OTKA K 109737 Final Report

1. Summary

Obesity and metabolic syndrome are major risk factors of ischemic heart diseases including myocardial infarction. Obesity is associated with disturbed myocardial function. It induces apparent myocardial contractile abnormalities, while endogenous ischemic adaptation of the heart is markedly impaired. Therefore, to investigate molecular alterations that induce functional and structural abnormalities in the heart in obesity are of high clinical importance.

The main objective of the present project was to develop and study rat models of metabolic co-morbidities with myocardial dysfunction, and to identify novel pharmacological tools aimed on the modulation of autophagy and mitophagy, which could be utilized to treat or prevent obesity or obesity-induced myocardial dysfunction.

In this project we studied 4 rat models of hyperlipidemia, obesity, insulin resistance, or acute hyperglycemia. We described that acute or chronic disturbances in lipid and carbohydrate metabolism deteriorate cardiac functions, negatively influence cardiac mitochondrial homeostasis and dynamics, increase oxidative stress, blunt pro-survival pathways such as autophagy and mitophagy, and enhance cardiac apoptosis. These novel findings evidence that modulation of mitochondrial homeostasis and/or autophagy are viable therapeutical targets in treating or preventing cardiac consequences of metabolic co-morbidities. We also investigated therapeutic tools, such as the monoamine oxidase-B inhibitor selegiline or chloramphenicol, an antibiotic, and assessed their effects on autophagy and metabolic parameters. We discovered that enhancing autophagosome formation is essential for cardioprotection against ischemia/reperfusion injury, and that selegiline also induces autophagy and reduces adiposity, however its mitochondrial effect are blunted in obesity. Furthermore, we collaborated in projects on the metabolic aspects of polycystic ovary syndrome and the assessment of molecular pathways of angiotensin receptor-1 antagonism and its effect on oxidative pathways.

The results of these investigations resulted in a high number of journal publications (20 published, 1 submitted, 2 under preparation), and contributed to 2 PhD theses. The scientific output exceeded initial expectations (sum of impact factors: 109). Furthermore, the results of the grant has a potential for use in further drug development programs.

2. Aims

The hypothesis of this grant was that pathological obesity interferes with the endogenous stress adaptation mechanisms of the heart, in which process impaired myocardial autophagy/mitophagy may play a central role.

Therefore, our aims were:

i) To assess which cellular pathways/proteins play key role in the obesity-induced alteration of autophagy/mitophagy
ii) To isolate cardiomyocytes from the obese animals, to test potential inducers of autophagy/mitophagy in vitro.
iii) To assess whether altered autophagy/mitophagy plays a pivotal role in the impairment of endogenous ischemic stress adaptation in obesity.

3. Performed experiments, major achievements

To enable our experiments first we set up models of diet-induced obesity in rats to study the effect of metabolic alterations and their effect on cardiac functions. As our first model, Long-Evans rats were fed with high-fat diet (HFD, 70% of total calorie intake from fat) and were injected with a low dose of streptozotocin (20 mg/kg on week 4) to challenge pancreatic insulin production. After 20 weeks of diet, significant weight gain was detected in the HFD group which was associated with altered glucose tolerance (impaired insulin sensitivity), however, an overt diabetes did not develop. We performed cardiac ultrasound imaging, whole-body CT scanning, intracardiac pressure-volume catheterization, analysis of mitochondrial morphology and functions by electron microscopy and enzyme activity measurements, and we investigated several molecular pathways related to autophagy, apoptosis, heat shock proteins, reactive oxygen species production and cardiac calcium handling. These results proved that in this model of obesity, diastolic function deteriorated which was associated with cardiac ultrastructural changes, increased oxidative stress and mitochondrial abnormalities. Thus, in this project, we developed and characterized a new, diet-induced model for early-stage heart failure with preserved ejection fraction (HFpEF). We published these data on national and international conferences and in an international peer-reviewed journal (11).

In our next experiment, we set up another model of diet-induced obesity to study more advanced forms of metabolic cardiac abnormalities. Here, Long-Evans rats were fed with 20 fat and 15% sucrose-enriched (HFHSD) or control diet for 25 weeks. In order to study the influence of mitochondrial monoamine oxidase enzymes (MAO) on obesity-induced cardiac disturbances, a group of rats received 0.25 mg/kg selegiline as intraperitoneal injection once daily from the 16th week of diet. Similarly to our previous model, here we assessed an overweight, which was confirmed by a significant elevation in body fat ratio, as assessed by CT scans and fat pad weights. However, no elevation in plasma cholesterol or lipoprotein levels was detected. Metabolic cage measurements showed that HFHSD animals consumed lower amount of chow, and that the total ingested calories were equal between groups, showing that altering food composition alone can induce obesity in rats without overt abnormalities in blood lipids. To test if changes in calorie intake was due to behavioral changes, we performed novel object recognition and locomotor activity tests and did not detect any difference between treatment groups. Here we also found that selegiline treatment significantly reduced body fat ratio while not affecting calorie intake in HFHSD animals. Similarly to our previous model, here we also evidenced a disturbance in glucose homeostasis by altered glucose and insulin tolerance in HFHSD groups, however, selegiline did not affect these parameters. Here we concluded that MAO inhibition might be a potential treatment option to limit obesity and its cardiac consequences. We published these results in national and international conferences and a manuscript is under revision in a peer-reviewed journal (16).

In high-fat high-sucrose diet (HFHSD)-induced metabolic syndrome animals we also characterized cardiac parameters. We observed that as opposed to our expectation, HFHSD did not induce more severe cardiac functional alterations than our previous model of fat-feeding combined with STZ treatment. We assessed cardiac parameters by echocardiography and pressure-volume catheterization and observed a slight deterioration in diastolic function, which was only mildly influenced by selegiline. Cardiac mitochondrial morphology, function and dynamics were assessed by electron microscopy, and reactive oxygen species and mitochondrial enzyme activities were measured. Subsarcolemmal mitochondria from hearts of animals treated with selegiline demonstrated a decreased state 4 respiration, which was accompanied by lower mitochondrial ROS production. However, these beneficial mitochondrial effects of selegiline were lost in HFHSD animals. We also evaluated the effect of selegiline on mitochondrial dynamics and auto- and mitophagy. We

found that selegiline is a powerful inducer of autophagy and plausibly of mitophagy in cardiac myocytes, which might explain its beneficial effect on cardiac mitochondrial functions. However, selegiline did not induce mitochondrial biogenesis in the heart or in the liver in either control or HFHSD conditions. To further study the effect of inhibition of the MAO-B enzyme by SEL on cardiac mitochondrial dynamics and functions, we developed a fluorescent microscopy-based screening system, with which we are currently assessing mitophagy in H9c2 myocyte cell line. We are also going to assess the effect of selegiline on mitophagy in cells cultured under conditions modelling disturbed lipid metabolism and to study why selegiline did not exert its mitochondrial effects in HFHSD. We plan to publish these results early 2018.

We also studied the status of cardiac autophagy in another model of disturbed lipid metabolism. We found that in Wistar rats fed with high cholesterol-chow for 12 weeks cardiac mTOR signalling was upregulated and autophagy was blunted, while apoptosis was increased and necroptotic pathways were unaffected. These data evidence that disturbed cholesterol metabolism also influences cardiac pro-survival and pro-death pathways. We published these results in national and international conferences and in a peer-reviewed journal (18).

By resources provided by this grant, we also investigated the effect of acute metabolic disturbances on cardiac functions and cardioprotective interventions. We discovered that even an acute hyperglycemia blunts cardioprotection by remote ischemic preconditioning. We uncovered that this effect was accompanied by an abrupt downregulation of cardiac autophagy and a parallel induction of the mTOR pathway. These results further demonstrated that modulation of cardiac autophagy may provide us a valuable target to tackle cardiac functional consequences of various metabolic disturbances. We published these data on national and international conferences and in an international peer-reviewed journal (4).

We assessed the possible beneficial effects of chloramphenicol, an antibiotic agent previously shown to induce cardioprotection against ischemia/reperfusion injury. We found that chloramphenicol treatment induced cardiac autophagy, increased phosphorylation of Erk1/2 in the myocardium and significantly reduced infarct size. Here we demonstration that autophagosome formation but not autophagosomal clearance is required for chloramphenicol-induced cardioprotection, which might serve as a valuable information for the development of future cardioprotective treatments. We published these data on national and international conferences and in an international peer-reviewed journal (19).

To maximize scientific output we initiated numerous international and domestic collaborations. With Dr. Adriana Adameova (Bratislava, Slovakia) we investigated the role of cardiac CaMKII signalling in the potential cardioprotective effect of losartan, a selective AT1 blocker. We found that losartan did not prevent the elevated cardiac lipoperoxidation due to ischemia and reperfusion and did not influence NOX2 expression in isolated hearts. We also described that oxidative activation of CaMKIIδ is not elevated at the end of reperfusion, and that NOX2-oxCAMKIIδ signaling is unlikely to be involved in cardioprotective action of angiotensin AT1 receptor blockade. These results were published in international peer-reviewed journals (12, 13). In another collaboration with Dr. Szabolcs Várbíró (Budapest, Hungary) we investigated polycystic ovary syndrome (PCOS), where carbohydrate metabolism is often disturbed. We described that in a rat model of PCOS vascular insulin resistance developed, which was mostly corrected by feeding animals with chow enriched by vitamin D. We also assessed the levels of microRNA 487-b in PCOS, a miRNA involved in the pathomechanism of diabetes, and found that its expression was unchanged in the aorta of the insulin-resistant PCOS animals, suggesting that it does not play a role in the pathomechanism of PCOS. We plan to publish these findings in an international peer-reviewed journal in early 2018.

Between 2014 and 2017 we also published a number of reviews (1, 5-10, 14, 17, 20, 21) in the field of interference of metabolic comorbidities and oxidative stress with cardioprotection and on proposed therapeutical options. These reviews generated a high impact (331 independent citations up to date) indicating that the topics researched in the above projects are in the highlight of the international community of cardiovascular research and evidencing the successful completion of this grant.

4. Further experiments

Our current results on modulation of cardiac autophagy in metabolic co-morbidities generated by the help of this grant serve a solid basis of development of novel cardioprotective therapies. Publishing these results helped us secure a significant grant (NVKP-16-1-2016-0017) in which, amongst other aims, we further study molecular pathways related to autophagy and mitophagy, and where we will test candidate molecules in cell-based and in-vivo models of metabolic co-morbidities. We also plan to apply non-hypothesis driven approaches, where we will perform high-throughput functional genomic investigations, such as miRNA- and mRNA sequencing, etc. The dataset obtained in these experiments will be analysed with methods of multi-level network biology to highlight novel pathways with a possibility for therapeutic exploitation. We expect to have multiple high-impact publications in this field during the following years.

5. Education, involvement of early career investigators

The above projects continuously attracted a high number of undergraduate students to our workgroup, who presented their findings with success on local, national and international forums of undergraduate researchers every year.

We also recruited PhD students who are involved in the investigations of the project. From 2013, Gabor Koncsos, from 2014, Csilla Nagy works primarily on projects funded by this grant under the supervision of Dr. Zoltan Giricz. Results generated here are the basis of their theses, the defences of which are expected in 2018.

6. Research output

The majority of 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 number of **journal publications** (**20 published, 1 submitted, 2 under preparation**), and to **two PhD theses**. The output exceeded initial expectations (**sum of impact factors: 109**), in part, due to the high number of international and Hungarian collaborations. The number of publications is going to be further increased since manuscripts of numerous experiments will be submitted in the near future due to additional experimental needs.

7. Published articles:

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10. Hausenloy DJ, Barrabes JA, Bøtker HE, Davidson SM, Di Lisa F, Downey J, Engstrom T, Ferdinandy P, Carbrera-Fuentes HA, Heusch G, Ibanez B, Iliodromitis EK, Inserte J, Jennings R, Kalia N, Kharbanda R, Lecour S, Marber M, Miura T, Ovize M, Perez-Pinzon MA, Piper HM, Przyklenk K, Schmidt MR, Redington A, Ruiz-Meana M, Vilahur G, Vinten-Johansen J, Yellon DM, Garcia-Dorado D.: Ischaemic conditioning and targeting reperfusion injury: a 30 year voyage of discovery., Basic Res Cardiol. 111(6):70., 2016
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8. PhD Theses:

1. **Csilla Nagy** has received her PhD absolutorium in 2017 and she is in the preparation of her thesis with the title: **Mechanisms of remote cardioprotection in metabolic diseases**. She is going to defend her thesis in 2018.
2. **Gábor Koncsos** received his PhD absolutorium in 2017 and he is in the preparation of his thesis with the title: **Cardiac consequences of metabolic derangements: role of mitochondrial oxidative stress and autophagy**. He is going to defend his thesis in early 2018.