OTKA ANN 107803 project - Final report

Summary

To limit the size of infarction during ischemia/reperfusion injury of the heart, development of cardioprotective strategies is of great importance. The main objective of the present OTKA- and FWF-funded bilateral project was to investigate the efficacy of cardioprotective interventions such as ischemic postconditioning (IPostC), ischemic remote perconditioning (RIC) and ischemic preconditioning (IPreC) in a clinically relevant, close-chest swine models, and to identify pivotal microRNAs (miRNAs) in the mechanism of ischemic cardioprotection. Here we identified that in a porcine model of acute ischemia/reperfusion injury IPostC and RIC interventions may protect the coronary microvasculature even without reducing myocardial necrosis. We successfully identified key miRNAs modulated by the investigated cardioprotective interventions and used bioinformatic analysis methods to pinpoint pivotal miRNAs. Therapeutic potential of these miRNAs are under investigations. Furthermore, here we assessed mRNA targets of the identified cardioprotective miRNAs. We observed an early activation of Ca-, adipocytokine and insulin signaling pathways and a late activation of antigen immunomodulatory pathways with upregulation of STAT1 and downregulation of neprilysin. In addition, we discovered that exosomes, potential carriers of miRNAs, are necessary for cardioprotection by ischemic conditioning. We published 15 articles in peer-review high rank journals, 11 conference presentations, and 2 PhD theses are based on the results of this program. Our achievements show a greatly successful conclusion of the project that opened up paths for new projects and international collaborations.

Aims Proposed in the project:

1. To identify miRNAs responsible for cardioprotection by ischemic postconditioning.
2. To identify molecular targets of miRNAs relevant to postconditioning
3. To isolate exosomes and analyze whether they contain miRNAs involved in cardioprotection
4. To modulate the targets of the identified miRNAs, and to assess whether this treatment confers cardioprotection similarly to ischemic postconditioning.
5. To identify miRNAs characteristic on the efficacy of cardioprotection by ischemic postconditioning.

Performed experiments

During the first year of the project, we performed most of the proposed porcine cardiac interventional procedures to investigate the role of miRNA in cardioprotection and collected biological samples for further studies. We induced acute myocardial infarction in 45 swine, 14 of which were control infarctions, 14 IPostC, 8 RIC, 3 control limb ischemia and 6 sham operated

controls. In the second year of our project we performed the remaining interventions, hence, we enrolled 51 swine, 11 of which were control infarction, 6 IPostC, 10 RIC, 1 sham-operated control. We had to exclude 3 animals due to severe adverse events. Based on our interim results and on novel literature on the efficacy of IPostC in humans, a major alteration was introduced to the scientific plan to increase the impact of our study. Accordingly, in the second year of the study, we included an additional experimental group treated with IPreC (12 animals), a well-characterized, effective positive control of cardioprotection. Eight animals were lost during intervention. Therefore, the total number of animals used in this study was 96. We performed the experiments at the Kaposvar University diagnostic center.

To establish efficacy of the cardioprotective interventions, MRI and echocardiographic measurements were performed in animals in the 3 day-survival arm of the study. MRI data and cardiac functional measurements were evaluated in collaboration with two (one Austrian and one Hungarian) cardiac MRI experts in a blinded fashion to exclude the possibility of misinterpretation. Echocardiographic measurements were performed and evaluated by an expert from University of Szeged. Furthermore, we have assessed the infarct size with the gold standard triphenyltetrazolium chloride (TTC) staining in addition to MRI in the 3h-survival arm of the study. This measurement was necessary since discrepancies have been recently shown in the literature between MRI and TTC data and these results increased the value of our experimental model and data.

To carry out molecular, histological and biochemical analyses, e.g. miRNA, mRNA, protein and enzyme activity measurements and exosome isolation, we collected more than 7000 samples according to pre-defined protocols. We harvested heart samples (ischemic, border, non-ischemic samples from all chambers), liver, aorta and lung samples and stored them flash-frozen, in formalin, RNALater and glutaraldehyde, collected venous blood samples at 4-7 time points, blood plasma from the coronary sinus during and after ischemia to capture the most possible miRNA and exosomes released from the myocardium.

To investigate the effect of ischemic conditioning on myocardial electrical activity, NOGA endocardial mapping was performed on a number of animals by our Austrian partner.

We organized further collaborations to maximize the outcome of the current project, i.e with Dr. Péter Hamar (Semmelweis University) to investigate the effect of remote conditioning on the kidney, with Prof. Manuel Mayr (University College London) to assess the effect of cardioprotective interventions on platelet functions, with Dr. Adriana Adameova (Comenius University, Bratislava) to assess CaM kinase and necroptosis in ischemic conditioning, with Dr. Zsuzsanna Helyes (University of Pécs) to assess the role of somatostatin signaling in cardioprotection, and with Prof. Rainer Schulz (University of Essen) to assess the effect of ischemic conditioning on post-infarct remodeling and cardiac inflammation. These collaborations will greatly increase the scientific output of the current project and may lead to multiple further side projects.

Major achievements

Cardiac MRI investigations showed that in our translational porcine model of acute ischemia/reperfusion injury IPostC and RIC reduced the extent of myocardial edema, a potential marker for cardioprotection (Fig 1b), although, infarct size was not affected (Fig 1a). Furthermore, microvascular obstruction volume was significantly decreased by IPreC and IPostC, but not by RIC as compared to the Isch group, which evidence that the clinically applicable conditioning interventions protect cardiac vasculature (Fig 1c).

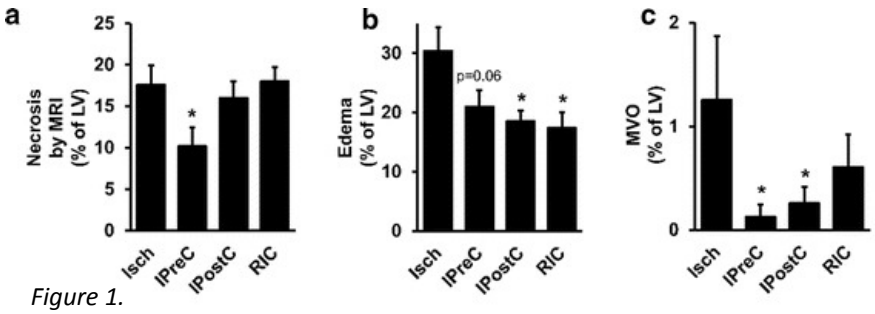

Figure 1.

Based on our histological measurements, (TTC staining), IPostC and RIC have failed to reduce infarct size, however, IPreC, a positive control treatment reduced infarct size significantly, which validated that cardioprotection could be achieved in our models (Figure 2).

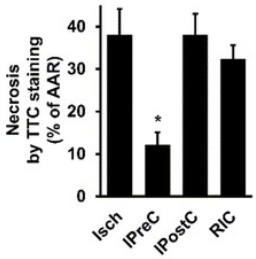

Figure 2.

Myocardial hemodynamic and electrophysiologial function was analyzed by cardiac MRI and echocardiography by NOGA mapping, and was not different between groups after either 3 h or 3 days of reperfusion. Biochemical analyses have shown that IPreC, the positive control treatment, attenuated the release of creatin-kinase MB (CK-MB), a cardiac necroenzyme. IPostC has also shown a tendency to attenuate CK-MB release, however, RIPerC have failed to do so. These results further evidence that IPostC and RIC protected the microvasculature against ischemia/reperfusion injury and that myocardial edema and microvascular obstruction may change independently from myocardial necrosis. Our results support findings of clinical trials that myocardial edema may change due to various interventions and it does not represent area at risk. We published our these results in 2 papers (Pavo et al, JACC Cardiovasc Imaging. 7(9):956-8, 2014. and Baranyai et al, J Transl Med. 1;15(1):67, 2017).

In order to evaluate cardiac ultrastructural alterations, we initiated collaboration with László Deres (University of Pécs), an expert in myocardial electron microscopy, evidencing large-scale structural degradation of contractile elements and mitochondria in ischemic tissues of IPostC, RIC and Ischemic groups (Fig 3).

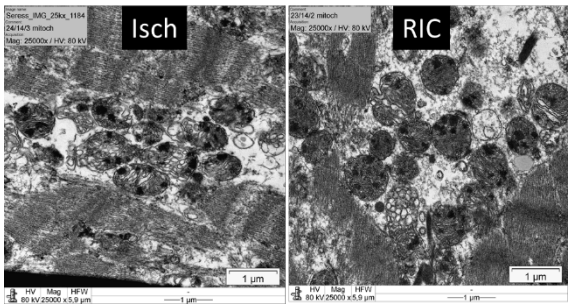

Figure 3. Representative EM images (Ischemic vs. RIC) showing disorganized ultrastructure

To characterize miRNA expression in the heart, we have run a high-throughput qPCR experiment in collaboration with Dr. László Puskás (Biological Research Center of Hungarian Academy of Sciences, Szeged) and analyzed expression of more than 200 miRNAs in our samples. Due to the complexity of high-throughput RNA analyses, we employed a PhD student to perform bioinformatics network analyses. The analysis indicates that several miRNA can be connected to at least 2 types of cardioprotective interventions. We identified these miRNAs as most likely candidates to mediate cardioprotection by ischemic conditioning. The identity of these miRNAs will be disclosed after the evaluation of the need for intellectual property protection.

Our Austrian partner performed gene expression analysis of the heart in remote and infarcted areas by using next generation sequencing, network dynamical stimulation and attractor landscape analyses after 3 hours or 3 days post-AMI. We analyzed the expression of genes involved in pro-survival kinase pathways, which were mostly similar in IPostC and Isch experimental groups in both time windows and tissue regions. However, significant deregulation of gene sets enriched in focal adhesion pathway was observed in the late window of cardioprotection after IPostC. Transcripts involved in adhesion and activation of blood cells, cardiac hypertrophy and cardiomyopathy were downregulated, further evidencing a beneficial effect of IPostC on microvasculature (Pavo et al, Sci Rep, 2017).

To enhance the predictive value of our bioinformatics-based analyses, our workgroup is currently building a network analysis protocol to incorporate and correlate mRNA data from our Austrian partner with our miRNA network information. From the combined, multi-level network analysis we expect to better identify pivotal signaling mechanisms of ischemic conditioning, the pharmacological modulation of which may reveal novel tools to mimic cardioprotection.

Endogenous survival pathways have been also assessed at protein level in the infarcted regions and border zones of the heart by Western blot. In these experiments we assessed that IPostC

induces the phosphorylation of Akt in ischemic tissue, and that of Erk in the border zone, as compared to ischemic hearts. These results further evidence that cardioprotective signaling cascades are triggered in IPostC.

In a collaboration with Prof. Manuel Mayr, we also performed proteomic analysis of heart samples. These investigations revealed that 12, 11, and 27 proteins are differentially expressed in the ischemic regions of IPC, RIC, and IPostC hearts, respectively, as compared to non-conditioned ischemic samples. 2D-DIGE analyses were performed (Fig 4), and the identification of the proteins with differential expression is being investigated.

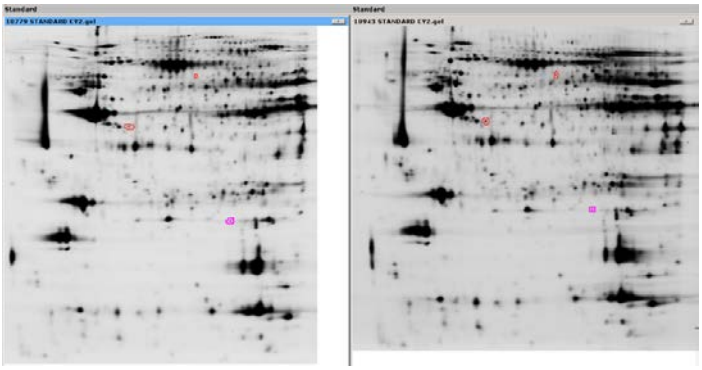

Figure 4. Example gels (sham vs. ischaemia) demonstrating differentially expressed proteins

Since our main objectives included the characterization of exosomal transfer of cardioprotection, we optimized the parameters for the isolation and storage of exosomes from plasma samples, which was essential for the downstream analyses (Baranyai et al, PLoS One. 2015 21;10(12):e0145686.). Furthermore, we performed an exploratory study in rats and proved that ischemic conditioning induces a release of exosomes from the heart and that exosomes are necessary for ischemic conditioning (Giricz et al, J Mol Cell Cardiol. 2014;68:75-8.). Accordingly, we were the first in the literature to show that remote ischemic preconditioning is transferred by exosomes in the rat heart. Currently we are analyzing miRNA and protein content of exosomes isolated from the blood from Ischemic, IPC, RIC, and IPostC animals to assess diagnostic potentials of exosomal cargo and to seek for novel therapeutic targets.

Further experiments and future perspectives

The unique opportunity of this project to finance such a volume of large animal experiments and sample collections enabled us to build new national and international collaborations and secure further financial support. Therefore, we designed several additional experiments currently being performed, where we further explore cellular and vesicular mechanisms of cardioprotective interventions and its therapeutic potential. In addition, we are currently further validating our miRNAs and their mRNA and protein targets in small- and large animal models to develop therapeutic tools with translational potential to elicit cardioprotection. Moreover, the several thousand of biological samples collected in this program will lead to further discoveries in cardioprotection.

Research output

Goals set in our grant proposal have been achieved by our scientific programs, and several further aims have been investigated. The grant has contributed to a high number (15) of publications in mostly high-rank peer-reviewed journals and conference presentations (11). Moreover, the results are the basis of two PhD theses. The scientific output exceeded initial expectations, mostly due to the high number of new international and national collaborations. The number of publications is going to be further increased in the near future since manuscripts of numerous experiments have not yet been submitted due to additional experimental needs. We have to emphasize here, that largely based on this program, the project leader of this program was able to organize writing 2 position papers on behalf of the European Society of Cardiology Working Group on Cellular Biology of the Heart (see publications 13 and 15).

Published peer-reviewed articles:

1. Csonka C, Kupai K, Bencsik P, Görbe A, Pálóczi J, Zvara A, Puskás LG, Csont T, Ferdinandy P.: Cholesterol-enriched diet inhibits cardioprotection by ATP-sensitive K+ channel activators cromakalim and diazoxide, Am J Physiol Heart Circ Physiol. 306(3):H405-13., 2014
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3. Giricz Z, Varga ZV, Baranyai T, Sipos P, Pálóczi K, Kittel Á, Buzás EI, Ferdinandy P.: Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles., J Mol Cell Cardiol. 2014;68:75-8., 2014
4. Pavo N, Emmert MY, Giricz Z, Varga ZV, Ankersmit HJ, Maurer G, Hoerstrup SP, Ferdinandy P, Wu JC, Gyöngyösi M.: On-line visualization of ischemic burden during repetitive ischemia/reperfusion., JACC Cardiovasc Imaging. 2014 Sep;7(9):956-8., 2014
5. Varga ZV, Zvara A, Faragó N, Kocsis GF, Pipicz M, Gáspár R, Bencsik P, Görbe A, Csonka C, Puskás LG, Thum T, Csont T, Ferdinandy P.: MicroRNAs associated with ischemia-reperfusion injury and cardioprotection by ischemic pre- and postconditioning: protectomiRs., Am J Physiol Heart Circ Physiol. 2014 Jul 15;307(2):H216-27., 2014
6. Baán JA, Varga ZV, Leszek P, Kuśmierczyk M, Baranyai T, Dux L, Ferdinandy P, Braun T, Mendler L.: Myostatin and IGF-I signaling in end-stage human heart failure: a qRT-PCR study., J Transl Med. 2015 Jan 16;13:1. doi: 10.1186/s12967-014-0365-0., 2015
7. Madonna R, Cadeddu C, Deidda M, Giricz Z, Madeddu C, Mele D, Monte I, Novo G, Pagliaro P, Pepe A, Spallarossa P, Tocchetti CG, Varga ZV, Zito C, Geng YJ, Mercuro G, Ferdinandy P.: Cardioprotection by gene therapy: A review paper on behalf of the Working Group on Drug Cardiotoxicity and Cardioprotection of the Italian Society of Cardiology., Int J Cardiol. 2015 Jul 15;191:203-10., 2015
8. Pickard JM, Bøtker HE, Crimi G, Davidson B, Davidson SM, Dutka D, Ferdinandy P, Ganske R, Garcia-Dorado D, Giricz Z, Gourine AV, Heusch G, Kharbanda R, Kleinbongard P,

MacAllister R, McIntyre C, Meybohm P, Prunier F, Redington A, Robertson NJ, Suleiman MS, Vanezis A, Walsh S, Yellon DM, Hausenloy DJ.: Remote ischemic conditioning: from experimental observation to clinical application: report from the 8th Biennial Hatter Cardiovascular Institute Workshop., Basic Res Cardiol. 2015 Jan;110(1):453., 2015

9. Schulz R, Görge PM, Görbe A, Ferdinandy P, Lampe PD, Leybaert L.: Connexin 43 is an emerging therapeutic target in ischemia/reperfusion injury, cardioprotection and neuroprotection, Pharmacol Ther. 2015 Sep;153:90-106., 2015
10. Varga ZV, Giricz Z, Bencsik P, Madonna R, Gyöngyösi M, Schulz R, Mayr M, Thum T, Puskás LG, Ferdinandy P.: Functional genomics of cardioprotection by ischemic conditioning and the influence of comorbid conditions: implications in target identification., Curr Drug Targets., 2015
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12. Baranyai T, Giricz Z, Varga ZV, Koncsos G, Lukovic D, Makkos A, Sárközy M, Pávó N, Jakab A, Czimbalmos C, Vágó H, Ruzsa Z, Tóth L, Garamvölgyi R, Merkely B, Schulz R, Gyöngyösi M, Ferdinandy P.: In vivo MRI and ex vivo histological assessment of the cardioprotection induced by ischemic preconditioning, postconditioning and remote conditioning in a closed-chest porcine model of reperfused acute myocardial infarction: importance of microvasculature., J Transl Med. 2017 Apr 1;15(1):67., 2017
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Conference presentations:

1. Mariann Gyongyosi, Noemi Pavo, Katrin Zlabinger, Inna Sabdyusheva, Zsolt Petrasi, Ors Petnehazy, Peter Ferdinandy, Gerald Maurer: Effect Of Brief Repetitive Ischemia/Reperfusion On Myocardial Electrical Activity Visualized And Quantified By 3D

Endocardial Electroanatomical Mapping, Presented at the Scientific Session of the American Heart Association, Dallas, USA, 2013

2. Ferdinandy P: Plenary Valsalva lecture, XX National Congress of the Italian Society of Cardiovascular Research 2015.11.26-11.29.- Imola, 2015
3. Ferndinandy P: Role of micrornas and exosomes in cardioprotection, 11th International Congress on Coronary Artery Disease 2015.11.29.-12.02. - Florence, 2015
4. Giricz Z: Are extracellular vesicles mediators of remote conditioning, European Society of Cardiology Congress 2015, 2015.08.29.- 09.02.- London, 2015
5. Macejovska D, Pavo N, Zlabinger K, Ferdinandy P, Mauer G, Gyöngyösi M: Difference in cardioprotective effects of ischemic post-conditioning and remote conditioning in porcine closed-chest reperfused myocardium, Annual meeting of the Austrian Society of Cardiology, Salzburg, Wien Klin Wochenschr. 2015; 127:S42-S3, 2015
6. T. Baranyai, Z. Giricz, M. Sárközy, H. Vágó, C. Czimbalmos, L. Tóth, B. Merkely, M. Gyöngyösi, P. Ferdinandy: Ischemic postconditioning prevents ischemia-induced acute myocardial dilation and limits cardiac edema in a clinically relevant, closed-chest pig model of acute myocardia, CARDIOLOGIA HUNGARICA 45:(Suppl. D) p. D21. (2015), 2015
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8. T. Baranyai, Z. Giricz, Z. V. Varga, G. Koncsos, M. Sárközy, H. Vágó, C. Czimbalmos, L. Tóth, B. Merkely, M. Gyöngyösi, P. Ferdinandy: Ischemic postconditioning prevents ischemia-induced acute myocardial dilation and limits cardiac edema in a clinically relevant, closed-chest pig model of acute myocardia, Myocardial Function & Cellular Biology of the Heart Meeting 2015 Varenna, Italy, 30.04-03.05.2015., 2015
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10. Giricz Z: Az iszkémiás pre-, poszt- és távoli kondícionálás összehasonlítása egy klinikailag releváns, sertés, akut miokardiális infarktus modellben, Magyar Farmakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok Közös Tudományos Konferenciája, Pécs, 2016
11. Lukovic D, Pavo N, Zlabinger K, Gugerell A, Winkler J, Giricz Z, Baranyai T, Ferdinandy P, Gerald Mauer, Mariann Gyöngyösi: Ischemic postconditioning modulates focal adhesion signaling pathway in porcine model, Annual meeting of the Austrian Society of Cardiology, Salzburg, Wien Klin Wochenschr. 2016;128:S231-S2, 2016

PhD Theses

1. Dominika Lukovic (maiden name Macejovska) has submitted her PhD thesis: Mechanisms involved in ischemic conditioning of the heart evaluated in reperfused porcine model of myocardial infarction. The final PhD defense will be done in 2017 (exact time depending from the commission members).
2. Dr. Tamas Baranyai has received his PhD absolutorium in 2016 and he is in the preparation of his thesis with the title: Remote ischemic conditioning and its molecular mechanism. He is going to defend his thesis in 2017, local defence has been already successful.