Pre-clinical investigation of cardioprotective therapies for acute myocardial ischemia/reperfusion-injury and for pressure-overload-induced chronic heart failure

PhD thesis

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Introduction

Despite the continuous improvement of preventive, diagnostic, and therapeutic approaches for cardiovascular diseases, this disease group is the leading cause of mortality and disability worldwide. When broken down into the causes of cardiovascular disease, acute myocardial infarction (AMI) and heart failure (HF) are two of the leading causes of morbidity and mortality.

To mitigate the burden of AMI and HF, and to improve the survival and quality of life of patients, there is an unmet need for novel pharmacological or non-pharmacological therapeutic interventions. Thus, a deeper understanding of pathophysiology and the identification and testing of novel modifiable targets are essential.

To this end, reproducible pre-clinical studies with a high translational value are of paramount importance, as such investigations have profoundly shaped the treatment strategies in both AMI and HF. Although the number of pre-clinical studies continues to rise intensively, the reproducibility and translatability remain to be improved.

Objectives

The main objectives of this doctoral work are to establish pre-clinical approaches aiming to enhance reproducibility, and thus, translatability in studies (i) investigating the cardioprotective efficacy of RIPC in acute myocardial I/R-injury (Study no. 1)[1], or (ii) identifying novel GPCR targets for HF (Study no. 2)[2].

Methods

All the investigations performed comply with the regional, Hungarian, European, and global ethical standards. All studies were conducted in accordance with the ARRIVE 2.0 guidelines. For all studies, animals were randomly assigned to experimental groups. Evaluation of the results was performed in a blinded manner. Animals were excluded from further analyses if any event occurred during or after surgery that was pre-defined.

Methods for Study no. 1.

Remote ischaemic preconditioning (RIPC) is a robust cardioprotective intervention in preclinical studies reportedly showing a robust reduction in myocardial infarct size (IS). We aimed to establish a working and efficacious RIPC protocol in our laboratories as a basis for studying cardioprotective mechanisms on a reliable pre-clinical model.

To this end, we performed a three-center, individually designed, randomized, and blinded pre-clinical study in study sites of Budapest, Szeged, and Amsterdam on male Wistar rats to test the cardioprotective efficacy of limb RIPC in a model of acute myocardial ischemia/reperfusion (I/R) injury. Induction of acute myocardial I/R-injury was performed by ligating the left anterior descending coronary artery (LAD) for various durations (20-45 minutes), followed by relieving the ligation and continuing reperfusion for 120 minutes. RIPC was elucidated by 3 or 4 × 5−5 min occlusion/reperfusion of

one or two femoral vessels by clamping, tourniquet, or pressure cuff, before the LAD ligation.

After the completion of myocardial reperfusion, myocardial infarct size/area at risk (IS/AAR), microvascular obstruction size (MVO)/total left ventricular area were measured on 2 mm thin cardiac slices by computer planimetry. The severity and duration of I/R-induced arrhythmias were analyzed on continuous ECG records of each animal.

To verify that the study settings of the current in vivo studies are in good alignment with previously published in vivo rat studies of acute myocardial I/R-injury measuring cardioprotective efficacy of limb RIPC, we performed a systematic review and meta-analysis, according to the PRISMA guidelines. We assessed the reporting frequency of methodological parameters and their values. Data items were collected using a pre-defined data sheath. The primary outcome of the meta-analysis was defined as the unstandardized, weighted mean differences between IS/AAR% of the RIPC and control groups. As the included studies were found to be highly heterogeneous, random-effects DerSimonian-Laird model was used for the analysis. Publication bias was estimated using Egger's regression test.

Methods for study no. 2.

Male Wistar rats were randomly assigned to sham-operated (SHAM) or transverse aortic constriction (TAC) groups. TAC was performed through a mini thoracostomy,

and the transverse aorta was constricted to the size of a 21G needle. SHAM animals underwent the same procedure, without the completion of the aortic constriction. Animals were followed up for 15 weeks and transthoracic echocardiography was performed before termination to calculate left ventricular dimensions and function. After humane euthanasia, cardiac samples were obtained for further analyses.

Cardiac mRNA deep sequencing was performed, followed by the bioinformatics analysis of the data, and differential expression analysis was done. From the whole transcriptome, data was screened for non-olfactory G-protein-coupled receptors GPCRs.

From the isolated RNA of the same hearts, droplet digital polymerase chain reaction (ddPCR) analyses were performed using a pre-designed assay kit allowing for the measurement of 288 GPCRs.

In vitro model of neonatal rat cardiomyocyte (NRCM) hypertrophy was performed using angiotensin-II (Ang-II). ANG-II treated cells received either no additional treatment or AL-8810, a selective inhibitor for prostaglandin F2α at the concentrations of 1 μM or 10 μM. 24 hours after the start of treatment, cardiomyocyte cell surface area was measured.

RNA-Scope in situ hybridization method was used to visualize the localization of selected GPCRs on major cardiac cell types (i.e. cardiomyocyte, endothelial cells, smooth muscle cells and macrophages).

Results

Results for Study no. 1

Unexpectedly, for the first time in the literature, we demonstrated no cardioprotective effects of limb RIPC, as we found (i) no decrease in myocardial IS, (ii) no decrease in the size of MVO, and (iii) no change in duration and occurrence of reperfusion arrhythmias in neither of the study sites. As our experimental results were in contrast to those in the literature, we hypothesized that some of the key methods (e.g. in performing the RIPC procedure) in our in vivo study differs from the previously published studies. To explore this, we performed a systematic review of similar RIPC studies in rats.

We analysed parameters ranging from animal husbandry characteristics to preoperative or intraoperative procedures, including quality control measures, as these parameters are pertinent for pre-clinical reproducibility. Overall, we found that the majority of the included studies reported 50-60% of the data items, and when reported, methods were heterogeneous. Of note, the methodological settings in our in vivo experiments were within the boundaries of the published literature.

Although data items for the "interventions" regarding myocardial I/R-injury and limb RIPC were generally well-reported, perioperative measures ranging from details in anaesthetic regimes to monitoring vital parameters were poorly reported.

In addition, we found that ~40% of the included studies did not report on randomization, and ~70% did not report on blinded evaluation of the results. Of interest, one quality criterion specific to studies assessing cardioprotection in myocardial I/R-injury is the reporting on AAR/LV, as this parameter provides information on the consistency of the location of coronary ligation. Nevertheless, only 70% of the included studies gave information on this data, and when reported, the AAR/LV sizes were highly heterogeneous between studies. These findings also question the reliability and reproducibility of RIPC studies in rats, in general.

Of interest, with the Egger's regression test, we have found a tendency for publication bias towards positive results, raising the concern that studies with neutral or negative results might be withheld from publication.

Results for Study no. 2

As expected, TAC resulted in a significant systolic dysfunction, LV dilation, and LV hypertrophy vs. sham-operated animals, as assessed by echocardiography. After obtaining cardiac samples from both groups, we performed a systematic screening for novel cardiac GPCR targets of HF by using the gold standard RNAseq, as well as ddPCR.

In TAC vs. SHAM hearts, RNAseq identified 69, and ddPCR identified 27 significantly differentially expressed GPCR mRNAs. From these results, 8 cardiac GPCR mRNAs were identified to be significantly differentially

expressed in failing vs. healthy rat hearts by both methods, with a good correlation between the two methods. We aimed to further characterize those GPCRs that (i) have commercially available modifiers, and (ii) have not been described in the context of HF previously.

We have found, for the first time, that Prostaglandin-F2α receptor (Ptgfr) matched the above criteria, as (i) the expression of this GPCR gene was significantly higher in TAC vs. SHAM hearts, (ii) it has commercially available modifiers, and (iii) has not been directly brought in context with HF. In line with these pre-clinical results on rats, single-nucleus transcriptomic data of human failing hearts has also identified a significantly increased level of Ptgfr expression compared to healthy hearts.

We demonstrated that Ptgfr is primarily expressed in cardiac muscle- and fibroblast cells using RNA-Scope.

Finally, in an in vitro model of ANG-II-induced cardiomyocyte hypertrophy, we have demonstrated that the pharmacological inhibition of Ptgfr by AL-8810 prevented cardiomyocyte hypertrophy in a dose-dependent manner.

Conclusions

Based on the above studies, to improve the success of translation of potential cardioprotective approaches for acute myocardial I/R -injury and for HF from pre-clinical testing into clinical reality, reliably conducted, well-reported, and methodologically reproducible studies should be performed, irrespective of the outcome. as without such studies, clinically translatable cardioprotective interventions could not be identified. In addition, we emphasize that screening for molecular targets that have already available pharmacological modifiers, i.e. that can be subjects for drug repositioning, could also improve the translational success rate.

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