Extracellular vesicles in hypercholesterolemia-induced cardiotoxicity and helium-induced cardioprotection

PhD thesis

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1 Introduction

The global burden of cardiovascular diseases (CVD) has significantly declined in the last few decades (Mensah et al., 2023) due to the successful development of pharmacological and non-pharmacological therapies. However, it still remains the leading cause of deaths worldwide (Murray, 2022). These data indicate our incomplete understanding of the pathomechanism of CVDs. Gaining further insight into the regulation of cardiovascular homeostasis could result in the development of novel therapeutics.

Treatments to reduce myocardial damage, or specifically necrosis after ischemia reperfusion injury, are called as cardioprotective interventions. So far, the most efficient cardioprotective therapy is ischemic preconditioning (IPC), consisted of a series of short ischemic periods applied to the myocardium before a prolonged ischemic event (Heusch, 2020; Murry et al., 1986). Numerous biological processes associated with cardioprotection have been described (Heusch, 2020), however, discrepancies in efficacy of cardioprotective interventions (Sayour et al., 2023) highlight the incomplete understanding of these pathways. One of the less-studied cardioprotective intervention is

helium conditioning (HeC) (Pagel et al., 2007). Understanding of how HeC regulates myocardial functions could reveal novel mechanisms involved in cardiac stress adaptation.

A better understanding of cardiac homeostasis could be achieved by analyzing not only cardioprotective interventions, but cardiotoxic conditions. One of the primary comorbidity of CVDs is hypercholesterolemia (HC) (Murray, 2022). HC has direct negative effects on the myocardium (Andreadou et al., 2017), disrupts the physiological function of the heart and result in impaired systolic and diastolic functions (Huang et al., 2004; Ónody et al., 2003). However, mechanisms that connect HC and CVDs are yet not fully elucidated.

Extracellular vesicles (EV) are nano-sized, cell-secreted membrane particles (van Niel et al., 2018), which transport a wide variety of molecular information between cells, establishing intercellular communication. EVs seem to contribute to the pathomechanism of both HC and CVDs (Akbar et al., 2019; Giricz et al., 2014; Martínez & Andriantsitohaina, 2017; Sluijter et al., 2018). Furthermore, HeC modulates EV secretion from various sources (Weber et al., 2019) (Thom et al., 2014) (Smit et al., 2015).

However, as EVs have been studied extensively in the last few decades only, their exact role in maintaining cardiac homeostasis is yet unknown.

In this thesis, we aimed to investigate how EVs contribute to the maintenance of cardiac homeostasis by addressing the effect of a cardiotoxic condition, HC, and a cardioprotective intervention, HeC, on EVs. We investigated the metabolomic profile of plasma-derived EVs (pEV) of rats fed with high-cholesterol chow, and we performed in-depth analysis of cardiomyocyte (CM) small EVs (sEV) under HC conditions. In the context of HeC, we analyzed medium-sized EVs (mEV) derived from cardiac fibroblasts (CF) and the effect of CF secretome on endothelial cell function.

2 Objectives

We hypothesized that HC dysregulates the composition of both circulating and cardiac-derived EVs contributing to the progression of CVDs. Furthermore, we hypothesized that HeC modifies EV secretion of CFs, which could contribute to its cardioprotective effect. By analyzing EVs in both cardiotoxic and cardioprotective mechanisms we aim to gain deeper understanding of the role of EVs in maintaining cardiac homeostasis. According to our hypotheses, we have established the following objectives:

1) Analyze how HC modifies circulating EV metabolome

2) Analyze the effect of HC on CM EV secretion

3) Analyze whether CM EVs induce inflammation in HC

4) Analyze the effect of HeC on CF EVs

3 Methods

3.1 Metabolomic analysis of circularing extracellular vesicles of hpyercholesterolemic rats

Male Wistar rats were fed with standard or high-cholesterol chow for twelve weeks. Then animals were anesthetized with pentobarbital intraperitoneally and arterial blood was collected into Anticoagulant Citrate Dextrose-A vacuum tubes. Platelet-free plasma was prepared by centrifugation and stored at -80 °C. pEVs-were isolated with iodixanol density gradient ultracentrifugation followed by size-exclusion chromatography with a HiScreen Capto Core 700 column as described earlier(Onódi et al., 2018). Metabolomics analysis was conducted on plasma and pEV samples using a Biocrates MxP Quant 500 kit.

3.2 In-depth analysis of cardiomyocyte-derived extracellular vesicles and their immune cell activation in hypercholesterolemia

AC16 human cardiomyocytes were treated with Refeed® hypercholesterolemic supplement or with its vehicle (3 µL/mL 96% ethanol) for 48 hours, when sEVs were isolated from the cell culture supernatant with differential centrifugation with a final gravitational force of 174.900×g. sEV particle size and concentration was measured with

nanoparticle tracking analysis (NTA). Protein concentration was measured with 280 nm light absorbance, while lipid concentration was measured according to the protocol of Visnovitz *et al*. (Visnovitz et al., 2019). To analyze total phosphatidylcholine (PC) content, a commercial colorimetric assay was used. Atomic force microscopy (AFM) was used to measure sEV elastic modulus. sEV proteomics was measured using liquid chromatography coupled tandem mass spectrometry.

To analyze the immune cell activation of CM sEVs in HC, THP1 monocytes expressing CARD domain fused by green fluorescent protein (ASC-GFP) were treated with AC16 sEVs with multiple doses for 16 hours. Then GFP expression was measured with flow cytometry and interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) and interleukin 10 (IL-10) gene expression were measured with quantitative PCR.

3.3 Analysis of the effect of helium conditioning on cardiac fibroblast extracellular vesicles

To analyze the effect of HeC on CF EV secretion, neonatal rat cardiac fibroblasts (NRCF) were isolated and HeC treatment was conducted on them according to our established protocol (Jelemenský et al., 2021) consisted of

glucose deprivation and 4 repeats of one hour 95% helium + 5% CO_2 culturing conditions followed by one hour normal gas conditions.

NRCF mEVs were isolated with differential centrifugation with a final gravitational force of 13.500×g. Isolates were analyzed with western blot, electron microscopy and NTA. To analyze the effect of NRCF mEVs on the endothelium after HeC, supernatant of HeC-treated NRCFs were transferred to HUVEC-TERT2 and in-vitro migration assay and tube formation assay was performed.

4 Results

4.1 Plasma extracellular vesicle metabolome is dysregulated in hypercholesterolemia

When we analyzed the metabolite composition of pEVs, we identified that the amounts of several PCs were reduced by HC. Interestingly, some of these PCs showed opposite changes in plasma. To further analyze the connection between plasma and pEV metabolites, we correlated intensities of metabolites detected both in plasma and in pEVs. Surprisingly, there was no correlation in most of the metabolite groups; only a moderate correlation was identified in the concentration of glycerophospholipids. This analysis highlights that HC dysregulates pEV metabolite composition and these changes are differentially regulated from plasma metabolome.

4.2 Hypercholesterolemia dysregulates cardiomyocyte extracellular vesicle secretion that do not result in inflammation

Our in vitro experiments on AC16 cells revealed that HC increases particle and protein concentration of sEVs isolated from the cells without affecting its lipid-to-protein ratio or its PC content. Furthermore, elastic modulus of sEVs were unaffected by the treatment. These results

suggest that HC increases CM sEV secretion without having major effect on its metabolite composition.

When we treated THP1-ASC-GFP monocytes with AC16 EVs, we did not observe change in GFP intensity or IL-1β, TNF-α or IL-10 gene expression of the monocytes. These results suggest that CM sEVs do not regulate monocyte function in HC.

To understand how HC can affect CM EV-derived mechanisms, we analyzed the proteome of AC16 sEVs. We identified 110 proteins dysregulated by HC. Within downregulated proteins, we observed extracellular matrix proteins, and proteins that are part of the *endosomal sorting complex required for transport*. Most of the proteins found upregulated are associated with RNA binding, some of them are part of the ribosomal or RNA splicing complexes. Furthermore, multiple upregulated proteins are involved in EV-mediated tissue remodeling. These results indicate that HC substantially modifies CM sEV proteome and potentially affects EV RNA content as well. These changes can result in tissue remodeling and thus CVD development.

4.3 Helium conditioning does not have major effect on cardiac fibroblast extracellular vesicle secretion

When we isolated mEVs from NRCFs after HeC, we observed that a mild, however statistically not significant ($p = 0.062$) reduction in particle concentration. Furthermore, HUVEC-TERT2 treated with EV-rich cell culture supernatant of NRCFs after HeC treatment did not have different migration speed. Similarly, we did not observe differences in the number of branches, mean mesh size and in mean tube length in in-vitro tube formation. These results suggest that, however EVs contribute to cardioprotection in certain interventions (Giricz et al., 2014), HeC do not have major effect of HeC on NRCF mEV secretion.

5 Conclusions

To gain further insight into the role of EVs in cardiac physiology, we examined the effects of both cardiotoxic HC and cardioprotective HeC on EVs. We specifically focused on the metabolome of pEVs in HC and investigated how HC affects CM sEV secretion and whether the changes contribute to inflammation. Furthermore, we analyzed how HeC affects CF mEV secretion and its effect on endothelial function.

Our findings that HC significantly alters metabolome of pEVs, which is regulated differently from the plasma metabolome, strengthens the original hypothesis that analyzing EVs can reveal information about the disease states that would remain hidden if plasma metabolome was investigated alone.

Moreover, we demonstrated that HC increases CM sEV secretion. However, unlike in other stressed conditions, such as hypertrophy (Yu et al., 2021), CM EVs do not modulate immune cells in HC. Instead, our proteomics analysis suggests that it may induce cardiac tissue remodeling. Together with our results on pEVs, we conclude that EVs can play significant role in altered intercellular communication in HC. The identified changes

can regulate intracardiac and systemic changes that can contribute to the development and progression of CVDs.

In contrast to our results in HC conditions, we observed limited effect of HeC on CF mEVs. While HeC might slightly affect the quantity of secreted mEVs, the vesicles do not transmit activation signals to endothelial cells. Taking into account that HeC affects EVs from certain sources (Smit et al., 2015; Thom et al., 2014; Weber et al., 2019), we suggest that EVs are involved in cardioprotective mechanisms, however CF mEVs have limited role in HeC.

In conclusion, we demonstrated that EVs are important players in conditions with high cardiovascular risks, such as HC, as well as in certain cardioprotective mechanisms. These results demonstrate that EVs contribute to cardiac homeostasis and further understanding of their functional roles can facilitate the development of novel diagnostics and therapeutics for CVDs. Our observations, such as the neutral effect of HeC on CM EVs, highlights the complexity of EV-mediated transcellular communication. Therefore, to fully understand the role of EVs in cardiac physiology, future experiments with specific focus on EV subpopulations are needed.

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