Adaptation mechanisms of the myocardium: adverse effects of hypercholesterolemia and cardioprotection with skeletal muscle electrical stimulation

Summary of PhD thesis

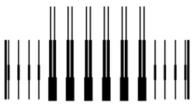
Márton Richárd Szabó

Supervisors:

Tamás Csont MD, PhD and Csaba Csonka MD, PhD

Doctoral School of Multidisciplinary Medical Sciences

Metabolic Diseases and Cell Signaling Research Group
Department of Biochemistry
Albert Szent-Györgyi Medical School
University of Szeged



2023

1 List of publications

Full papers directly related to the subject of the thesis

I. Szabó, M. R., Gáspár, R.; Pipicz, M.; Zsindely, N.; Diószegi, P.; Sárközy, M.; Bodai, L.; Csont, T., Hypercholesterolemia Interferes with Induction of miR-125b-1-3p in Preconditioned Hearts. *International Journal of Molecular Sciences* **2020,** 21, (11):3744. [D1, IF: 5.924]

II. Szabó, M. R., Pipicz, M.; Sárközy, M.; Bruszel, B.; Szabó, Z.; Csont, T., Diet-Induced Hypercholesterolemia Leads to Cardiac Dysfunction and Alterations in the Myocardial Proteome. *International Journal of Molecular Sciences* **2022,** 23, (13):7387. [D1, IF: 5.6]

III. Szabó, M. R., Csont T; Csonka C., The effect of electrical stimulation of skeletal muscle on cardioprotection and muscle-derived myokine levels in rats: a pilot study *Physiology International* **2023,** 110, (2):135-149 [Q3, IF:1.4]

Cumulative impact factors of papers directly related to the subject of the thesis: 12.924

Other publications

I. Faragó A., Zsindely N., Farkas A., Neller A., Siági F., Szabó M. R., Csont T., Bodai L., Acetylation State of Lysine 14 of Histone H3.3 Affects Mutant Huntingtin Induced Pathogenesis *International Journal of Molecular Sciences* **2022,** 23(23):15173 [D1, IF: 5.6]

II. Mitra, A.; Sarkar, A.; Szabó, M. R.; Borics, A., Correlated Motions of Conserved Polar Motifs Lay out a Plausible Mechanism of G Protein-Coupled Receptor Activation. *Biomolecules* **2021,** 11, (5). [Q2, IF: 6.064]

III. Gopisetty, M. K.; Adamecz, D. I.; Nagy, F. I.; Baji, Á.; Lathira, V.; Szabó, M. R.; Gáspár, R.; Csont, T.; Frank, É.; Kiricsi, M., Androstano-arylpyrimidines: Novel small molecule inhibitors of MDR1 for sensitizing multidrug-resistant breast cancer cells. *European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences* **2021,** 156, 105587. [Q1, IF: 5.112]

IV. Sárközy, M.; Márványkövi, F. M.; Szűcs, G.; Kovács, Z. Z. A.; Szabó, M. R.; Gáspár, R.; Siska, A.; Kővári, B.; Cserni, G.; Földesi, I.; Csont, T., Ischemic preconditioning protects the heart against ischemia-reperfusion injury in chronic kidney disease in both males and females. *Biology of sex differences* **2021,** 12, (1), 49. [D1, IF: 8.811]

V. Szabó, M. R.; Pipicz, M.; Csont, T.; Csonka, C., Modulatory Effect of Myokines on Reactive Oxygen Species in Ischemia/Reperfusion. *International Journal of Molecular Sciences* **2020,** 21, (24). [D1, IF: 5.924]

VI. Demján, V.; Kiss, T.; Siska, A.; Szabó, M. R.; Sárközy, M.; Földesi, I.; Csupor, D.; Csont, T., Effect of Stellaria media Tea on Lipid Profile in Rats. *Evidence-based complementary and alternative medicine: eCAM* **2020,** 2020, 5109328-5109328. [Q1, IF: 2.629]

VII. Adamska-Bartłomiejczyk, A.; Janecka, A.; Szabó, M. R.; Cerlesi, M. C.; Calo, G.; Kluczyk, A.; Tömböly, C.; Borics, A., Cyclic mu-opioid receptor ligands containing multiple N-methylated amino acid residues. *Bioorganic & Medicinal Chemistry Letters* **2017,** 27, (8), 1644-1648. [Q2, IF: 2.442]

VIII. Váradi, A.; Marrone, G. F.; Palmer, T. C.; Narayan, A.; Szabó, M. R.; Le Rouzic, V.; Grinnell, S. G.; Subrath, J. J.; Warner, E.; Kalra, S.; Hunkele, A.; Pagirsky, J.; Eans, S. O.; Medina, J. M.; Xu, J.; Pan, Y.-X.; Borics, A.; Pasternak, G. W.; McLaughlin, J. P.; Majumdar, S., Mitragynine/Corynantheidine Pseudoindoxyls As Opioid Analgesics with Mu Agonism and Delta Antagonism, Which Do Not Recruit β-Arrestin-2. *Journal of Medicinal Chemistry* **2016,** 59, (18), 8381-8397. [D1, IF: 6.259]

IX. Kotormán, M.; Simon, M. L.; Borics, A.; Szabó, M. R.; Szabó, K.; Szögi, T.; Fülöp, L., Amyloid-like Fibril Formation by Trypsin in Aqueous Ethanol. Inhibition of Fibrillation by PEG. *Protein and peptide letters* **2015,** 22, (12), 1104-10. [Q2, IF: 1.069]

Total cumulative impact factors: 56.834

2 Introduction

2.1 Epidemiological burden of ischemic heart diseases

Ischemic heart diseases (IHD) contribute significantly to the high mortality rate of cardiovascular diseases. IHD is a group of disorders characterized by reduced oxygen and nutrient supply to the heart muscle, followed by functional and structural deterioration of the myocardium.

2.2 The concept of ischemia/reperfusion injury

Acute myocardial infarction is the most severe manifestation of IHD. The reduced blood flow to the myocardium results in cardiac arrhythmias, contractile impairment, and irreversible myocardial cell damage. In-time readmission of blood flow, termed reperfusion therapy, is mandatory to salvage the ischemic myocardium. Paradoxically, prompt and full restoration of oxygen and nutrient supply contributes to further structural damage and contractile impairments; therefore, the sum of the resulting cellular and functional myocardial damage is called ischemia/reperfusion (I/R) injury.

2.3 Cardiac adaptation to ischemic injury

The heart has been shown to possess a remarkable ability to withstand the detrimental effects of ischemic injury. One of the most powerful strategies to trigger endogenous cardioprotective mechanisms in the myocardium is ischemic preconditioning (IPre), i.e., when brief, repetitive cycles of I/R are applied before the sustained lethal ischemia. Additionally, alternative approaches that mimic the ischemic conditioning mechanism without direct impact on the myocardium have been also developed.

2.3.1 The role of microRNAs in ischemic preconditioning

Several studies demonstrated that microRNAs (miR) play an important role in the pathophysiology of myocardial infarction and have emerged as regulators of preconditioning possibly through the modulation of reactive oxygen species production. Recently members of the miR-125b family were also implicated in the pathomechanism of IHD with a special emphasis on miR-125b-1-3p. Although pieces of evidence suggest a protective role for miR125b-1-3p in the ischemic heart, little is known about whether these beneficial effects are also manifested in the presence of risk factors predisposing to myocardial infarction.

2.4 Hypercholesterolemia is a major risk factor for ischemic heart diseases

IHDs are a complex group of diseases that are associated with certain well-known risk factors and comorbidities like high blood pressure, aging, sedentary lifestyle, and metabolic diseases (e.g.: hyperlipidemia, diabetes, insulin resistance), respectively. Among the many

risk factors increased blood cholesterol, termed hypercholesterolemia, has a significant role in the development of IHD. Elevated circulating cholesterol is associated with endothelial dysfunction and subsequent atherosclerosis of the blood vessels.

2.4.1 Direct effects of hypercholesterolemia on the myocardium

Independently from its proatherogenic effect, direct cardiac consequences are also responsible for the adverse effects of elevated blood cholesterol levels such as enhanced formation of reactive oxygen species, decreased nitric oxide bioavailability, and impaired mitochondrial function in the myocardium. Additionally, the development of cardiac dysfunction and disturbed cardiac stress adaption mechanisms are characteristic outcomes of hypercholesterolemia. Although the adverse cardiac effects of hypercholesterolemia are well known, the precise underlying molecular mechanisms and involved pathways are still not fully understood. Nonetheless, testing alternative conditioning approaches might provide the basis for the elaboration of applicable methods that can restore the endogenous adaptive mechanisms of the hypercholesterolaemic heart.

2.5 An alternative approach for cardioprotection – electromyostimulation (EMS)

Apart from the ischemic conditioning techniques, direct cardioprotection can be elicited remotely through distant organs and tissues. It is well-established that physical activity has potent protective and therapeutic effects against heart diseases. However, performing regular exercise is often limited due to various health reasons. For those, electrical muscle stimulation or electromyostimulation (EMS), the rhythmical muscle activation triggered with electrical impulses, might provide an alternative way to partially gain the benefits of exercise. EMS is a widely used method in sport- and rehabilitation therapy and is a far more attractive clinical application for subjects unable to perform regular exercise. Nevertheless, improvements in exercise capacity and quality of life are established outcomes of EMS treatment. To date, little is known whether these beneficial effects of EMS can trigger cardiac preconditioning and protection against I/R injury.

2.5.1 The role of myokines in the skeletal muscle-mediated cardioprotection

Both humoral and neural factors are implicated in the favorable effects of physical activity on the general health of the individual. Recently, skeletal muscle-derived myokines are emerged as the molecular mediators of the systemic beneficial effects of exercise through endocrine signaling pathways. The term myokine or exerkine is collectively used for a wide variety of cytokines and proteins, which are predominantly produced and released by contracting skeletal muscles. However, little is known whether muscle contractions evoked by electrical stimuli can trigger the expression and secretion of different myokines.

3 Aims

The present thesis aimed to investigate different aspects of cardiac adaptation mechanisms against I/R injury. In our work, we focused on the detrimental effects of hypercholesterolemia on the heart as well as whether electrical stimuli-triggered skeletal muscle contraction evoked cardiac preconditioning in normocholesterolemia. The specific aims of the thesis are the followings:

1. The recent advances in the field of 'omics' techniques provide a great and effective tool for high throughput screening and molecular profiling of different conditions and diseases. Therefore we aimed to utilize proteomics and subsequent bioinformatic analyses to characterize the global left ventricle proteome in the settings of hypercholesterolemia. Additionally, we were also interested in identifying enriched pathways and protein interaction networks concerning the adverse cardiac effects of hypercholesterolemia.

2. Cardioprotection conferred by IPre is lost in diet-induced hypercholesterolemia, however, limited literature data is available focusing on the precise molecular background and the possible amelioration strategies against the deleterious myocardial effects of hypercholesterolemia. Hence we aimed to address whether is there any association between the blunted cardioprotection of IPre in the settings of hypercholesterolemia and IPre-induced upregulation of miR-125b-1-3p in the heart.

3. The development of alternative conditioning approaches to confer cardioprotection in the presence of different comorbidities has great importance. Taking together the promising therapeutic potential of EMS, we also applied a pilot experimental setup in normocholesterolemic rats to test the remote preconditioning manner of short-term EMS treatment. Moreover, the investigation of skeletal muscle-derived myokine expression and secretion, as the possible mediators of EMS-associated cardioprotection, was also in the scope of the present thesis.

4 Materials and Methods

4.1 Animals

All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Animal Research Ethics Committee of Csongrád County (XV.1181/2013, XV.2153/2022) and the local animal ethics committee of the University of Szeged. Male Wistar rats, weighing between 300-350 g were used in this study. The animals were kept in pairs in individually ventilated cages in a temperature-controlled room with 12 h:12 h light/dark cycles. Laboratory chow and water were supplied ad libitum throughout the study.

4.2 Effect of hypercholesterolemia on the left ventricular proteome

4.2.1 Experimental setup

Male Wistar rats were fed with 2% (w/w) cholesterol and 0.25% (w/w) sodium-cholate enriched laboratory chow for eight weeks. The control animals were fed with standard rat chow. At the end of the diet period, hearts were isolated from anesthetized animals, then left ventricular tissue samples were rapidly frozen in liquid nitrogen and stored at -80 °C until proteomics analyses.

4.2.2 Transthoracic echocardiography

Cardiac morphology and function were assessed by transthoracic echocardiography with a Vivid IQ ultrasound system using a phased array 5.0-11 MHz transducer.

4.2.3 Blood lipid measurement

At the end of the diet period, blood samples from the thoracic aorta were collected to determine total cholesterol and triglyceride concentrations.

4.2.4 Proteomics analysis by liquid chromatography-mass spectrometry

Frozen left ventricular tissue samples were homogenized and proteins were precipitated. Digested samples were pooled to build a spectral library for quantitative liquid chromatography–mass spectrometry analysis. The separation of the digested samples was carried out on a nanoAcquity Ultra Performance Liquid Chromatograph and coupled to a high-resolution Q Exactive Plus quadrupole-orbitrap hybrid mass spectrometer. Quantitative analysis of the spectral library was performed in Encyclopedia v0.9.

4.2.5 Bioinformatic analyses of proteomics data

Pathway enrichment analysis was performed with the web-based g:Profiler public server and EnrichmentMap v3.3.2. application of Cytoscape v3.8.2. Protein-protein interactions analysis among the identified proteins was performed with STRING v11.5

database and modified using the STRINGApp v1.7.0 and STRING Enrichment applications. Cluster analysis was performed with clusterMaker2. Enriched gene sets from the whole, unfiltered proteomics data were explored with Gene Set Enrichment Analysis (GSEA) software v4.1.0. The results of the GSEA analysis were further analyzed according to the Kyoto Encyclopedia of Genes and Genome resource using Pathview Web.

4.3 Influence of hypercholesterolemia on preconditioning-induced miR-125b-1-3p expression

4.3.1 Experimental setup

In a separate set of experiments, hypercholesterolemia was induced as described above. At the end of the eight-week feeding period, hearts from both feeding groups were isolated and perfused according to Langendorff. Isolated hearts were divided into global I/R or IPre subgroups. The time-matched I/R control group hearts were equilibrated for 45 min before 35 min global ischemia and 120 min reperfusion. In the IPre group after 15 min equilibration time, three intermittent cycles of 5 min no-flow ischemia, separated by 5 min aerobic perfusion was applied before the onset of global ischemia to induce IPre. At the end of the reperfusion, either infarct size was determined or miR analysis was performed.

4.3.2 Infarct size determination

Ventricles were cut into equal slices and incubated in triphenyl tetrazolium chloride (TTC) solution. Digitalized images from the stained heart slices were evaluated with planimetry method.

4.3.3 Creatine-kinase release measurement

Coronary effluents were collected after 2, 5, 30, and 120 minutes of the beginning of the reperfusion to measure the release of cardiac-specific creatine kinase (CK-MB) enzyme.

4.3.4 Measurement of cardiac miR-125b-1-3p level with miR-sequencing

Total RNA was isolated from left ventricles and next-generation sequencing was performed with Illumina MiSeq instrument to investigate miR-125b-1-3p expression. Sequencing reads were quality checked and analyzed with the mirdeep2 package.

4.4 The effect of skeletal muscle electrical stimulation on cardioprotection and muscle-derived myokine levels

4.4.1 Experimental setup

EMS treatment was performed in sedated normocholesterolemic rats. Three EMS sessions were applied; each included 10 Hz frequency continuous stimulation for 35 minutes

daily. Twenty-four hours after the last EMS treatment, rats were anesthetized, then blood and *gastrocnemius* muscle samples were collected for myokine level measurements. Hearts from EMS-treated and untreated animals were isolated and perfused according to Langendorff; equilibrated for 15 min followed by 30 min global ischemia and 120 min reperfusion. During reperfusion, coronary effluents were collected and used to measure cardiac lactate-dehydrogenase (LDH) and CK-MB release. At the end of the reperfusion, infarct size was determined by TTC staining as described above.

4.4.2 Electrical muscle stimulation

EMS treatment was performed with a portable electrostimulation device. Bilateral EMS was applied to target the *gastrocnemius* muscles with stimulating electrodes once a day for three consecutive days.

4.4.3 Determination of skeletal muscle myokine expression and secretion

Total RNA was isolated from the *gastrocnemius* muscles and used as the template for qPCR measurement of myokine expression levels. Double-antibody sandwich ELISA kits specific for rat Irisin, Decorin, Myonectin, Myoglobin, IL-6, IL15, and FSTL1 proteins, respectively, were used to measure protein content in gastrocnemius and serum samples.

4.4.4 Western Blot analysis

From left ventricular samples phosphorylation of protein kinase B (AKT), extracellular signal-regulated kinase (ERK), protein kinase B (AKT), and signal transducer and activator of transcription 3 (STAT3) proteins were detected by Western blot.

4.5 Statistics

Student's t-test was used to evaluate blood lipid levels, echocardiographic parameters, and to evaluate the effect of EMS on infarct size. Two-way analysis of variance (ANOVA) was used to evaluate the infarct size limiting effect of IPre. For proteomic data, statistical significance was tested using an unpaired Welch test. Multiple testing correction was applied in the pathway-enriched analysis. Wald test was performed in differential miR expression analysis. Repeated measures ANOVA was used for evaluation of LDH and CK-MB releases. Relative expression levels of myokines were determined with the 2^-ΔΔCt method. For all statistical evaluations through the experiments, a p-value < 0.05 was considered as an indicator of significant difference among the groups.

5 Results

5.1 Characterization and network analysis of the left ventricular proteome in the settings of hypercholesterolemia

5.1.1 Effect of cholesterol-enriched diet on plasma lipid levels

At the end of the eight-week feeding period, total plasma cholesterol showed a marked elevation in the cholesterol-fed group, supporting the manifestation of diet-induced hypercholesterolemia. The plasma total triglyceride level also increased significantly in the hypercholesterolemic animals.

5.1.2 Cardiac function assessed by transthoracic echocardiography

Early (E) and late (A) ventricular filling velocities showed a trend toward a decrease, thus significantly elevating the E/A ratio in the hypercholesterolemic hearts, suggesting impaired diastolic function. The presence of diastolic dysfunction was further supported by significantly decreased mitral annulus velocity (e') and mitral valve deceleration time.

5.1.3 Proteomic characterization of the hypercholesterolemic left ventricle

Differential expression analysis revealed the upregulation of 23 proteins and downregulation of 22 proteins with at least a 1.2-fold change value in the left ventricle of hypercholesterolemic animals compared with the normocholesterolemic controls.

5.1.4 Pathway enrichment analysis of the significantly altered proteins

Based on Gene Ontology (GO) and subsequent pathway enrichment analyses the significantly altered proteins are associated with contractile function and cytoskeletal organization. Additionally, a minor enrichment of mitochondrial proteins was also observed.

5.1.5 Functional interaction analysis of the differentially expressed proteins

Beta-actin (ACTB) was downregulated in the hypercholesterolemic myocardium and established a prominent hub within the functional interactions of the significantly altered proteins. Additionally, the cluster analysis revealed other minor subnetworks among the resulting interactions, which might implicate disturbed metabolic functions and subsequent energy production.

5.1.6 Protein-specific gene set enrichment analysis

Gene set enrichment analysis (GSEA) identified downregulated expression patterns among the proteins associated with mitochondrial complexes, with particular emphasis on the elements of the respiratory chain complexes as well as in proteins with important roles in normal cardiac contractile function.

5.2 Testing the effect of hypercholesterolemia on ischemic preconditioning-induced miR-125b-1-3p upregulation and cardioprotection

5.2.1 Effect of hypercholesterolemia on ischemic preconditioning

In the hearts of normocholesterolemic rats, IPre significantly decreased infarct size compared to the I/R control group, however, IPre failed to significantly attenuate infarct size in the hearts of hypercholesterolemic animals. Additionally, at the end of the reperfusion, IPre significantly decreased CK-MB enzyme release activity only in normocholesterolemic but not in hypercholesterolemic hearts.

5.2.2 The effect of preconditioning on miR-125b-1-3p levels

At the end of reperfusion, IPre significantly upregulated miR-125b-1-3p in normocholesterolemic hearts compared to I/R controls. In contrast, IPre failed to increase significantly miR-125b-1-3p levels in the hearts of hypercholesterolemic animals.

5.3 The effect of electrical stimulation of skeletal muscle against cardiac ischemia/reperfusion and on muscle-derived myokine levels

5.3.1 Effect of skeletal muscle EMS on ex vivo perfused hearts

Cardiac CK-MB and LDH release were significantly lower upon EMS at the end of reperfusion. Although the mean value of infarct size tended to be lower in the EMS group compared to the nonstimulated control group (approximately by 20%), the applied EMS treatment failed to attenuate infarct size significantly.

5.3.2 Assessment of myokine expression levels in the stimulated muscle

Among the investigated myokines the applied EMS treatment upregulated *Fstl1, Il6,* and *Igf1* mRNA expression in the gastrocnemius muscle. Additionally, Irisin, Decorin, Myonectin, FSTL1, and Myoglobin proteins were upregulated as a consequence of EMS.

5.3.3 Measurement of serum myokine levels upon EMS

At the time of serum sampling none of the measured myokines showed significant differences in the blood compared to the untreated control animals.

5.3.4 Effect of EMS on cardiac conditioning-associated pathways

Phosphorylation of ERK1 and ERK2 showed a trend toward an increase in the hearts of EMS-treated animals compared to the untreated controls. Additionally, phosphorylation of STAT3 and AKT proteins was not affected in the left ventricles.

6 Discussion and conclusion

In the present thesis, we investigated different aspects of myocardial infarction and its major risk factor, hypercholesterolemia. Based on our results, hypercholesterolemia alters the expression of proteins related to the maintenance of the contractile and cytoskeletal structure and energy generation processes. We have also shown an inverse correlation between the attenuated cardioprotective effect of IPre in hypercholesterolemia with diminished miR-125b-1-3p induction, suggesting that miR-125b-1-3p may be an important activator of IPre-induced cardioprotection and its decreased expression level seems to interfere with the infarct size limiting effect of IPre in hypercholesterolemia. Furthermore, despite the lack of significant infarct size reduction in normocholesterolemia, EMS application seems to influence the course of cellular damage due to I/R and alters the expression levels of several myokines in the skeletal muscle, which might be potential mediators of the beneficial effects of EMS in the cardiovascular system.

6.1 New findings

The novel findings of the present thesis can be summarized as follows:

- Hypercholesterolemia is associated with an altered left ventricular proteome.
- Pathway enrichment and interaction analyses revealed hypercholesterolemia-associated protein changes in contractile and cytoskeletal systems as well as in the protein components of the mitochondrial respiratory chain.
- Upregulation of miR-125b-1-3p induced by preconditioning is lost in settings of hypercholesterolemia.
- EMS treatment seems to alleviate the I/R damage of ex vivo perfused normocholesterolemic hearts
- EMS is associated with modified myokine mRNA and protein levels in the targeted gastrocnemius muscle tissue

6.2 *Alterations in the contractile and cytoskeletal system and mitochondrial respiratory chain possibly contribute to hypercholesterolemia-associated cardiac dysfunction*

In the present work, we utilized the advantages of downstream bioinformatics analyses of the proteomics dataset to clarify the underlying protein expression changes associated with the direct cardiac effects of hypercholesterolemia. Mild diastolic dysfunction developed in hypercholesterolemic rats as reported previously. In line with the impaired cardiac function,

the proteome scale analyses of the hypercholesterolemic myocardium revealed modest quantitative changes in the left ventricular proteome. Possible rearrangements of subcellular structures and macromolecular complexes in the left ventricle upon hypercholesterolemia is further supported by our GO analysis of the differentially expressed proteins as the resulting network implicates alterations in the contractile apparatus and cytoskeletal system of the hypercholesterolemic left ventricle. Protein-protein interactions network analysis highlighted many interactions among the differentially expressed proteins, of which ACTB formed the hub of the revealed network, thus forming many interactions with other accessory proteins, suggesting cytoskeletal rearrangements.

A deeper analysis of the whole, unfiltered left ventricular proteome turned out that hypercholesterolemia negatively influenced many protein components of the mitochondrial respiratory chain system in the heart. Impaired mitochondrial function is implicated in the adverse cardiac effects of hypercholesterolemia, which might be associated with the downregulation of the elements of the respiratory chain complex. Based on the findings of the present thesis we can conclude that hypercholesterolemia induced quantitative changes in the left ventricular proteome, affecting both the contractile and cytoskeletal apparatus as well as the mitochondrial respiratory chain system. These alterations might provide a feasible explanation for the mild cardiac dysfunction observed in hypercholesterolemia. Nevertheless, these results of our network and enrichment analyses might contribute to a better understanding and development of further therapeutic approaches mitigating cardiac dysfunction in the presence of metabolic risk factors.

6.3 The attenuated cardioprotective effect of ischemic preconditioning upon hypercholesterolemia is correlated with diminished miR-125b-1-3p induction

IPre is one of the most powerful endogenous cardioprotective approaches as it markedly enhances the ability of the heart to withstand ischemic injury, however, the exact mechanism is still not entirely clear. Previous findings suggested that miR-125b-1-3p upregulation might be an important mediator of IPre-induced cardioprotection. In our study, the application of IPre in the presence of hypercholesterolemia failed to upregulate miR-125b-1-3p expression as compared to I/R. This is in line with previous reports demonstrating that hypercholesterolemia abolished the infarct size-limiting effect conferred by IPre. Additionally, hypercholesterolemia *per se* seems to alter cardiac miR expression profile, which is possibly associated with the alleviated protective effect of ischemic conditioning.

Although several studies revealed relationships between IPre and cardiac miRNA levels in normocholesterolemic subjects, this is the first demonstration that hypercholesterolemia influences miRNA expression changes induced by IPre. Upregulation of miR-125b-1-3p is considered an adaptive response of IPre, however, the mRNA targets in the myocardium are still not clear. Our study suggests that the upregulation of miR-125b-1-3p in response to IPre may be an important element in the cardioprotective mechanism, however, further studies need to confirm a direct causative relationship.

6.4 Electromyostimulation treatment seems to mitigate I/R injury on ex vivo perfused heart – the possible role of myokines

EMS has been long utilized to either supplement or substitute muscle strengthening in several rehabilitation settings. To test whether EMS could be a feasible alternative for cardiac conditioning, an ex vivo heart perfusion method was applied. In our perfusion model, LDH and CK-MB release during the reperfusion was significantly decreased in the ex vivo perfused hearts of animals that received EMS. However, EMS before global I/R failed to significantly decrease the infarcted area. Our results suggest that despite the lack of significant infarct size reduction EMS treatment might initiate different cardioprotective mechanisms which were partially retained during the ex vivo heart perfusion. These observations are in accordance with previous studies where targeted electrical stimulation of peripheral nerves of the limbs straight before I/R mitigated myocardial infarct size and improved the post-ischemic cardiac performance in rodents. For this reason, electrical stimulation of the skeletal muscle might provide an alternative remote preconditioning approach; nevertheless, further optimization of the protocol is warranted before deeper investigations of the method.

Several hundred cytokines and oligopeptides, termed myokines, are produced and released by muscle in response to muscle contractions. These molecules may act as mediators which link muscle exercise to the whole body physiology, nevertheless, the majority of myokines were also shown to exert protection against ischemia. Based on our findings, the applied EMS protocol altered the expression of several myokines. Similarly to voluntary exercise, myokines might be also involved in the cardioprotective effects of EMS, however, verification of their mechanistic role remains for the scope of future studies.

7 Acknowledgment

Our studies were supported by the grants OTKA-NKFIH (K115990), EFOP 3.6.2-16-2017-00006, GINOP 2.3.2-15-2016-00006, GINOP-2.3.2-15-2016-00040 and TKP2021-EGA-32 project. Furthermore, the present work was supported by the ÚNKP-19-3-SZTE-269 grant provided by the New National Excellence Program of the Ministry for Innovation and Technology.

I would like to express my sincere gratitude to Prof. Dr. László Dux for providing me with the possibility to join the Department of Biochemistry.

I owe my greatest gratitude to my supervisors, Dr. Tamás Csont and Dr. Csaba Csonka for their scientific guidance, encouragement, and loads of helpful suggestions through the past years in both professional and personal aspects.

I am also thankful to the senior members of our research group, Dr. Márton Pipicz, Dr. Márta Sárközy, and Dr. Gergő Szűcs for their practical suggestions and advice.

This endeavor would not have been possible without the invaluable contribution of Dr. Renáta Molnár-Gáspár as well as my fellow Ph.D. students, Dr. Andrea Apjok-Sója, Dr. Virág Demján, and Petra Diószegi.

I owe a special thanks to all the members of the Department of Biochemistry especially Atina Čolić, Dóra Csóré, Éva Plechl, Réka Somogyi, Ildikó Engi, Tünde Bodnár, Imre Ocsovszki, and József Papp for their technical support and willingness to help me anytime. I am also grateful to my former undergraduate students, Dr. Réka Marik and Dr. Kamilla Nagy for their enthusiasm during our common scientific work.

I would like to thank our cooperation partners Dr. László Bodai and Dr. Nóra Zsindely to make us possible to perform miRNA sequencing, also for Dr. Zoltán Szabó and Bella Bruszel for the quantitative proteomics.

I am deeply indebted to my mentor during my undergraduate years, Dr. Attila Borics whom I learned the scientific perspective.

I could not have undertaken this journey without the emotional support of my mother, my father, my mother's husband Árpi, my sister Tina, my brothers Patrik, Oli, and Levi, as well my best friend Tomi. Above all, words cannot express my gratitude to my wife, Anikó; her never-ending love and enormous effort to provide stable background to our children have always kept my spirits and motivation high during this process.