CARDIOPROTECTION IN PRECLINICAL ISCHEMIA/ REPERFUSION MODELS

PhD thesis

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Budapest
2021

INTRODUCTION

Ischemic heart diseases

Acute myocardial infarction (AMI), commonly known as heart attack, is a major consequence of ischemic heart disease and is considered as the single most common cause of death. AMI is defined by the evidence of myocardial injury with necrosis in a clinical setting consistent with myocardial ischemia. The result of such insult to the heart muscle cells could alter not only the metabolism of the cells, but the structure or function of the involved area of myocardium significantly, depending on the location of the occlusion/stenosis and time of starvation.

Reperfusion injury is an additional factor influencing infarct size resulted by myocardial infarction. The underlying mechanism of ischemia/reperfusion injury (I/R) is complex and have been studied extensively in the past few decades. Reactive oxygen-nitrogen species have been found to be potential mediators as well as matrix metalloproteinase-2 enzymes. Cells surface receptors could also lead to activation of intracellular signalling pathway resulting cell death.

Cardioprotection: intervention and available treatments

For efficient treatment of acute myocardial infarction, the aim is to reduce ischemia and reopen the coronary vessels by unblocking them and/or preventing further clotting. Several strategies, including pharmacological and mechanical therapies, have shown a reduction of infarct size by reducing ischaemia/reperfusion injury and microvascular obstruction. Primary percutaneous coronary intervention is the gold standard. However, it is only beneficial during a window of 90-120 minutes beginning upon contact with a medical professional. Coronary angiography could also be considered.

The pharmacological treatment includes antithrombotic therapy and anticoagulants (heparin, aspirin and prasugrel/ticagrelor) and the immediate and simultaneous management of pain and blood pressure with morphine, nitroglycerine beta-blockers, angiotensin convertase enzyme inhibitors.

There is an unmet clinical need for cardioprotective therapies against myocardial I/R injury. Therefore, novel potential cardioprotective therapies are much sought for. In line with this need, plenty of substances including natural

biomolecules have been extensively tested for their cardioprotective potential.

Possible cardioprotective candidates

L- alpha-glycerylphosphorylcholine

L-alpha-glycerylphosphorylcholine (choline alphoscerate, GPC) is a natural endogenously produced choline derivative and acetylcholine precursor in the brain which (in the form of a synthetic compound) is widely used as a food supplement. GPC is converted metabolically to phosphatidylcholine, the active form of choline that is able to increase acetylcholine levels in the brain. GPC is known for its protective effects against I/R injury in the brain and in the liver. However the cardioprotective effect of GPC is not known.

Decorin

Decorin is natural component of the connective tissue. Decorin is included in a broad range of cellular processes including collagen fibrillogenesis, wound repair, angiostasis, tumor growth, and autophagy. In cardiovascular system it is proposed to play a key role in the proper tissue scar formation following myocardial infarction.

Decorin and biglycan are proteoglycans and as Gáspár et al. have previously shown that exogenous administration of biglycan, protects myocardial cells from simulated ischemia and reperfusion (SI/R) injury, we hypothetized that another proteoglycan from small leucine-rich proteoglycan family, decorin, could also exert cardioprotection. However, it is not known if decorin exerts acute cardiocytoprotective effect.

Matrix metalloproteinase inhibitors

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases and the gelatinase-type MMP-2 isoenzyme occurs physiologically in the heart and synthesized by cardiac myocytes, fibroblasts, and endothelial cells. MMP-2 has a critical role as an intracellular mediator of cardiac I/R injury contributing to the acute mechanical dysfunction. MMP-2 could be activated intracellularly during I/R induced myocardial injury, therefore the inhibition of MMP-2 seems to be a promising target in therapy of AMI.

The imidazole and thiazole carboxylic acid-based compounds (MMP inhibitor: MMPI-1154, MMPI-1261 and MMPI-1248) have shown cardioprotection in vitro.

AIMS

The aim of this thesis is to achieve cardioprotection by using different potentially protective compounds (Fig.1.),

1) to test the hypothesis that GPC could prevent ischemia-induced cell death and oxidative stress in cardiac myocytes subjected to simulated I/R and

2) to test if decorin exerts cardioprotective effects against simulated ischemia/reperfusion injury in primary cultures of isolated neonatal and adult rat cardiomyocytes, and to reveal molecular pathways involved in these effects and

3) to test cardioprotective effect of MMP inhibitors (MMPI-1154, -1260, and -1248) in an *in vivo* rat model of acute myocardial infarction in presence or absence of hypercholesterolemia.

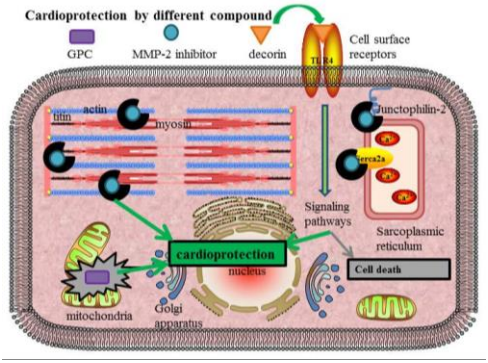

Figure 1. Visual summary about the possible targets of investigated compounds to achieve cardioprotection.

MATERIAL AND METHODS

Our experiments were performed in accordance with the EU directive guidelines for the care and use of laboratory animals, published by the European Union (2010/63/EU). Methods were also reviewed by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority of Hungary) and were approved by the Animal Welfare Committee of the University of Szeged (I-74-52/2012 MAB).

Culturing primary neonatal rat cardiac myocytes

Neonatal rat cardiac myocytes (NRCMs) were isolated from newborn Wistar rats. The cells were cultured for 3days and maintained at 37 °C in a standard CO_2 incubator.

Treatments of primary neonatal rat cardiac myocytes

Cells were treated with different concentration of GPC (1, 10, 80, 100 µM) or decorin (1, 3, 10, 30, 100 nM) under normoxic condition or simulated ischemia/reperfusion injury. Vehicle was used as control.

Simulated ischemia/reperfusion injury in primary neonatal rat cardiac myocytes

To induce simulated ischemia/reperfusion the cells were placed in a tri-gas incubator gassed through with a mixture of 95 % N_2 and 5 % CO_2 for 240 min at 37 °C and the medium was changed to hypoxic solution. Control cells were kept in normoxic incubator. After simulated ischemia or normoxia, the cells were placed to normoxic incubator; hypoxic or normoxic medium was then replaced by culture medium (Simulated reperfusion) for 2 hours at 37°C.

End point measurements of primary neonatal rat cardiac myocytes

Cell viability measurement was assessed by calcein staining, and for oxidative stress measurement dihydroethidium and 2'-7'-dichlorodihydrofluorescein diacetate staining were used. Caspase and TUNEL assays were performed for the measurement of apoptosis. Fluorescence intensity was measured using plate reader (FluoStar Optima, BMG Labtech) in well scanning mode at the appropriate wavelength. Unbiased mRNA deep seq analysis was done in case of decorin treatment.

In vivo surgical induction of acute myocardial infarction in rats

Male Wistar rats weighing 260-340 g were anesthetized by intraperitoneal injection of pentobarbital sodium. In open chest surgery the coronary artery was occluded for 30 min followed by 120 min of reperfusion.

In separate experiments hypercholesterolemia was induced in the animals via 12 weeks of cholesterol-rich diet. Comorbid model was validated by lipid panel measurement from baseline serum samples.

In vivo treatments of MMP inhibitors against acute myocardial infarction

MMP inhibitor molecules (dose of 0.3, 1, 3 µmol/kg for MMPI-1154, MMPI-1260 and 1, 3, 10 µmol/kg for MMPI-1248) were given at the 25th minute of ischemia through intravenous injection.

Determination of myocardial infarct size and microvascular obstruction

At the end of the 120 min reperfusion period, the heart was isolated and attached to Langendorff retrograde perfusion system. First washed with Krebs solution, followed by thioflavine-S staining, then the LAD was re-occluded and the

heart was perfused with 4 ml of 0.25 % (w/v) Evans-blue dye to delineate the area at risk. Stained hearts were rapidly frozen cut into 2 mm thick slices. Thioflavine-S was detected under UV light, the dark patches indicated where the staining could not penetrate through the coronary arteries. After photos were taken, each slice was incubated with 1 % 2,3,5-Triphenyltetrazolium-chloride (TTC) at 37 ºC. TTC penetrated the cardiac myocytes and living cells reduced the TTC into a brick-red compound, while in the dead cells the stain remained colorless. Photos were taken from both sides of the heart slices. The differently stained areas of the heart images were quantified by digital planimetry (Infarctsize™ 2.5, Pharmahungary 2000 Ltd).

Statistical analysis

Data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Fisher LSD/Dunnett's multiple comparison *post-hoc* tests were used to analyse differences in mean values of the experimental groups versus appropriate control. Lipid panel measurement was compared between normo- and hypercholesterolemic animals with Student's t-test. Significance value was chosen *$p<0.05$.

RESULTS

Effect of GPC and decorin in cultured cardiac myocytes under normoxic contition

15 minutes or 3 hours pretreatment with different concentration of GPC in normoxic conditions did not change the cell viability of NRCMs and, the overall ROS production was significant reduced after 3-hour treatment with 100 µM GPC compared to the vehicle treated group.

On the other hand the long-term GPC treatment, which was applied for 24 hours, a profoundly induced cell death in NRCMs, as compared to the vehicle-treated group

Applying decorin was safe, the different concentrations (1-100nM) of decorin treatment of NRCMs for 4+2 hours under normoxic conditions showed that decorin applied in 3 nM and 10 nM concentrations significantly increased cell viability, while 1 nM, 30 nM and 100 nM concentrations has not changed that when compared to the vehicle-treated normoxic group.

Cardioprotection in cardiac myocytes under simulated ischemia/reperfusion

Most of the applied concentrations of GPC had no effect on cell death compared to the vehicle. The 80 µM GPC

pre-treatment; however, significantly improved cell viability after the SI/R injury

1 nM, 3 nM and 10 nM of decorin treatment significantly increased the cell viability of NRCMs after 4 h/2 h of SI/R in comparison to vehicle-treated group, but the rate of apoptosis showed no difference compared to the vehicle-treated group after 3nM decorin treatment combined with simulated ischemia/reperfusion injury.

Eif4enif1 and Zmynd19 mRNA significantly upregulated due to the decorin treatment.

Cardioprotective effects of Matrix metalloproteinase inhibitor compounds against acute myocardial infarction

Two of the MMP inhibitor compounds showed significant reduction in infarct size: MMPI-1154 at 1 µmol/kg and MMPI-1260 at 3 µmol/kg decreased infarct size significantly as compared to the vehicle group, from 63.68±1.91 % to 53.53±3.36 % and 56.64±2.46 %, respectively. The third inhibitor, MMPI-1248, showed no reduction of infarct size in any of the applied doses when compared to the ischemic control group.

Cardioprotective effects of Matrix metalloproteinase inhibitor compounds against acute myocardial infarction in hypercholesterolemic and age-matched normocholesterolemic rats

In a separate experiment MMP inhibitors were tested in a comorbid model, AMI was combined with hypercholesterolemia. Male Wistar rats developed hypercholesterolemia due to 12 weeks cholesterol-enriched diet. In the age-matched normocholesterolemic group, ischemic preconditioning significantly reduced infarct size (26.23±6.16 %) as compared to the vehicle-treated ischemic group (55.58±3.41 %) and both MMP inhibitor molecules provided cardioprotection (MMPI-1154: 40.61±3.38 % and MMPI-1260: 36.75±4.63 %).

Based on the results of the first in vivo experimental set up, the infarct size limiting effect of the compound was reproducible, as expected. In the presence of hypercholesterolemia, ischemic preconditioning (36.77±6.6 %) and both MMP inhibitors, MMPI-1154 at 1 µmol/kg (44.82±6.1 %) and MMPI-1260 at 3 µmol/kg (44.03±2.4 %) failed to reduce infarct size when compared to the vehicle-treated control group (45.59±4.8 %).

Microvascular obstruction was not affected by matrix metalloproteinase inhibitors

The percentage of MVO was significantly reduced in the positive control, ischemic preconditioning both in time-matched normocholesterolemic and hypercholesterolemic groups (Nchol: 4.45±0.75 %, Hchol: 5.35±1.68 %) compared to the vehicle-treated ischemic group (Nchol: 9.67±1.47 %, Hchol: 12.57±2.70 %). The tested MMP inhibitor molecules showed similar amount of MVO as vehicle-treated ischemic group (Nchol MMPI-1154: 7.49±1.56 % and MMPI-1260: 9.19±2.23 %; Hchol MMPI-1154: 11.09±2.34 % and MMPI-1260: 13.45±2.07 %).

CONCLUSION

In this thesis relevant in vitro and in vivo animal experimental models are presented for discovering protective compounds against acute myocardial infarction. In conclusion, we have shown for the first time that choline donor L-alpha-GPC (which is widely used as a food supplement and generally considered as safe for human use) had ambiguous effects on cardiac cells. It may be beneficial in short-term administration to maintain the physiological balance of ROS-production under normoxic, healthy conditions and could be also protective in I/R conditions, but could, in fact, be cytotoxic if it surrounded the cells for long enough. Besides the duration of the treatment, the correct dosage can also be a crucial factor, as a fine-tuning effect seemed to occur in a small, but dietary-relevant concentration range. Thus (despite many limitations of this *in vitro* study), our results indicate the need for a comprehensive cardiac safety testing of GPC.

The small leucine-rich proteoglycan, decorin exerts cardiocytoprotective effects against SI/R, suggesting a therapeutic potential of exogenously administered decorin for the treatment of acute myocardial infarction. The molecular mechanism of its action still remains to be uncovered.

In addition, the protective effect of matrix metalloproteinase inhibitors has been demonstrated in vivo in

a rat model of acute myocardial infarction. Matrix metalloproteinases are able to degrade a number of intracellular and extracellular proteins, therefore the novel inhibitors could protect the sarcomere proteins responsible for myocardial cell contractility. MMPI-1154 and MMPI-1260 previously shown to be cardiocytoprotective in vitro and ex vivo and have been further proved here to be cardioprotective in vivo when administered before the onset of reperfusion, which is a clinically relevant therapeutics approach in a rat model of AMI. The use of the new inhibitory molecules can be used in normocholesterolemic animals at safe, clinically relevant timing and doses, however, further research is needed in the presence of comorbidities. This is the first demonstration of the dose-dependent cardioprotective effect of novel MMP inhibitors MMPI-1154 at 1μmol/kg and MMPI-1260 at 3μmol/kg in an in vivo rat model of AMI when administered before reperfusion.

However, in the presence of hypercholesterolemia, their infarct size-limiting effect was not seen in a single dose that showed cardioprotective effects in normal rats. Whether hypercholesterolemia inhibits their cardioprotective effect or may only shift the dose–response relationship of these compounds remains unknown.

Finding cardioprotective molecules are complex because the duration of the treatment and the correct dosage are both crucial factors. We were aiming to test 5 different molecules, from which we have found 4 to be cardioprotective.

Dissertation-related publications

L-Alpha-glycerylphosphorylcholine can be cytoprotective or cytotoxic in neonatal rat cardiac myocytes: a double-edged sword phenomenon.
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Independent publications

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Acknowledgments.

Firstly, I would like to express my sincere gratitude to my supervisor Dr. Anikó Görbe for the continuous support of my Ph.D study and related researches.

My sincere thanks also goes to Dr. Péter Ferdinandy (SE) and Dr István Baczkó (SZTE), Head of the Department of Pharmacology and Pharmacotherapy, for providing me an opportunity to work at the departments.

Besides my supervisor, I would like to thank to Dr. Péter Bencsik for the help, advices and his insightful comments.

I am also thankful to our collaborator Dr. György Dormán, Dr Eszter Tuboly, Dr Petra Hartmann and Dr Renáta Gáspár.

This Ph.D project would not have been possible to complete without the assistance and work of the Cardiovascular Research Group, Judit Pipis, Éva Kenyeres, Tamara Szabados, Ágnes Szántai and Dr. János Pálóczi. I thank my fellow labmates from Cardiovascular and Metabolic Research Group, especially to Bernadett Kiss, Dr. András Makkos and Dr. Bence Ágg.

I would like to thank my friends and family, especially Dr. Tímea Raffai and Brian Hernandez for their support through these years, who had always helped and reinforced me.

Last but not at all the least, I dedicate this PhD thesis to my beloved mother, who was my biggest supporter, who passed away in 2019, before seeing me attain my doctorate degree.