. Cortese-Krott, M.M. Rodriguez-Mateos A, Sansone R, Kuhnle GG, Thasian-Sivarajah S, Krenz T, Horn P, Krisp C, Wolters D, Heiß C, Kröncke KD, Hogg N, Feelisch M, Kelm M., 2012. Human red blood cells at work: Identification and visualization of erythrocytic eNOS activity in health and disease. Blood 120, 4229–4237. https://doi.org/10.1182/blood-2012-07-442277
2. Cortese-Krott, M.M. and Kelm, M., 2014. Endothelial nitric oxide synthase in red blood cells: key to a new erythrocrine function? Redox.Biol 2,251–258. https://doi.org/10.1016/j.redox.2013.12.027
3. Mori, M., 2007. Regulation of nitric oxide synthesis and apoptosis by arginase and arginine recycling. J. Nutr. 137, 1616S-1620S. https://doi.org/137/6/1616S [pii]

The research was carried out within the framework of the **GINOP-2.3.2.-15-2016-00035**.

List of publications MTMT ID: 10040727

Publications in refereed journals

1. **Krisztina Dugmonits**, Ágnes Ferencz, Zsanett Jancsó, Renáta Juhász, and Edit Hermesz. 2013. "Major Distinctions in the Antioxidant Responses in Liver and Kidney of Cd2 +-Treated Common Carp (Cyprinus Carpio)." Comparative Biochemistry and Physiology - C Toxicology and Pharmacology 158 (4): 225–30. https://doi.org/10.1016/j.cbpc.2013.07.005. **IF: 2.892**
2. **Krisztina N. Dugmonits**, Ágnes Ferencz, Szabolcs Zahorán, Renáta Lázár, Petra Talapka, Hajnalka Orvos, and Edit Hermesz. 2016. "Elevated Levels of Macromolecular Damage Are Correlated with Increased Nitric Oxide Synthase Expression in Erythrocytes Isolated from Twin Neonates." British Journal of Haematology 174 (6): 932–41. https://doi.org/10.1111/bjh.14156. **IF:5.518**
3. **Krisztina N. Dugmonits**, Payal Chakraborty, Réka Hollandi, Szabolcs Zahorán, Gabriella Pankotai-Bodó, Péter Horváth, Hajnalka Orvos, and Edit Hermesz. 2019. "Maternal Smoking Highly Affects the Function, Membrane Integrity, and Rheological Properties in Fetal Red Blood Cells." Oxidative Medicine and Cellular Longevity. https://doi.org/10.1155/2019/1509798. **IF: 5.076**
4. Payal Chakraborty*, **Krisztina N. Dugmonits***, Attila G. Végh, Réka Hollandi, Péter Horváth, József Maléth, Péter Hegyi, Gábor Németh, and Edit Hermesz. 2019. "Failure in the Compensatory Mechanism in Red Blood Cells Due to Sustained Smoking during Pregnancy." Chemico-Biological Interactions. https://doi.org/10.1016/j.cbi.2019.108821. **IF:3.723**
5. Gábor Balogh*, Payal Chakraborty*, **Krisztina N. Dugmonits***, Mária Péter*, Attila G. Végh, László Vígh, and Edit Hermesz. 2020. "Sustained Maternal Smoking-Associated Changes in the Physico-Chemical Properties of Fetal RBC Membranes Might Serve as Early Markers for Vascular Comorbidities." Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids. 1865(4):158615. doi: 10.1016/j.bbalip.2020.158615. **IF: 4.519**
6. Payal Chakraborty, **Krisztina N Dugmonits**, Hajnalka Orvos, Edit Hermesz. 2020. "Mature

Twin Neonates Exhibit Oxidative Stress via Nitric Oxide Synthase Dysfunctionality: A Prognostic Stress Marker in the Red Blood Cells and Umbilical Cord Vessels." Antioxidants (Basel). 10;9(9):E845. doi: 10.3390/antiox9090845**. IF:4.610**
* shared first author **Total impact factor: 26.338**

Posters presentations

1. ZsanettJancsó, **Krisztina Dugmonits**, Edit Hermesz: Metal-induced alteration in the expression of two heme-oxygenase genes. FEBS3+ meeting, From molecules to life and back Opatija, Croatia 13-16th June 2012. Poster number: PIII-140
2. RenátaJuhász, Ágnes Ferencz, Zsanett Jancsó, **Krisztina Dugmonits** and Edit Hermesz: Comparative study on the expression of glutathione peroxidase, glutathione reductase, glutathione synthetase and metallothionein genes in common carp during cadmium exposure. Society for Free Radical Research International 16th Biennial Meeting, London, 2012. Sept. 6-9, Poster number: P1.114
3. Edit Hermesz, **Krisztina Dugmonits**, Renáta Juhász, Ágnes Ferencz: Heavy metal induced alternative splicing of glutathione synthetase mRNA in the brain of common carp (Cyprinus carpio). 22nd IUBMB, 37th FEBS Congress, Spain, 04-09th September 2012, Poster number: P08-78
4. **Krisztina Nikoletta Dugmonits**, Renáta Lázár, Ágnes Ferencz, Szabolcs Zahorán, Hajnalka Orvos, Edit Hermesz: Smoking and Pregnancy: Molecular Study Of Oxidative Stress Response In The Human Umbilical Cord. 14Th FEBS-Young Scientist's Forum, 50th FEBS-EMBO Congress, Paris, 30th August-04 September 2014. Poster number of 14Th FEBS-Young Scientist's Forum: P19, Poster number of 50th FEBS-EMBO Congress: SUN-115
5. ÁgnesFerencz, **Krisztina N. Dugmonits**, Szabolcs Zahorán, Edit Hermesz: Antioxidant response in the umbilical cord of neonates with intrauterine growth restriction. FEBS3+ Meeting, Molecules of Life, Portorož, Slovenia 2015. Book of Abstracts. p. 252.,
6. **Krisztina N Dugmonits**, Szabolcs Zahorán, Edit Hermesz. Novel Insights of Nitric Oxide Signaling: The Importance of Red Blood Cells Activity in Twin Neonates. In: 16th International Conference of Biochemistry and Molecular Biology: Signaling pathways in development, disease and aging. 130 p. Konferenciahelye, ideje: Vancouver, Kanada, 2016.07.17-2016.07.21. Vancouver: p. 73.
7. Payal Chakraborty**, Krisztina N Dugmonits**, Ágnes Ferencz, Szabolcs Zahorán, Edit Hermesz. P 097 - Is Twin Pregnancy being a Medical Boon? –Comparative Evaluation on Oxidative Stress in Multiple Pregnancy. (2017)
8. Szabolcs Zahorán, **Krisztina Nikoletta Dugmonits**, Nikolett Bódi, Edit Hermesz: Cord endothelial dysfunction can be sensed by circulating red blood cells? (The FEBS Journal 284 (Suppl. 1) (2017) 102–103 DOI: 10.1111/febs.14174), 17th FEBS Young Scientists' Forum (YSF), and at the 42nd FEBS conference, Jerusalem, Israel
9. Payal Chakraborty, **Krisztina N. Dugmonits**, Szabolcs Zahorán, Edit Hermesz: Mapping the Morphological and Functional Alterations of the Red Blood Cells in Active Maternal Smoking during Pregnancy. FEBS OPEN BIO 8 : S1 Paper: P.13-004 (2018)
10. Paya lChakraborty, **Krisztina, N. Dugmonits**; Edit, Hermesz: In vivo investigation on the morphological and functional alterations of RBCs due to active tobacco smoke during pregnancy. In:Free Radical Biology & Medicine : Abstract Book. (2018) Paper: P-323.