

**Cytoprotective approaches to protect
myocardium against
ischemia/reperfusion injury**

Ph.D. Thesis

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LIST OF PUBLICATIONS

1. List of full papers directly related to the subject of the thesis:

- I.** Bencsik P*, Pálóczi J*, Kocsis GF, Pipis J, Beleczi I, Varga ZV, Csonka C, Görbe A, Csont T, Ferdinandy P. Moderate inhibition of myocardial matrix metalloproteinase-2 by ilomastat is cardioprotective. *Pharmacol Res.* 2014; 80:36–42. **IF.: 3.976**

*the authors contributed equally to the present work.

- II.** Görbe A, Varga ZV, Pálóczi J, Rungarunlert S, Klincumhom N, Purity MK, Madonna R, Eschenhagen T, Dinnyés A, Csont T, Ferdinand P. Cytoprotection by NO-donor SNAP against ischemia/reoxygenation injury in mouse embryonic stem cell-derived cardiomyocytes. *Mol Biotechnol.* 2014; 56:258–64. **IF.: 2.275**

Cumulative impact factor of papers directly related to the thesis: 6.251

2. List of full papers indirectly related to the subject of the thesis:

- III.** 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.* 2014; 306:H405–13. **IF.: 4.012**

- IV.** Monostori P, Kocsis GF, Ökrös Z, Bencsik P, Czétényi O, Kiss Z, Gellén B, Bereczki, C, Ocsovszki I, Pipis J, Pálóczi J, Sárközy M, Török S, Varga IS, Kiss I, Fodor E, Csont T, Ferdinandy P, Túri S. Different administration schedules of darbepoetin alfa affect oxidized and reduced glutathione levels to a similar extent in 5/6 nephrectomized rats. *Clin Exp Nephrol.* 2013; 17:569–74. **IF.: 1.708**

- V.** Sz cs G, Murlasits Z, Török S, Kocsis GF, Pálóczi J, Görbe A, Csont T, Csonka C, Ferdinandy P. Cardioprotection by Farnesol: Role of the Mevalonate Pathway. *Cardiovasc Drugs Ther.* 2013; 27:269–77. **IF.: 2.952**

- VI.** Varga ZV, Kupai K, Sz cs G, Gáspár R, Pálóczi J, Faragó N, Zvara A, Puskás LG, Rázga Z, Tizlavicz L, Bencsik P, Görbe A, Csonka C, Ferdinandy P, Csont T. MicroRNA-25-dependent up-regulation of NADPH oxidase 4 (NOX4) mediates hypercholesterolemia-induced oxidative/nitrative stress and subsequent dysfunction in the heart. *J Mol Cell Cardiol.* 2013; 62:111–21. **IF.: 5.218**

- VII.** Kocsis GF, Sárközy M, Bencsik P, Pipicz M, Varga ZV, Pálóczi J, Csonka C, Ferdinandy P, Csont T. *Am J Physiol Heart Circ Physiol.* 2012; 303:H1229–36. **IF.: 3.629**

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1. INTRODUCTION

Ischemic heart disease (IHD) is one of the leading cause of death in the industrialized world. IHD by itself is the single most common cause of death in the European Union: accounting for over 681 000 deaths in each year (Nichols *et al.* 2012). In Hungary, more than 6000 patients die due to the consequences of acute myocardial infarction (AMI) in each year (according to the official statement of the Hungarian Central Statistical Office, released in 2012). AMI leads to massive cardiac cell loss, rapid decline of cardiac contractility, structural and functional cardiac remodeling and eventually to the development of heart failure. Therefore, it is highly important to salvage the ischemic region of heart via its reperfusion as soon as possible to improve the clinical outcomes of myocardial infarction. However, reperfusion may lead to intense generation of reactive oxygen (ROS) and nitrogen species (RNS) and activation of numerous cascade mechanisms which may result in further myocardial damage (Zhu *et al.* 2007). Additionally, despite of a decade of intense research on the cellular mechanism of cardioprotective interventions aiming to reduce reperfusion injury, the underlying mechanisms of these protective pathways are still poorly understood. Therefore, no drug targets could be identified so far that would lead to the development of a cardioprotective compound until market launching. This fact urges to identify novel targets and to find effective drugs for cardioprotection.

Over the last decade, a novel therapeutic approach has been offered; stem cell derived cardiomyocytes may be used as a cell source for cardiac repair after myocardial infarction (Lundy *et al.* 2013). Despite the encouraging results and the enormous potential of human stem cell-derived cardiomyocytes, several complications and ethical concerns need to be overcome towards clinical translation. Therefore, the improvement of cell replacement therapy is one of the most important aims of further investigations.

1.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a large family of zinc dependent endopeptidases. They were firstly identified by Gross *et al.* and named according to their ability to remodel extracellular matrix (Gross and Lapiere 1962). MMPs are synthesized in an inactive form (zymogen or pro-MMP), and are activated by proteolytic cleavage of an N-terminal domain or by conformational changes induced by denaturing agents or ROS. Basically, They are involved in the remodeling of extracellular matrix during various physiological processes. On the other hand, MMPs have been shown to play a crucial role in several pathologies as well. Moreover, gelatinase types of MMPs, (MMP-2 and MMP-9) are implicated with numerous

cardiovascular diseases such as ischemia/reperfusion injury. It has been previously reported that MMP-2 is constitutively expressed in cardiomyocytes and stored intracellularly in an inactive pro-MMP-2 (also referred as 72 kDa MMP-2) form. Increasing evidences suggest that ischemic stimulus can activate the intracellular pro-MMP-2 which is able to proteolytically cleave several structural proteins including titin, alpha-actinin, or troponins (for recent review see DeCoux *et al.* 2014). Degradation of these proteins could lead to contractile dysfunction and cell death. The activation of pro-MMP-2 may be due to the increased peroxynitrite production (Kupai *et al.* 2010). In addition, active MMP-2 is released from necrotic cells thereby exacerbating the outcome of ischemic injury (for review see Hughes and Schulz 2014).

We have previously reported that MMP-2 activity was moderately decreased during ischemic preconditioning and that exogenous inhibition of MMPs by ilomastat, a non-selective MMP inhibitor diminished ischemia-induced MMP-2 activity in isolated rat hearts (Giricz *et al.* 2006). Furthermore, we have demonstrated that the activities of MMP-2 and MMP-9 were decreased significantly in an *in vivo* rat model of ischemic late preconditioning (Bencsik *et al.* 2010). Moreover, we and others have shown that the non-selective MMP inhibitor ilomastat reduced infarct size in rats and mice (Bencsik *et al.* 2010; Bell *et al.* 2013). Taken together, MMP-2 became a major target for drug development in acute cardiovascular pathologies including AMI (Dormán *et al.* 2012). However it is unknown whether ilomastat-induced cardioprotection is due to MMP-2 inhibition and what extent of this inhibition is required for sufficient cardioprotection. Moreover it is still unclear whether the ilomastat-induced cardioprotection occurs via the inhibition of the intracellularly active MMP-2.

1.2 Stem cell-derived cardiomyocytes: a new therapeutic approach to treat myocardial infarction

Thrombolytic therapy and other coronary interventions are widely used clinical strategies to reduce infarct size after myocardial infarction (for review see Bagai *et al.* 2014). However, if there is no adequate intervention to minimize myocardial damage, the prolonged ischemic injury would increase the risk of mortality. Recently, an alternative approach has come into the focus of cardiovascular regenerative medicine. Over the last few years, various cell types have been used in preclinical studies for cell replacement therapy (for review see Dai *et al.* 2005). Embryonic stem cells might be a promising cell source, because of their capability to provide unlimited expansion and differentiation into cardiomyocytes *in vitro*. However, applications of embryonic stem cell-derived cardiomyocytes have ethical

considerations; therefore, better potential of these cardiomyocytes is testing cardioprotective compounds *in vitro*. Moreover, generation of induced pluripotent stem cells and their differentiation into functional cardiomyocytes provides new perspectives in personalized cell replacement therapy (for review see Kawamura *et al.* 2012).

Despite the increasing number of promising results, several complications need to be overcome towards clinical translation. One of the most important limitation regarding their clinical utilization is that the survival of implanted cells is reduced after transplantation (Qiao *et al.* 2009). A plausible reason for this effect is the unfavourable microenvironment the grafted cells face when grafted into the ischemic myocardium. Thus, *in vitro* preconditioning strategies might be beneficial to improve and optimize long-term survival and maturation of the cell grafts. Furthermore, characterization of these cells in an ischemia/reperfusion test system would be important, since little is known about the ischemic tolerance of stem cell-derived cardiomyocytes.

1.3 Role of nitric-oxide

Nitric oxide (NO) is a gaseous substance that regulates a wide range of biological processes. NO is synthesized at low physiological levels by NO synthase (NOS) enzymes. NO has a well-known cardioprotective effect against ischemia/reperfusion injury that is conducted by activating the cyclic guanosine monophosphate (cGMP)/ protein kinase G (PKG) axes, as a key modulator of the RISK (reperfusion injury salvage kinase) and SAFE (survivor activating factor enhancement) pathways, and by inducing S-nitrosylation of proteins (for review see Schulz and Ferdinandy 2013; Murphy *et al.* 2014). However, excess of NO in the presence of the co-existing free radicals have detrimental effect on cell survival.

We have previously shown that the exogenously administered NO donor S-nitroso-n-acetyl-penicillamine (SNAP) and the particulate guanylate cyclase activator B-type natriuretic peptide (BNP) exert cytoprotective effect against simulated ischemia/reoxygenation (SI/R) injury in primary neonatal rat cardiomyocytes (Gorbe *et al.* 2010). This protection acts via the activation of cGMP/PKG signaling pathway, thereby stimulating its multiple downstream signal transduction cascade which leads to increased cell viability against SI/R injury (Burley and Baxter 2007; Gorbe *et al.* 2010). Moreover, our previous findings also suggest that the present screening platform is a useful tool for discovery of cardiocytprotective molecules and investigate their cellular mechanisms. Nevertheless, the importance of the cardioprotective effect of the activation of cGMP-PKG axes in mouse ESC (mESC)-derived

cardiomyocytes is still unknown. Thus, it would be beneficial to test this pathway in the previously established SI/R test system.

2. AIMS

In study 1, we aimed to test whether ilomastat-induced cardioprotection is due to (and what extent of) MMP-2 inhibition, we performed gelatin zymography and *in situ* zymography followed by immunostaining of MMP-2 in primary neonatal rat cardiomyocytes exposed to SI/R injury. Furthermore, our goal was to investigate the dose-response effect of ilomastat administered before the onset of ischemia as well as before the onset of reperfusion in an *in vivo* rat model of myocardial infarction.

The aim of study 2 was to establish a mouse embryonic stem cell-derived cardiomyocyte-based drug screening platform by investigating whether the cytoprotective NO-donor SNAP and B-type natriuretic peptide is able to protect these cells against SI/R injury. We also investigated the downstream pathways of the protection in this platform.

3. MATERIALS AND METHODS

Animal handling and the investigation was in conjunction with Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (National Institutes of Health publication 85-23, revised 1996), and it was approved by a local animal ethics committee.

3.1 Experimental design of Study 1

Male Wistar rats (Charles-River, Germany) weighing 280-370g were used in the experiments housed in individually ventilated cages. Animals were fed with standard murine chow and unlimited access to water was ensured prior to the surgical intervention. For the cell culture experiments, neonatal Wistar rats were purchased from the local live-stock of the University of Szeged.

3.1.1 Inhibition of gelatinase activity by ilomastat

In order to determine the source of the gelatinolytic activity and its inhibition by ilomastat, gelatin zymography was performed on cardiac tissue homogenate of a non-treated control rat. Gelatinolytic activities of MMP-2 were examined as previously described in detail (Kupai *et al.* 2010). Gelatinolytic activities were detected as transparent bands against the dark-blue background. Band intensities were quantified (Quantity One software, Bio-Rad,

Hercules, CA) and expressed in arbitrary units. The gelatin zymography protocol does not contain any component or step, which may inhibit proteases including other MMPs.

3.1.2 Simulated ischemia/reoxygenation in cardiomyocytes

Neonatal rat cardiomyocytes were cultured in 48-well plates. Cell isolation, culturing method and the SI/R drug screening platform were described before in detail (Csont *et al.* 2010; Gorbe *et al.* 2010). Cardiomyocytes were kept under normoxic conditions (95% air and 5% CO₂ at 37°C) for three days prior to SI/R experiments. We used a combination of hypoxic chamber and hypoxic solution to simulate tissue ischemia (Li *et al.* 2004; Gorbe *et al.* 2010). In the SI groups, the medium of the cultures were replaced with a hypoxic solution (Li *et al.* 2004)(Gorbe *et al.* 2010) and plates were kept in a hypoxic chamber (gassed with 95% N₂ and 5% CO₂ at 37°C) for 240 min in the presence or absence of ilomastat (5 nM, 50 nM 500 nM and 5 μM). The vehicle group was treated only with 0.2% Dimethyl sulfoxide (DMSO). During reoxygenation, cells were covered with differentiation medium (containing 1% fetal bovine serum) and kept in a normoxic incubator for 120 min.

3.1.3 Cell viability assay

Cell viability was assessed by a calcein assay performed in each group after 2 h simulated reperfusion. Then calcein fluorescence of each well was detected by a fluorescent plate reader. Calcein data were normalized to total cell count obtained by propidium iodine + digitonin staining. The fluorescence of propidium iodine (PI; Sigma, St. Louis, MO) was detected in the same way.

3.1.4 In situ zymography and MMP-2 co-localization

To detect *in situ* MMP-2 activity, neonatal rat cardiomyocytes were cultured in 24-well tissue culture plate for 3 days. The medium of cells was replaced with hypoxic solution supplemented with DQTM gelatin (Invitrogen, Life Technologies Hungary Ltd, Budapest, Hungary) at 40 μg/mL concentration. Cells were then subjected to 240 min simulated ischemia in the presence of ilomastat (at 500 nM concentration) or its vehicle. Other series of cells were covered with normoxic solution and kept in normoxic incubator for 240 min. Subsequently, all groups were subjected to reoxygenation: the hypoxic, or normoxic solution of the cells was replaced with differentiation medium supplemented with DQTM gelatin and cells were placed into a normoxic incubator for 120 min. Finally cells were washed, and fixed with paraformaldehyde. MMP-2 fluorescent immunostaining was performed by using anti-

proMMP-2 antibody (Chemicon, MAB3308; Merck Ltd, Budapest, Hungary) to detect co-localization of MMP-2 with gelatinolytic activity. Nuclei of the cells were stained with Hoechst 33342 (Invitrogen, Life Technologies Hungary Ltd, Budapest, Hungary). Fluorescence was detected by a confocal laser microscope in sequential scanning mode (Olympus Fluoview 1000, Olympus Hungary Ltd, Budapest, Hungary). Assessment of the gelatinolytic activity was carried out by quantifying different parameters of fluorescent particles from 10 randomly selected fields by using ImageJ 1.45 software (National Institutes of Health, Bethesda, MD).

3.1.5 Surgical procedure of coronary occlusion

Rats were anesthetized then intubated and mechanically ventilated (Model 683, Harvard Apparatus, Holliston, MA) with room air according to their body weight. Rats were placed onto a heating pad to maintain body core temperature in physiological range ($37.0^{\circ}\text{C}\pm 1.0^{\circ}\text{C}$). Mean arterial blood pressure and electrocardiogram (ECG) was continuously monitored throughout the experiments (Haemosys, Experimetria Inc., Budapest, Hungary). Right jugular vein was cannulated for fluid substitution and drug administration. Left anterior descending coronary artery (LAD) occlusion was induced for 30 minutes. Appearance of ischemia was confirmed by ST segment elevation and arrhythmias. After 30 min ischemia, hearts were reperfused for 120 min by releasing the ligature. Restoration of blood flow was confirmed by arrhythmias observed in the first minutes of reperfusion.

3.1.6 Experimental groups

In first series of *in vivo* experiments, ilomastat was administered before the onset and during the 30-min ischemia. Animals were divided into five groups. Ilomastat (at the dose of 0.3, 0.75, 1.5, and 3.0 $\mu\text{mol/kg}$) or its vehicle (DMSO) was administered intravenously in slow bolus 5 minutes before ischemia. In order to maintain serum level of ilomastat, additional 3 boluses of vehicle or ilomastat with half dose (0.15, 0.375, 0.75; and 1.5 $\mu\text{mol/kg}$, respectively), were given at the 10th, 25th min of ischemia and at the 10th min of reperfusion. In the second series of *in vivo* experiments ilomastat (at the dose of 0.75, 1.5, 3.0, and 6.0 $\mu\text{mol/kg}$) or its vehicle (DMSO) was injected at the 25th min of ischemia. Maintaining boluses (0.375, 0.75, 1.5, and 3.0 $\mu\text{mol/kg}$, respectively) were administered at the 10th, 25th and 40th min of reperfusion to maintain constant ilomastat concentration in blood.

3.1.7 Determination of infarct size

At the end of reperfusion hearts were isolated for infarct size measurements. Hearts were retrogradely perfused in a Langendorff perfusion system with 37°C Krebs-Henseleit buffer (Csonka *et al.* 1999) to remove blood from the coronary vessels. After 5 min of perfusion, risk area was re-occluded, and hearts were perfused with 0.1% Evans blue dye through the ascending aorta. Following Evans staining, hearts were cut into 5 transversal slices and incubated in 1% triphenyl-tetrazolium-chloride for 10 min at 37°C followed by formalin fixation for 10 min. Planimetric evaluation was carried out to determine infarct size using InfarctSize™ software (Csonka *et al.* 2010).

3.1.8 Statistical analysis

Statistical analysis was performed using Sigmaplot 11.0 software. All data were given as mean ± standard error of the mean (SEM). One-way ANOVA followed by Fisher-LSD post hoc tests were performed to show differences among groups. P values of 0.05 were accepted as statistically significant difference compared to vehicle control.

3.2 Experimental design of Study 2

3.2.1 Mouse Embryonic Stem Cell Culture

Undifferentiated mouse embryonic stem cells (mESCs) (Nkx2.5/EGFP transgenic C57BL/6 mouse embryonic stem cell line; Tg^{Nkx2.5/EGFP} C57BL/6; passages 10-12) were cultured on feeder layers of mitomycin C-inactivated mouse embryonic fibroblasts (MEFs). mESCs were cultured on feeder layers for at least two passages after thawing and subsequently were cultured without feeder cells on gelatin-coated tissue culture plates in the presence of mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon International, Budapest, Hungary). mESCs were usually passaged every 1-2 days prior reaching 70% confluences.

3.2.2 Embryoid Body (EB) Formation and Cardiomyocyte Differentiation

mESCs were dissociated from monolayer culture with 0.05% trypsin-EDTA into a single cell suspension. EBs were produced by using the hanging drop method (Mummery, Ward, and Passier 2007). Two days later, the EBs were transferred and seeded into 24 well plates on gelatin coated coverslips. 0.1 mg/ml ascorbic acid was presented in the medium to induce cardiac differentiation. mESC-derived cardiomyocytes were used at 6-8 day-old stage

for SI/R experiments. At this stage, the ratio of Nkx2.5-eGFP positive cells, an early marker for cardiac differentiation, exhibited $52.5\pm 10\%$ eGFP positivity (n=30). The cells were fluorescently imaged and analyzed by using Digital Image Processing Software (AxioVision 4.8.1, Carl Zeiss MicroImaging GmbH, Germany).

3.2.3 Experimental Groups

For cell SI/R experiments, mESC-derived cardiomyocytes were tested under normoxic condition or were subjected to SI. The normoxic mESC-derived cardiomyocytes were kept under normoxic conditions; the growth medium was changed to a normoxic solution (Li *et al.* 2004; Gorbe *et al.* 2010) and the cells were incubated under 95% air and 5% CO₂ at 37 °C for 150 min. In the second series of experiments, mESC-derived cardiomyocytes were subjected to SI by incubating cells in hypoxic solution (Li *et al.* 2004; Gorbe *et al.* 2010) and placing the plates in a humidified 37°C hypoxic chamber exposed to a constant flow of a mixture of 95% N₂ and 5% CO₂ for 150 min. The cells were then subjected to the following treatments during SI or normoxic condition: (1) untreated control; (2) SNAP (0.1 μM, 1 μM, 10 μM; Sigma, St. Louis, MO); (3) selective PKG inhibitor KT-5823 (60 nM; Sigma, St. Louis, MO); (4) SNAP (1 μM, a concentration found here protective) in combination with KT-5823 (60 nM); (5) the particulate guanylate cyclase activator BNP (1 nM, 10 nM, 100 nM; American Peptides, Sunnyvale, CA); (6) the nitric oxide synthase (NOS) inhibitor N-Nitro-L-arginine (L-NNA; 100 μM, 10 μM; Sigma, St. Louis, MO); (7) the non-selective K_{ATP} channel inhibitor glibenclamide (1 μM; Sigma, St. Louis, MO); (8) SNAP (1 μM) and glibenclamide (1 μM); and DMSO (Sigma, St. Louis, MO) control groups. Either normoxic or SI treatments were followed by 120 min reoxygenation with growth medium without ascorbic acid and superfusion with 95% air and 5% CO₂ at 37 °C.

3.2.4 Cell Viability Assay

Cell viability was assessed by a PI assay performed in each group after 120 min reoxygenation. Each experiment included a digitonin (Sigma, St. Louis, MO) treated positive control well and PI control (cells without PI staining). Then PI fluorescence intensity of each EB was detected by a fluorescent plate reader. PI intensity reflecting the cell death was evaluated manually by detecting eGFP expression driven by the promoter of the early cardiac myocyte marker Nkx2.5. The cytoprotective effect of different compounds was compared to simulated ischemic control groups.

3.2.5 Statistical Analysis

Results are expressed as mean \pm SEM. One way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post-hoc tests was used to determine differences in mean values between groups. In case comparison of two groups, unpaired t-test was used. Differences were considered significant at $p < 0.05$.

4. RESULTS

4.1 Study 1

4.1.1 Effect of ilomastat on cardiac gelatinolytic activity

In a preliminary series of studies, the *in vitro* MMP-inhibitory dose range of ilomastat was estimated in rat cardiac tissue homogenate by gelatin zymography. We have found that the IC_{50} of ilomastat was 0.83 nM. Gelatinolytic activity was detectable only at 72 kDa in cardiac homogenate suggesting that only MMP-2 activity was present in the heart tissue at a significant level.

4.1.2 Effect of ilomastat on ischemia/reoxygenated cardiomyocytes

In order to test if a direct cardiocytoprotection by MMP-2 inhibition of ilomastat is involved in its cardioprotective effect, we examined ilomastat-induced cytoprotection in primary neonatal rat cardiomyocytes exposed to normoxia or SI/R. Ilomastat at a dose range of 0.5 nM up to 5 μ M did not influence cell viability in normoxic conditions. However, ilomastat at 500 nM and 5 μ M significantly increased cell viability as compared to vehicle treated group in neonatal rat cardiomyocytes exposed to SI/R injury.

4.1.3 In situ MMP-2 inhibition by ilomastat in ischemia/reoxygenated cardiomyocytes

To test the *in situ* MMP inhibitory efficacy of the cardiocytoprotective concentration of ilomastat, we performed *in situ* zymography on isolated neonatal rat cardiomyocytes subjected to SI/R. SI/R injury significantly increased gelatinolytic activity as compared to normoxic control. The cardiocytoprotective concentration of ilomastat (500 nM) moderately inhibited the *in situ* gelatinolytic activity approximately by 25%. Moreover, MMP-2 immunostaining showed over 90% co-localization rate with gelatinolytic activity in all groups.

4.1.4 Effect of ilomastat on infarct size in vivo

The cardioprotective effect of ilomastat administered before the onset of ischemia or before the onset of reperfusion was studied in an *in vivo* myocardial infarction model induced by coronary occlusion in rats. When administered before the onset of ischemia, ilomastat at 0.75 and 1.5 $\mu\text{mol/kg}$ doses significantly reduced infarct size as compared to vehicle-treated group showing a U-shaped dose-response relationship. When administered before the onset of reperfusion, only higher dose of ilomastat (6.0 $\mu\text{mol/kg}$) was able to reduce infarct size significantly, however, lower doses were ineffective. There were no significant differences in the area at risk among the groups.

4.2 Study 2

4.2.1 Cell Viability after SI/R treatment of mESC-derived cardiomyocytes

We applied SI/R to mimic *in vivo* ischemia/reperfusion injury in mESC-derived cardiomyocytes. 150 min of SI followed by 120 min reoxygenation caused significantly higher cell death in mESC-derived cardiomyocytes than time-matched controls kept under normoxic conditions. The cytoprotective effect of the NO donor SNAP that activates soluble guanylate cyclase was tested in this model of SI and reoxygenation-induced cell death in mESC-derived cardiomyocytes. Cell death was significantly decreased by SNAP in a concentration-dependent manner (1 μM and 10 μM compared to vehicle control) when applied during SI period. The contribution of endogenous NO production of mESC-derived cardiomyocytes to cell death during SI was tested by administration of the non-selective NOS inhibitor L-NNA at 10 μM and 100 μM concentration. The presence of L-NNA did not influence cell death after SI. BNP, an activator of particulate guanylate cyclase was also tested under SI condition at 1 nM, 10 nM and 100 nM concentrations. However, BNP did not influence cell death significantly. In separate experiments, the downstream pathways of SNAP-induced protection of mESC-derived cardiomyocytes were studied. The cytoprotective effect of SNAP (at 1 μM) was attenuated either by simultaneous administration of the selective PKG inhibitor KT-5823 (60 nM) or by simultaneous administration of K_{ATP} channel inhibitor glibenclamide (1 μM). Inhibitors administered alone, or their vehicle DMSO did not influence cell viability. In time-matched normoxic control groups, none of the above treatment influenced cell viability significantly.

5. DISCUSSION

5.1 Study 1: MMP-2 inhibition by ilomastat exerts cardioprotection

In *study 1* we have demonstrated that an approximately 25% inhibition of intracellular MMP-2 activity by the non-selective MMP inhibitor ilomastat confers significant cardiocytoprotection. Moreover, ilomastat reduced infarct size when administered either before the onset of ischemia or before the onset of reperfusion *in vivo* and revealed its cardioprotective dose-response relationship. This is the first demonstration that the cardioprotective effect of ilomastat may involve a cardiocytoprotective mechanism due to a moderate inhibition of MMP-2. It has been recently reported that ilomastat protects the heart against reperfusion injury independently from the well-known cardioprotective cellular RISK/mPTP modulating pathways (Bell *et al.* 2013). Since ilomastat is a non-selective MMP inhibitor, therefore, the question has arose, inhibition of which MMP isoenzyme was responsible for the cardioprotective effect of ilomastat. To answer this question, here we performed gelatin zymography from cardiac homogenates isolated from untreated rats and used purified MMP-2 enzyme to identify the MMP-2 specific activity in the zymogram. Gelatinolytic activities at 72 and 64 kDa were detected according to the molecular weights of the two active isoforms of MMP-2. Bands of other molecular weights were not observed on the zymogram which is in line with our previous finding (Kupai *et al.* 2010).

Recently, a large number of studies focused on the intracellular actions of MMP-2, that can degrade several newly identified intracellular targets (DeCoux *et al.* 2014). The degradation of myocardial contractile proteins is an early event in myocardial infarction: it may contribute to the induction of proapoptotic signals in cardiomyocytes and thus leads to cell death and contractile dysfunction. Therefore, here we tested the direct cytoprotective effect of ilomastat in a previously established drug screening platform using isolated neonatal rat cardiomyocytes exposed to S/R injury (Gorbe *et al.* 2010). Our results clearly show that ilomastat exerts direct cytoprotective effect via attenuating the intracellularly active MMP-2 activity as proved by *in situ* zymography whereas, gelatinolytic activity was co-localized with MMP-2 immunostaining. Therefore, the presenting results suggest that gelatinolytic activity in the heart is originated solely from MMP-2. Nevertheless, it cannot be excluded that inhibition of other non-gelatinolytic proteases may be involved in the cardiocytoprotective effect of ilomastat.

Moreover, here we further tested the magnitude of MMP-2 inhibition necessary for cardioprotection and found that the cardiocytoprotective dose of ilomastat inhibited MMP-2

activity only by 25%. Our present study suggests that a moderate MMP-2 inhibition is sufficient for cardioprotection. Due to the well-known side effects of MMP inhibitors including tendonitis-like fibromyalgia and musculoskeletal syndrome (Dormán *et al.* 2012), it is of great clinical importance that possibly there is no need for high efficacy MMP inhibitors to protect the heart against ischemia/reperfusion injury.

We further investigated the cardioprotective effect of ilomastat in an *in vivo* rat heart model of ischemia/reperfusion. Ilomastat reduced infarct size dose-dependently when administered either before ischemia or before reperfusion. We have found a different dose range between this two administration protocols. When administered before the onset of ischemia, the effective doses of ilomastat were 0.75 and 1.5 $\mu\text{mol}/\text{kg}$, however, higher doses of ilomastat were not significantly effective. Nevertheless, when ilomastat was administered before the onset of reperfusion, 6 $\mu\text{mol}/\text{kg}$ ilomastat was found to decrease infarct size, and lower doses were ineffective. This is the first demonstration that the cardioprotective dose ranges of ilomastat, when administered before ischemia or before reperfusion, were not overlapping *in vivo*.

Taken together, ilomastat at doses with moderate MMP-2 inhibition protects cardiomyocytes thereby reducing infarct size when administered either before the onset of ischemia or before the onset of reperfusion *in vivo*. Our results show that a moderate MMP-2 inhibition is sufficient for cardioprotection.

5.2 Study 2: the NO donor SNAP has cytoprotective effect on mESC-derived cardiomyocytes

In *study 2* we established an embryonic stem cell-derived cardiac myocyte-based *in vitro* drug screening system and showed that the NO-donor SNAP was protective against SI/R-induced cell death. In contrast to SNAP, the particulate guanylate cyclase stimulator BNP had no effect on cell viability during SI. This is the first demonstration that mESC-derived cardiomyocytes are a useful tool for screening cytoprotective agents and their cytoprotective signaling pathways against simulated ischemia/reoxygenation injury.

Currently used cell-based assays have limitations for screening cardioprotective compound (Xu *et al.* 2002; Walsh *et al.* 2009; Ozsvári *et al.* 2010). Therefore, advantages of ESC-based assays are the well-reproducible production of contracting myocardial cells and that they do not require sacrificing high number of animals. Therefore, here we validated a mESC-derived cardiomyocyte-based drug-screening platform using the NO donor SNAP. SNAP is a well-known cardioprotective compound. In the present study, SNAP showed a

concentration-dependent increase in viability of mESC-derived cardiomyocytes after SI/R insult. This finding indicates that mESC-derived cardiomyocytes are useful tools for testing cardioprotective agents and suggests that NO donors may also be cytoprotective for stem cell-derived cardiomyocytes implanted into ischemic areas of the myocardium. Moreover NO has also been shown to promote cardiac-committed differentiation of mESCs (Kanno *et al.* 2004).

It has been well established that NO donors including SNAP exert protective effect against myocardial ischemia/reperfusion injury via activation of soluble guanylate cyclase and increased cGMP signaling (for a review see Ferdinandy and Schulz 2003). We have recently shown that SNAP induces cytoprotection via the activation of soluble guanylate cyclase in neonatal cardiomyocytes (Gorbe *et al.* 2010). Moreover, the NOS inhibitor L-NNA did not affect cell viability after SI/R injury of mESC-derived cardiomyocytes showing that endogenous NO is not involved in cardiocytprotection. In order to test if activation of particulate guanylate cyclase can increase cell viability similar to SNAP, the effect of BNP was tested. Interestingly, in our present study, cell viability was not influenced by either concentration of BNP in mESC-derived cardiomyocytes. This finding may be due to a low expression of the BNP specific NPR-A receptor during mouse ESC differentiation (Abdelalim *et al.* 2009).

We further identified cardioprotective signaling pathways downstream of cGMP in mESC-derived cardiomyocytes. In the present study, the involvement of PKG in SNAP-induced protection was tested by the PKG inhibitor KT-5823 during SI, which interferes with PKG at the level of the ATP binding site of its catalytic domain. KT-5823 alone did not affect the viability of mESC-derived cardiomyocyte, but interfered with the cytoprotective effect of SNAP, which suggests that the mechanism of SNAP-induced protection involves PKG. Furthermore, here we investigated the involvement of K_{ATP} channels in SNAP-induced cytoprotection in mESC-derived cardiomyocytes. The nonselective K_{ATP} channel inhibitor glibenclamide alone did not affect mESC-derived cardiomyocyte viability, but abolished the cytoprotective effect of SNAP. This is in line with several earlier reports in other systems (Baharvand *et al.* 2006).

Taken together, our present study is the first demonstration that mESC-derived cardiomyocytes exposed to SI/R injury are a useful alternative tool for *in vitro* screening of potential cardioprotective agents and to study their downstream cellular signaling pathways. All the above-mentioned findings emphasize the necessity of detailed analyses of signal transduction pathways in ESC-derived cells both in physiological and pathological conditions

to establish well-reproducible ESC-derived drug screening platforms and to predict the viability of these cells after implantation into an ischemic region of a tissue.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Study 1 is the first demonstration that:

- the cardioprotective effect of ilomastat occurs via the moderate inhibition of MMP-2.
- ilomastat has direct cytoprotective effect via attenuating the intracellularly active MMP-2 activity as proved by *in situ* zymography. This finding was proven by the previously established drug screening platform that is suitable for testing cardioprotective compounds.
- ilomastat reduced infarct size when administered either before the onset of ischemia or before the onset of reperfusion *in vivo*. However, the cardioprotective doses of ilomastat are not overlapped, when administered before ischemia or before reperfusion.

We conclude that MMP-2 inhibition before the onset of reperfusion is a promising target in the treatment of AMI, since it may have high clinical relevance during recanalization therapy via percutaneous coronary intervention. Furthermore, moderate inhibition of MMP-2 might be a useful tool to reduce infarct size and improve clinical outcomes of AMI in patients without the occurrence of severe side effects derived from high efficacy MMP inhibition.

In *Study 2*, we demonstrated for the first time that:

- the NO-donor SNAP is protective against SI/R-induced cell death of mESC-derived cardiomyocytes. Moreover, we proved that the activation of cGMP-PKG signaling cascade is involved in this protection.
- mESC-derived cardiomyocytes exposed to SI/R injury can be a useful tool for predicting the viability of ESC-derived cardiomyocytes after implantation into the ischemic myocardium.
- this mESC-derived cardiomyocyte-based platform can be used for *in vitro* testing of potential cardioprotective drugs and to study their downstream signaling pathways.

In conclusion, stem-cell-derived cardiomyocytes may be a useful source for cardiac repair after AMI. The major limitation that needs to be overcome towards clinical translation is the low survival rate of cells after implantation. Therefore, the improvement of cell replacement therapy is one of the most important aims of our further investigations.

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