NOVEL APPROACHES OF PHARMACOLOGICAL PRECONDITIONING: the role of biglycan and miRNAs

Ph.D. Thesis
Summary

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List of publications

List of full papers directly releated to the subject of the thesis

1. **Renáta Gáspár**, Márton Pipicz, Fatime Hawchar, Dávid Kovács, Luna Djirackor, Anikó Görbe, Zoltán V. Varga, Mónika Kiricsi, Goran Petrovski, Attila Gácser, Csaba Csonka, Tamás Csont: The cytoprotective effect of biglycan core protein involves Toll-like receptor 4 signaling in cardiomyocytes. J Mol Cell Cardiol. (2016);99:138-150. [IF:4.874]

2. Márta Sárközy*, **Renáta Gáspár*,** Kamilla Gömöri, László Dux, Csaba Csonka, Tamás Csont: Effects of Proteoglycans on Oxidative/Nitrative Stress. Curr Org Chem (2016) accepted for publication [IF:1.949]

3. 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);307:H216-27. [IF:3.838]

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List of other full papers not related to the thesis

4. Kiscsatári L, Varga Z, Schally AV, **Gáspár R**, Nagy CT, Giricz Z, Ferdinandy P, Fábián G, Kahán Z, Görbe A.: Protection of neonatal rat cardiac myocytes against radiation-induced damage with agonists of growth hormone-releasing hormone. Pharmacol Res. (2016);111:859–866. [IF:4.816]

5. Márta Sárközy, Gergő Szűcs, Veronika Fekete, Márton Pipicz, Katalin Éder, **Renáta Gáspár**, Andrea Sója, Judit Pipis, Péter Ferdinandy, Csaba Csonka, Tamás Csont: Transcriptomic alterations in the heart of non-obese type 2 diabetic Goto-Kakizaki rats. Cardiovasc Diabetol (2016);15:110. [IF:4.534]

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7. Gergely S, Hegedűs C, Lakatos P, Kovács K, **Gáspár R**, Csont T, Virág L.: High Throughput Screening Identifies a Novel Compound Protecting Cardiomyocytes from Doxorubicin-Induced Damage. Oxid Med Cell Longev. (2015);2015:178513. [IF:4.44]

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10. Csont T, Sárközy M, Szűcs G, Szűcs C, Bárkányi J, Bencsik P, **Gáspár R**, Földesi I, Csonka C, Kónya C, Ferdinandy P.: Effect of a multivitamin preparation supplemented with phytosterol on serum lipids and infarct size in rats fed with normal and high cholesterol diet. Lipids Health Dis. (2013);12:138. [IF:2.31]

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Introduction

Cardiovascular diseases (CVDs) are the leading causes of death in the world and also in Hungary. CVDs are a group of disorders of the heart and blood vessels, including e.g. ischemic heart diseases (IHDs). IHD is the most common type within the group of CVDs being responsible for approximately 46% of deaths caused by CVDs. IHD is a group of diseases that includes stable angina, unstable angina, myocardial infarction, and sudden cardiac death. The common feature of these diseases is reduced blood supply to the myocardium with deficit of oxygen and nutrients. It could be emphasized that in Hungary acute myocardial infarction (AMI) has the highest mortality rate among IHDs. AMI is mainly caused by a thrombotic occlusion of a coronary artery following the rupture of a vulnerable atherosclerotic plaque. The most effective therapy for reducing AMI injury is timely and effective myocardial reperfusion. However, the reperfusion therapy – that is essential to salvage myocardium - can itself induce further cardiomyocyte death termed reperfusion injury. Currently there is no effective therapy for reducing AMI-induced cellular damage and death, therefore development of new therapeutic approaches for limiting myocardial infarct size has a great clinical potential.

Myocardial ischemia/reperfusion (I/R) injury is a phenomenon of cellular damage in the heart that is initiated during hypoxia and becomes exacerbated when oxygen delivery and tissue pH are restored. The exact pathomechanism of I/R has not been yet fully elucidated. The mechanisms contributing to the pathogenesis of I/R injury are multifactorial, complex and involve parallel regulation of cell death and cell survival processes. Two major forms of cellular death, apoptosis and necrosis can be observed in cardiomyocytes induced by I/R. Autophagy is a cell survival mechanism, a nonstop renewal process responsible for the degradation of damaged organelles and macromolecules. During I/R autophagy can also promote cell survival by generating reusable components to maintain function during nutrient-limited conditions.

Currently there is no effective cytoprotective therapy for I/R injury. It is known, that the heart has a remarkable ability to adapt to I/R stress and the investigation of these cardioprotective mechanisms has been in the focus of intensive research in the last three decades. Preconditioning markedly improves the ability of the heart to withstand a long ischemic insult. Ischemic preconditioning (IPre) is a well-described adaptive response in which there is a brief exposure to I/R before prolonged ischemia. This approach has limited clinical relevance and failed to be translated to useful clinical treatments. However,

elucidation of the underlying mechanisms of IPre helped to develop numerous pharmacological agents mimicking cardioprotection induced by IPre. Several studies have shown that IPre leads to alteration of global gene expression, and microRNAs (miRNAss) has been recently recognized as fine-tuning posttranscriptional modulators of gene expression. Therefore the modulation of gene expression and protein levels with posttranscriptional regulation via miRs might lead to increased cell survival or more efficient adaptation for stress situations and can be an alternative and effective therapeutic way of induction of cardioprotection. Besides modulation of gene expression, in pharmacological preconditioning, application of natural molecules modulating cell signaling via cell membrane receptors could be able to induce cardioprotection. Moreover, it has been shown recently that extracellular matrix-derived macromolecules also contribute to cardioprotection. Implantation of injectable ECM hydrogel derived from decellularized myocardial tissue after AMI, decreased cardiac remodeling and preserved ejection fraction. Moreover, in the hearts of transgenic mice overexpressing the ECM component small leucine-rich proteoglycan, biglycan was shown to increase expression of several genes and proteins associated with cardioprotection .

In this thesis, we aimed to investigate the potential pharmacological preconditioning effect of some macromolecules, i.e. biglycan and miRs against simulated ischemia/reperfusion (SI/R) injury in primary rat cardiomyocytes.

Biglycan consists of an approximately 43 kDa core protein and two high-molecular weight glycosaminoglycan (GAG) side chains that can be either dermatan or chondroitin sulfate. The role of biglycan in the cardiovascular system is controversial. It was shown to be involved in the initiation of atherosclerosis and in heart failure. Conversely, the lack of biglycan has led to abnormal long-term cardiac remodeling and increased mortality in biglycan-knock out mice subjected to coronary occlusion. Our research group has previously demonstrated that exogenously administered biglycan could protect neonatal cardiac myocytes against SI/R injury. Although these findings suggest that biglycan exerts cardioprotective effects, the exact molecular mechanism behind this phenomenon has not yet been elucidated. Biglycan has been reported to bind to several cell membrane receptors including integrin receptors, growth factor receptors, Toll-like receptors (TLR) and scavenger A receptors in macrophages, however, the role of these receptors in biglycan-induced cardiocytoprotection has not been investigated.

Growing evidence suggests that microRNAs (miRNAs) have an important role in various physiological and pathological processes in the heart including cardiac development, myocyte contractility and cellular response to different stress. MiRNAs are endogenously

expressed small – 21-25 nucleotides' long –non-coding RNA molecules, encoded in almost all organisms from viruses to humans and act as powerful posttranscriptional regulators of gene expression and have a pivotal role in cardiovascular functions.

Several studies demonstrated that the systemic use of chemically modified miRNA modulators (mimicking or inhibiting the miRNA function) has a great therapeutic potential in various disease states. Our research group has investigated the preconditioning-induced miRNAs expression profile in Langendorff-perfused rat hearts and suggested a set of therapeutically applicable miRNAs with potential cardioprotective features. In this present thesis we aimed to strengthen whether pharmacological modulation of selected preconditioning-associated miRNAs indeed protect primary neonatal cardiomyocytes against SI/R, thereby proving the causative role of these selected miRNAs in pharmacological preconditioning.

Aims

In the present thesis our aim was to investigate whether certain natural or modified macromolecules are able to protect the primary neonatal cardiomyocytes against simulated ischemia/reperfusion injury.

A) Our fist aim was to investigate the potential cardiocytoprotective effect of the small leucine rich proteoglycan biglycan.

Therefore we have assessed:

- whether the core protein or the glycosaminoglycan side chains (dermatan sulfate or chondroitin sulfate) are responsible for the cardiocytoprotective effect of native biglycan
- if the mechanism of action of biglycan involves Toll-like receptor 4 signaling, well-known cardioprotective mediators (e.g. nitric oxide, ERK, Protein kinase B), protection from oxidative stress, inhibition of cell death and induction of cell survival processes.

B) Our second aim was to test whether the modulation of selected preconditioning-associated miRNAs is able to protect cardiomyocytes against SI/R injury.

Materials and methods

All procedures used have been in accordance with the Directive 2010/63/EU of the European Parliament and were approved by the local animal ethics committee of the University of Szeged.

Characterization of the cytoprotective effect of biglycan

In pilot experiments, we tested whether the core protein obtained by enzymatic removal of GAG chains from biglycan induces cytoprotection. Two days old cardiomyocyte cultures were treated with 0 - 100 nM native (glycanated) or deglycanated biglycan for 20 h, and were subjected to 150 min SI followed by 120 min simulated reperfusion. Following simulated reperfusion, the ratio of dead cells was determined by Trypan blue staining. The native and deglycanated biglycan treatments were maintained throughout SI/R.

In order to further investigate which structural component is responsible for the cardiocytoprotective effect of biglycan, in a separate setup, cardiomyocytes were pretreated with 0-100 nM concentration of native biglycan, recombinant human biglycan core protein (rhBGNc) and the corresponding concentrations of dermatan sulfate or chondroitin sulfate for 20 h followed by 240 min SI and 120 min simulated reperfusion. During SI, the cells were covered with hypoxic solution and kept in a multigas incubator set to 0.4% O_2, 5% CO_2, 94.6% N_2. At the same time, to determine the rate of cell death due to SI/R, cells grown on two removable strips of each Strip well plate were covered with a normoxic solution and kept in a standard CO_2 incubator for 240 min followed by 120 min of simulated reperfusion. At the end of the reperfusion, cell viability was measured by calcein assay. In SI/R experiments, data obtained with various treatments were expressed as percentage of SI/R-induced cell death.

To determine if biglycan treatment directly increases cell count in the absence of SI/R, in separate experiments, the effects of rhBGNc treatment were investigated on cell proliferation with 5'-bromo-2'-deoxyuridine (BrdU) incorporation assay as well as on viability in normoxic control cultures.

To further strengthen the relevance of our findings we have subjected primary adult rat cardiomyocytes to 45 min simulated ischemia followed by 120 min simulated reperfusion. During SI/R the cells were treated with a vehicle or rhBGNc. SI/R was induced the same way as for primary neonatal cultures, however, since adult cells are more sensitive to SI/R-induced damage shorter duration of simulated ischemia (45 min) was applied. Cell viability was determined using calcein assay.

To examine if the cytoprotective effect of rhBGNc includes modulation of necrotic or apoptotic cell death or autophagy, cell cultures were subjected to SI/R with or without treatment with 10 nM rhBGNc as described above and compared to normoxic controls. Necrosis was assessed by measurement of lactate dehydrogenase (LDH) release to the medium, while apoptosis was assessed by activated caspase-3 immunostaining (overall apoptosis) and by estimation of mitochondrial membrane potential with JC-1 staining

(intrinsic apoptosis). Autophagy was assessed by LC3 I/II immunocytochemistry and western blot analysis.

To explore the mechanism of action of the biglycan core protein, first we tested if exogenously administered rhBGNc is capable to bind to cells. Cardiomyocyte cultures were treated with His_6-tagged rhBGNc followed by detection of the His_6-tag in the cellular fraction or supernatant using western blot.

To further investigate whether TLR4 signaling is involved in the protective mechanism of rhBGNc, 10 nM rhBGNc was applied with or without the TLR4 signaling inhibitors, TAK-242 or Lipopolysaccharide from *Rhodobacter Sphaeroides,* LPS-RS for 20 h pretreatment and during SI/R, then viability was measured by calcein assay.

To explore the downstream mechanisms of rhBGNc and TLR4, cardiomyocytes were treated with 10 nM rhBGNc in the presence or absence of inhibitors of IRAK-1/4, MEK/ERK, JNK and p38 MAPKs.

To further elucidate the possible downstream molecular mechanisms of biglycan core protein signaling, activation of well-known cardioprotective pathways, i.e. RISK and SAFE pathways were investigated in response to rhBGNc treatment. The alterations of phosphorylated or total Akt (Protein kinase B), ERK1/2 and Signal transducer and activator of transcription 3 (STAT3) proteins after 20 h rhBGNc treatment were determined by Western blot analysis. Activation of ERK was also assessed following 10 min, 1 h and 20 h of rhBGNc treatment.

For assessment of the role of nitric oxide (NO) and oxidative stress in the protective effect of rhBGNc, cardiomyocytes were treated with 10 nM rhBGNc for 20 h, then NO content and superoxide production was measured. In separate experiments, cells were treated with 10 nM rhBGNc in the absence or presence of NO synthase inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) for 20 h followed by SI/R. Then viability was measured by calcein staining. Superoxide production was also measured in cells subjected to normoxia or SI/R with or without rhBGNc treatment.

To investigate the potential effect of biglycan on oxidative stress, we set up a H_2O_2 cytotoxicity protocol. In preliminary experiments the concentration of H_2O_2 inducing approximately 50% all death was determined (50 μM H_2O_2). Then one-day-old primary neonatal cardiomyocytes were treated with biglycan for 20 h followed by 50 μM H_2O_2 treatment for 24 h during which appropriate biglycan treatments were maintained. At the end of the protocol, cell viability was measured by calcein assay.

Cytoprotective effect of selected preconditioning-associated miRNAs

Prior to test the potential cytoprotective effect of miRNAs against SI/R, miRNAs showing altered expression in response to ischemic preconditioning were identified. Isolated Langendorff-perfused hearts were subjected to I/R with or without ischemic preconditioning and differential expression of miRNAs were determined by miRNA microarray. Based on the microarray results three miRNAs were chosen for further investigations in neonatal cardiomyocyte cultures and transfected before SI/R. Thus the effects of mimics of rno-miR-139-5p and rno-miR-125b* and an antagonist of rno-miR-487b were tested on viability in neonatal cardiomyocytes subjected to SI/R.

Results

Both native biglycan and deglycanated biglycan protects against SI/R-induced cell death

We confirmed that treatment of cardiomyocytes with glycanated native biglycan attenuates SI/R-induced cell death in a dose-dependent manner. To test whether deglycanated biglycan obtained after enzymatic removal of GAG chains exerts cytoprotective effects, deglycanated biglycan was prepared by digesting native biglycan with Chondroitinase ABC. The deglycanated biglycan also exerted a similar dose-dependent attenuation of SI/R injury. These data suggest that the core protein plays an important role in the cardiocytoprotective effect of biglycan.

The biglycan core protein rather than the GAG chains is responsible for the protective effect of biglycan

To further investigate the role of the core protein and the GAG chains in the cardiocytoprotective effect of biglycan, a different approach was used to look separately at the effects of distinct structural components of biglycan. In this experimental setup, approximately 30% of the cells die due to SI/R.

Native biglycan protected cardiomyocytes against SI/R injury in a dose-dependent manner. In order to confirm whether the core protein of biglycan is responsible for the observed cardioprotective effect, a recombinant human GAG-free core protein (rhBGNc) was used for treatments.

SI/R-induced cell death was attenuated by rhBGNc in a dose-dependent manner showing a U-shaped curve. The 3 and 10 nM concentrations of rhBGNc decreased the cell death significantly compared to the vehicle group. To test the potential effects of GAG chains on SI/R injury, dermatan sulfate or chondroitin sulfate was applied in a dose range (0.105-

10.5 μg/mL GAGs), corresponding to the biglycan concentrations applied in the previous experiments (1-100 nM). Neither concentrations of the dermatan sulfate influenced significantly the SI/R-induced cell death.

Biglycan core protein protects primary adult cardiomyocytes from SI/R-induced cell death

Since the prevalence of I/R injury is higher in adults, we have verified if the cytoprotective effect of rhBGNc can be demonstrated in adult cells as well. Therefore we have prepared adult rat cardiomyocyte cultures and tested whether rhBGNc is able to protect primary adult rat cardiomyocytes against SI/R-induced cell death. While 45 min simulated ischemia followed by 120 min simulated reperfusion significantly decreased the cell viability compared to normoxic controls, 30 nM rhBGNc significantly attenuated SI/R-induced cell death.

Biglycan core protein does not increase cell proliferation

In order to assess whether the biglycan core protein-dependent increase in viability after SI/R is due to direct cytoprotection or enhanced cell proliferation, we determined the effect of rhBGNc on cell viability under normoxic conditions as well as on cell proliferation assessed by BrdU incorporation. BrdU incorporation was not increased and cell viability was not affected significantly by biglycan core protein under normoxic conditions. These data suggest that the cytoprotective effect of rhBGNc is not due to increased cell proliferation.

Biglycan core protein influences SI/R-induced necrosis and apoptosis

Release of the cytoplasmic enzyme LDH into the culture medium is a characteristic sign of necrotic cell death. LDH activity of culture supernatants was significantly increased due to SI/R as compared to normoxic controls, however, LDH release from rhBGNc-treated cells subjected to SI/R was not significantly different from that of normoxic controls.

To further characterize rhBGNc-related protection against SI/R-induced cell death, we studied the effect of rhBGNc on late events of apoptosis by performing cleaved caspase-3 immunostaining. Caspase-3 activation was significantly increased in the SI/R group compared to normoxic controls, while rhBGNc significantly attenuated SI/R-induced elevation of cleaved caspase-3. These results suggest a potential antiapoptotic effect of rhBGNc.

To reveal whether the protective effect of rhBGNc on SI/R subjected cells involves mitochondrial events, the changes in mitochondrial membrane potential were measured after SI/R by JC-1 staining. Cells subjected to SI/R had significantly decreased mitochondrial membrane potential compared to normoxic controls. The rhBGNc-treatment somewhat

improved mitochondrial membrane potential, as no significant difference in the red-to-green fluorescence was found when compared to the normoxic controls. However, based on these results, the contribution of mitochondrial apoptotic pathways to the antiapoptotic effect of rhBGNc during SI/R seems to be minor.

Biglycan core protein influences autophagy

To investigate whether the cytoprotective effect of rhBGNc against SI/R involves modulation of autophagy, presence of autophagic vacuoles was detected using immunocytochemistry. Autophagy was present in the cells exposed to normoxic conditions which effect was attenuated under SI/R and enhanced back by the cytoprotective effect of biglycan. In parallel, immunoblotting showed decreased conversion of LC3 I to LC3 II (a marker of decreased autophagy) under SI/R, which was recovered to the normoxic levels under the effect of biglycan.

Cytoprotection by biglycan core protein involves Toll-like receptor signaling

In order to assess whether exogenously administered rhBGNc is associated to cells indicating possible receptor binding, cardiomyocyte cultures were treated with His_6-tag labeled rhBGNc. Western blot analysis using an anti-His_6-tag antibody demonstrated the presence of exogenously administered rhBGNc in both the cellular and supernatant fractions indicating that biglycan core protein binds to cardiomyocytes.

To investigate the potential mechanisms of action of biglycan core protein, we tested if inhibition of TLR4 signaling interferes with the cytoprotective effect of rhBGNc against SI/R injury. The TLR4 signaling inhibitors TAK-242 and LPS-RS abolished the cytoprotective effect of rhBGNc, however, they did not affect cell death when administered alone.

In order to confirm the involvement of TLR4 signaling in the cardiocytoprotective effect of rhBGNc, specific pharmacological inhibitors of IRAK-1/4, MEK/ERK, JNK, and p38 – well known downstream members of TLR4 signaling – were applied alone or in combination with rhBGNc on cardiomyocytes subjected to SI/R. RhBGNc significantly decreased SI/R-induced cell death observed in the vehicle group, however, the cytoprotective effect of rhBGNc was abolished in the presence of the inhibitors of IRAK-1/4, MEK/ERK, p38 MAPK and JNK, respectively. The inhibitors alone did not affect significantly the viability of the cardiomyocytes. These results suggest that TLR4 signaling is involved in the protective effect of rhBGNc.

Biglycan core protein enhances Akt and ERK phosphorylation

To further elucidate the possible downstream molecular mechanisms of biglycan core protein signaling, activation of well-known cardioprotective pathways – RISK and SAFE pathways – were investigated in response to 20 h of rhBGNc treatment. Western blot analysis showed that 20 h of rhBGNc treatment significantly enhances Akt phosphorylation without affecting phosphorylation of STAT3 or ERK1/2. Since ERK1/2 is also a component of TLR4 signaling and known for its rapid phosphorylation/dephosphorylation kinetics, we have looked at ERK phosphorylation at earlier time points after rhBGNc treatment. Phosphorylation of ERK1 was markedly increased after 10 min rhBGNc treatment, however, rhBGNc-induced ERK1 phosphorylation was attenuated after 1 h, and was abolished after 20 h of treatment. Phosphorylation of ERK2 showed a similar tendency without reaching the level of statistical significance.

NO is involved in the cytoprotective effect of biglycan core protein

To assess the possible involvement of NO signaling in the cytoprotective effect of biglycan core protein, the NO content was measured in cardiomyocyte cultures after spin trapping. NO level of cardiomyocytes was elevated after 20 h of rhBGNc treatment. To confirm the causative role of NO signaling in the protective effect of biglycan core protein, in separate experiments, rhBGNc was applied in the presence or absence of the NO synthase inhibitor L-NAME. RhBGNc significantly decreased cell death compared to the untreated group, however, L-NAME abolished the cytoprotective effect of biglycan core protein.

The biglycan core protein attenuates the SI/R-induced superoxide production.

To investigate the effect of biglycan on superoxide level, cardiomyocyte cultures were treated with rhBGNc for 20 h, with or without SI/R injury and superoxide production was determined by DHE staining. RhBGNc had no effect on superoxide production in a stress free environment, however, it significantly attenuated the SI/R-induced increase in superoxide level.

The native biglycan attenuates the H_2O_2-induced cytotoxicity.

To assess the possible involvement of superoxide elimination in the cytoprotective effect of biglycan, we have set up an H_2O_2-induced cell death model. One day old cultures were treated with 0, 3, 10, or 30 nM biglycan for 20 hours followed by 50 μM H_2O_2 treatment for 24 h while the appropriate biglycan treatments were maintained. At the end of the protocol, cell viability was measured.

We found that H_2O_2 resulted in a marked loss of cells. Pretreatment with biglycan dose-dependently protected the cardiomyocytes and 30 nM biglycan significantly reduced the H_2O_2-induced cell death. These data suggest that biglycan might have a role in attenuation of oxidative stress in cardiac myocytes.

Preconditioning-associated miRNA modulators protect primary neonatal cardiomyocytes from SI/R injury

In the present thesis we aimed to demonstrate that modulation of the expression of preconditioning-associated miRNA with specific, exogenously administered, synthetic oligonucleotide modulators is able to protect cardiomyocytes from SI/R-induced cell death. Therefore, to validate the possible relationship between miRNAs and cardiocytoprotection, the preconditioning-associated rno-miR-139-5p as well as rno-miR-125b* mimick (agonist) and an inhibitor (antagonist) of rno-miR-487b were transfected into primary neonatal cardiomyocytes and the cells were then subjected to SI/R. The SI/R significantly decreased cell viability compared to the normoxic control. We found that the transfection of cardiomyocytes with any of the modulators of the three selected miRNA significantly decreased all death caused by SI/R. Therefore, these miRNAs can be used as potential therapeutic target molecules in the treatment of SI/R–induced cellular damage.

Discussion and conclusion

New findings

1. The biglycan core protein protects cardiomyocytes against cell death induced SI/R, while the GAG side chains do not exert a protective effect.

In the present study we have confirmed that biglycan attenuates cell death induced by SI/R in cardiomyocytes. In addition, we have demonstrated for the first time that the core protein of biglycan rather than the GAG chains of the molecule are responsible for the observed cytoprotection.

2. The rhBGNc modulates the effect of SI/R upon necrosis, apoptosis and autophagy.

We demonstrated that the LDH activity of culture supernatants (marker of necrotic cell death) was significantly increased due to SI/R as compared to normoxic controls, however, LDH release from rhBGNc-treated cells subjected to SI/R was not significantly different from that of normoxic controls. Caspase-3 activation (a marker of overall apoptosis) was significantly increased in the SI/R group compared to normoxic controls, while rhBGNc significantly attenuated SI/R-induced elevation of cleaved caspase-3. The rhBGNc treatment

somewhat improved mitochondrial membrane potential (marker of intrinsic apoptosis), but the contribution of mitochondrial apoptotic pathways seems to be minor in the antiapoptotic effect of rhBGNc during SI/R. The rhBGNc treatment recovered the SI/R-induced decrease autophagy to the normoxic level.

3. Cytoprotection by biglycan core protein involves Toll-like receptor signaling.

With the help of pharmacological inhibitors we have shown that the cytoprotective effect of biglycan core protein is mediated via activation of TLR4 signaling and involves the adaptor molecule IRAK-1/4 and its downstream targets, ERK, JNK and p38 MAP kinases.

4. Biglycan core protein enhances Akt and ERK phosphorylation and NO production.

The treatment of cardiomyocytes with rhBGNc resulted in increased Akt and ERK1 phosphorylation and enhanced production of NO which plays a role in the cardiocytoprotective effect of biglycan core protein.

5. Biglycan exerts antioxidant effects.

The native biglycan attenuated the H_2O_2-induced cell death and rhBGNc significantly attenuated SI/R-induced increase in superoxide production.

6. Modulation of selected preconditioning-associated miRNAs protect primary neonatal cardiomyocytes from SI/R injury.

The mimics of rno-miR-139-5p and rno-miR-125b* and an inhibitor of rno-miR-487b were able to significantly decrease the cell death caused by SI/R.

In conclusion, the core protein of biglycan mediates cytoprotection against SI/R injury in cardiomyocytes by modulating the effects of SI/R on necrosis, apoptosis, and autophagy. The molecular mechanism of action of biglycan core protein involves TLR4 signaling, activation of Akt, ERK, as well as JNK and p38MAP kinases and increased NO production. Biglycan is able to improve cell survival against H_2O_2 induced cell death and rhBGNc attenuates the SI/R-induced increase of superoxide level. These findings suggest an antioxidant role for biglycan, however, the mechanisms is still unclear. The novel cytoprotective effect of biglycan core protein may provide a basis for development of potential future therapeutic applications for the treatment of myocardial infarction as a tool for pharmacological preconditioning. Another way to elicit pharmacological preconditioning is the modulation of preconditioning-associated miRNA expression. The selected synthetic miRNA modulators protect the primary neonatal cardiomyocytes from SI/R injury. These

miRNAs serve as potential targets for modulation by specific miRNA mimics or inhibitors to achieve cardioprotection. In this present study we have demonstrated novel directions for pharmacological preconditioning to protect primary cardiomyocyte cultures from SI/R.

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Alulírott **Dr. Varga Zoltán** (felelős elsőszerző) kijelentem, hogy **Gáspár Renáta** (pályázó) PhD értekezésének tézispontjaiban bemutatott - közösen publikált - tudományos eredmények elérésében a pályázónak meghatározó szerepe volt, ezért ezeket a pontokat más, a PhD fokozat megszerzését célzó minősítési eljárásban nem használta fel, illetve nem kívánja felhasználni.

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dátum szerzõ

A pályázó tézispontjaiban érintett, közösen publikált közlemények:

MicroRNAs associated with ischemia-reperfusion injury and cardioprotection by ischemic pre- and postconditioning: protectomiRs.
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.
Am J Physiol Heart Circ Physiol. 2014 Jul 15;307(2):H216-27. doi: 10.1152/ajpheart.00812.2013. Epub 2014 May 23.
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