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, Belecz 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**
*these authors contributed equally to the work.

II. Görbe A, Varga ZV, Pálóczi J, Rungarunlert S, Klincumhom N, Pirity 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**

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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. Szűcs 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, Szűcs G, Gáspár R, Pálóczi J, Faragó N, Zvara A, Puskás LG, Rázga Z, Tiszlavicz 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

Cumulative impact factor of papers indirectly related to the thesis: 17.519

TABLE OF CONTENTS

1. LIST OF ABBREVIATIONS

AMI	acute myocardial infarction
ANOVA	analysis of variance
BNP	B-type natriuretic peptide
cGMP	cyclic guanosine monophosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
EBs	embryonic bodies
ECG	electrocardiogram
EDTA	ethylene-diamine-tetra-acetic acid
eGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
Glib	glibenclamide
I/R	ischemia/reperfusion
IHD	ischemic heart disease
ILO	ilomastat
K_{ATP}	ATP-sensitive potassium channel
LAD	left anterior descending coronary artery
LIF	leukemia inhibitory factor
L-NNA	N-Nitro-L-arginine
MEFs	mouse embryonic fibroblasts
mESCs	mouse embryonic stem cells
MMP	matrix metalloproteinase
mPTP	mitochondrial permeability transition pore
NEAA	nonessential amino acids
NFκB	nuclear factor kappa-B
Nkx2.5	homeobox protein Nkx2.5
NO	nitic oxide
NOS	nitric oxide synthase
NOS1 (or nNOS)	neural nitric oxide synthase
NOS2 (or iNOS)	inducible nitric oxide synthase
NOS3 (or eNOS)	endothelial nitric oxide synthase
PBS	phosphate buffer solution

PI	propidium iodine
PKG	protein kinase G
pro-MMP2	pro-form of matrix metalloproteinase type 2
RISK	reperfusion injury salvage kinase
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAFE	survivor activating factor enhancement
SEM	standard error of the mean
SI/R	simulated ischemia/reoxygenation
SNAP	s-nitroso-n-acetyl-penicillamine
SR	sarcoplasmic reticulum

2. 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 (according to the European Cardiovascular Disease Statistics, released in 2012) [1]. 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) [2]. AMI leads to massive cardiac cell loss, rapid decline of cardiac contractility, occurrence of lethal arrhythmias, and in the long run to scar formation, 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. In experimental studies, the rate of remodeling is highly affected by the extent of the infarcted area [3]. In humans, infarct size is a major determinant of mortality in myocardial infarction [4]. Therefore, limitation of infarct size by induction of reperfusion is an important therapeutic approach. 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 [5]. Additionally, despite of a decade of intense research on the cellular mechanism of cardioprotective interventions aiming to reduce reperfusion injury, such as ischemic or pharmacological postconditioning, 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 [6–9]. 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.

2.1 Pathophysiology of ischemia/reperfusion injury (I/R)

Ischemia is defined as an inadequate blood flow in the affected tissue or in an organ that is accompanied by the loss of oxygen and nutrients. It is well known that myocardial ischemia results in the impairment of contractile function and causes myocardial damage as a consequence of cell death from both necrosis and apoptosis [10]. Ischemia triggers the accumulation of intracellular sodium, hydrogen, and calcium ions, thereby facilitating tissue acidosis (Fig.1). The decline of pH stimulates Na^+-H^+ exchange and Na^+-HCO_3^- transporter, which leads to intracellular sodium accumulation and development of intracellular Ca^{2+} overload [11].

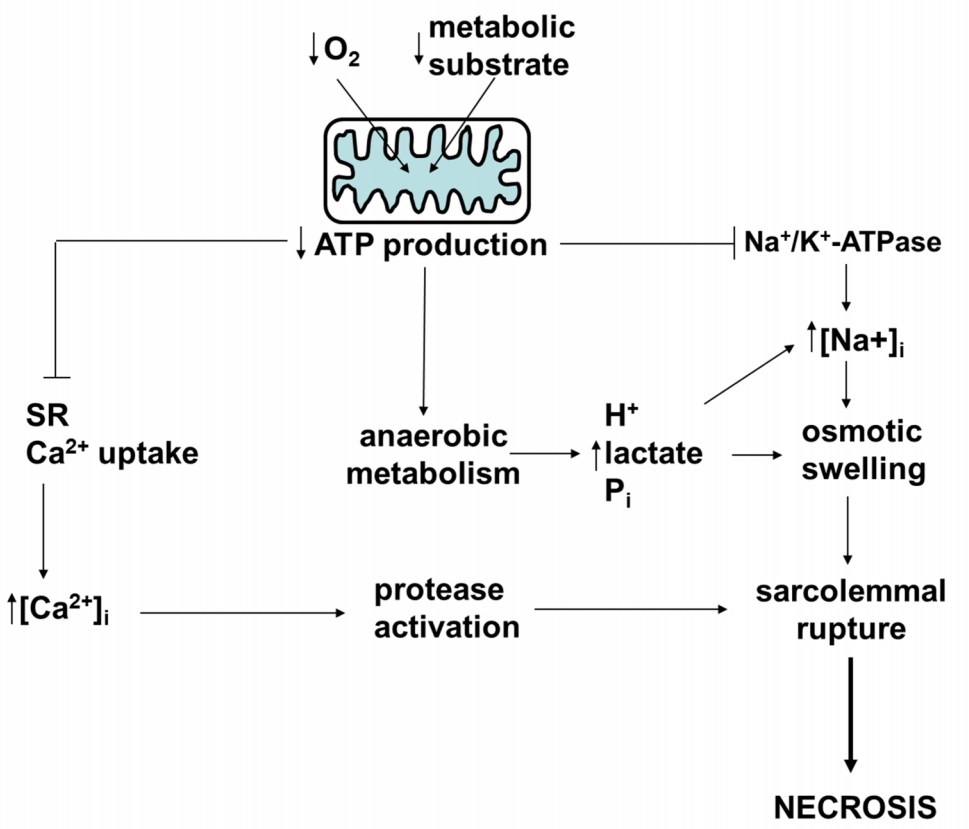

Fig.1: Ischemic injury of myocardium
(modified from Ferdinandy *et al.,* Pharmacol Rev. 2007).

Due to the impaired calcium handling, numerous intracellular enzymes are activated including proteases and endonucleases, which are involved in proapoptotic signaling [12], activating plasma membrane phospholipase A2, which leads to the formation of arachidonic acid derivatives (e.g. leukotriene B4, thromboxane A2). This event then facilitates neutrophil infiltration and perpetuates the generation of reactive oxygen radicals via stimulation of neutrophil oxidative burst. In addition, the absence of oxygen causes cellular ATP-depletion

that leads to mitochondrial dysfunction and initiates the translocation of Bax, a proapoptotic Bcl2 family member protein, from the cytosol to the outer mitochondrial membrane. This triggers mitochondrial swelling and induces the efflux of cytochrome c via opening of the permeability transition pore (mPTP) into the cytosol [13]. The intracellularly localized cytochrome c activates effector caspases and initiates apoptosis. The impairment of the mitochondrial electron transport chain stimulates the generation of ROS and development of oxidative stress in the ischemic heart. ROS react directly with cellular lipids, proteins and DNA exacerbating cell injury/death and leading to the activation of nuclear factor kappa-B (NFκB) [14]. Moreover, the excess of ROS impairs the antioxidant capacity of cell by depleting reduced glutathione. Additionally, it has been also reported that intracellular ROS (particularly peroxynitrite) are implicated with the activation of matrix metalloproteinases (MMPs), thereby playing a pivotal role in the progression of ischemic injury by contributing to the degradation of several extracellular matrix protein [15].

Although the re-establishment of adequate oxygen and nutrients supply is able to limit the extent of the infarction, restoration of normal blood flow results in a complex cascade of inflammation, sudden oxygen surplus and oxidative stress (Fig.2).

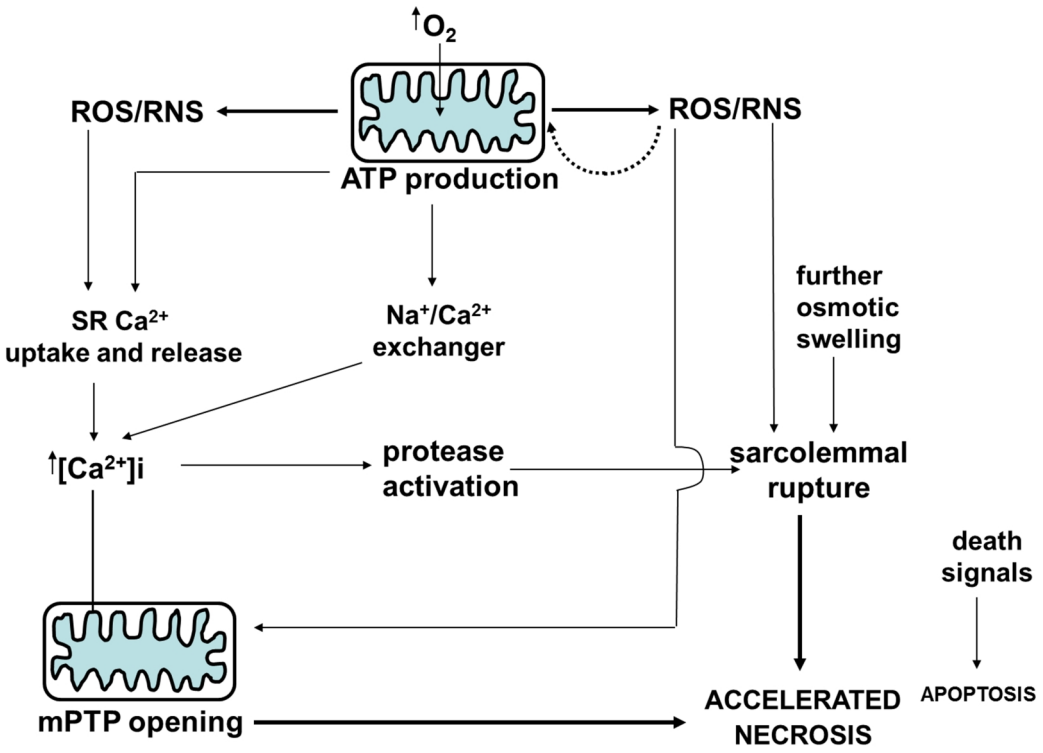

Fig.2: Reperfusion injury

(modified from Ferdinandy *et al*., Pharmacol Rev. 2007).

Increasing evidence points to the fact that reperfusion injury can lead to a rapid rising of cell necrosis and apoptosis. At reperfusion; endothelial cells up regulate the expression of adhesive proteins and release leukocyte attractants. Activation and accumulation of leukocytes (primarily neutrophils and monocytes) triggers multiple mediator cascades leading to cytokine release, increased vascular permeability, generation of ROS and liberation of proteolytic enzymes from granulocytes [10].

Additionally, during ischemia, the hydrolysis of ATP to AMP leads to the accumulation of hypoxanthine. Increased intracellular calcium enhances the conversion of xanthine dehydrogenase to xanthine oxidase which may produce superoxide and xanthine from the accumulated hypoxanthine upon reintroduction of oxygen. This leads to further oxidative stress that can jeopardize cellular function and survival [16].

2.2 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 [17]. 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, MMPs are activated by the removal of the autoinhibitory propeptide domain resulting in an active truncated MMP. They are involved in the remodeling of extracellular matrix during various physiological processes including embryonic development [18], angiogenesis [19], wound healing and tissue repair [20]. On the other hand, MMPs have been shown to play a crucial role in several pathologies such as metastatic cancers [21], arthritis [22], inflammation [23] and diabetes [24]. Moreover, gelatinase types of MMPs, (MMP-2 and MMP-9) are implicated with numerous cardiovascular diseases such as ischemia/reperfusion injury [25,26]. It has been previously reported that MMP-2 is constitutively expressed in cardiomyocytes [27] 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 ref. 28). 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 [26]. Peroxynitrite has been shown to activate pro-MMP-2 via the redox modification of the regulatory site of this enzyme [29,30]. This redox

modification leads to the undesired activation of 75 and 72 kDa isoforms of MMP-2, besides that only the 64 kDa isoform is active once MMP-2 is activated in the classical manner [31,32]. In addition, active MMP-2 is released from necrotic cells thereby exacerbating the outcome of ischemic injury [15]. Hence, the activation of MMP-2 during an ischemic insult is associated with decreased functional recovery and larger infarct size of the heart (for review see ref. 33).

We have previously reported that MMP-2 activity was moderately decreased during ischemic preconditioning [34] and that exogenous inhibition of MMPs by ilomastat, a non-selective MMP inhibitor diminished ischemia-induced MMP-2 activity in isolated rat hearts [35]. 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 [36]. Moreover, we and others have shown that the non-selective MMP inhibitor ilomastat reduced infarct size in rats and mice [36,37]. Taken together, MMP-2 became a major target for drug development in acute cardiovascular pathologies including AMI [38,39]. 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-iduced cardioprotection occurs via the inhibition of the intracellularly active MMP-2.

2.3 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 and facilitate rapid recanalization of the occluded coronary artery after myocardial infarction to limit infarct size (for review see ref. 40). 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 such as fetal or neonatal cardiomyocytes, skeletal muscle myoblasts, mesenchymal stem cells, hematopoietic stem cells, adult cardiac resident stem cells, embryonic stem cell-derived cardiomyocytes have been used in preclinical studies for cell replacement therapy following myocardial infarction (for review see ref. 41). Embryonic stem cells might be a promising cell source, because of their capability to provide unlimited expansion in undifferentiated state and

their ability to undergo inducible differentiation into cardiomyocytes in vitro [6–9]. However, applications of embryonic stem cell-derived cardiomyocytes have ethical considerations; therefore, better potential of these cardiomyocytes is testing cardioprotective compounds *in vitro*. Moreover, a recently discovered technique [42] gives new insight into stem cell biology: reprogramming of adult somatic cells into pluripotent stem cell lines (generation of induced pluripotent stem cells) and their differentiation into functional cardiomyocytes provides new perspectives in personalized cell replacement therapy (for review see ref. 43 and ref. 44).

Despite the increasing number of promising results [7,8,45], several complications need to be overcome towards clinical translation including the precise characterization of stem cell lines, such as the development of efficient protocols for proper cardiomyocyte differentiation, the establishment of strategies for cardiomyocyte selection, the development of scaling-up procedures to provide clinically-relevant number of cardiomyocytes, and the development of cell delivery strategies [43,44,46,47]. Another limitation regarding their clinical utilization is that the survival of implanted cells is reduced after transplantation [6,8]. Implanted cells undergo a significant cell death within the first 24 hours [48]. A plausible reason for this effect is the unfavorable microenvironment for the grafted cells when implanted 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 [49,50]. Furthermore, characterization of these cells and their hypoxic tolerance in a simulated ischemia/reoxygenation test system would be important, since little is known about the ischemic tolerance of stem cell-derived cardiomyocytes.

2.4 Role of nitric-oxide

Nitric oxide (NO) is a gaseous substance that regulates a wide range of biological processes. It typically exerts its physiological functions by binding directly to ferrous iron of heme proteins or indirectly by S-nitrosylation of protein thiol groups [51]. NO is synthesized at low physiological levels by NO synthase (NOS) enzymes. In this reaction, L-arginine is oxidized with molecular oxygen to form L-citrulline and NO [52]. Three main isoforms of NOS are widely present and named according to the tissues where they were first identified or to their mode of expression: neural NOS (nNOS or NOS1) is expressed mainly in neurons; inducible NOS (iNOS or NOS2), the NOS isoform with the highest rate of NO synthesis expressed in cell types of the immune system in response to infection; and endothelial NOS

(eNOS or NOS3) is presented in the vascular endothelium. 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 including sarco/endoplasmic reticulum Ca^{2+}-ATPase and several mitochondrial proteins (for review see ref. 51,53–57). 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 [58]. 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 [59,58,60]. Moreover, our previous findings also suggest that the present screening platform is a useful tool for discovery of cardiocytoprotective 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.

3. AIMS

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.

Study 2

The aim of the second study 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.

4. 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.

4.1 Experimental design of Study 1

4.1.1 Animals

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.

4.1.2 Experimental design of *in vitro* studies

4.1.2.1 Inhibition of gelatinase activity by ilomastat

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 by Kupai *et al.* [31]. Briefly, 8% polyacrylamide gels were copolymerized with gelatin (2 mg/mL, type A from porcine skin, Sigma-Aldrich, Budapest, Hungary), and 50 μg of protein per lane were loaded. After electrophoresis (90 V, 1.5 h), gels were washed with renaturation buffer (Bio-Rad, Hercules, CA; containing 2.5% Triton X-100) for 3×15 min then incubated in development buffer (Bio-Rad, Hercules, CA) for 20 min to eliminate Triton-X-100. Gels were sliced according to the lanes and the slices were incubated separately for 20 h at 37°C (pH 7.4) in development buffer in the presence of vehicle and/or different concentrations of ilomastat (0.5 nM and 5.0 nM). Recombinant, human MMP-2 was used as positive control. Gels were then stained with 0.05% Coomassie brilliant blue (G-250, Sigma-Aldrich,

Budapest, Hungary). 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.

4.1.2.2 Simulated ischemia/reoxygenation in cardiomyocytes

Neonatal rat cardiomyocytes were cultured in 48-well plates. Neonatal cardiomyocytes were chosen for this study, as they can be harvested on culture dishes without coating. The lack of coating is important in the present study, as coating materials (e.g. laminin, collagen, etc.) could influence the action of matrix metalloproteinases [61,62]. Cell isolation, culturing method and the SI/R drug screening platform were described before in detail [58,63]. Briefly, neonatal rats were sacrificed by cervical dislocation and hearts were placed into ice cold PBS solution. Ventricles were digested in 0.25% trypsin (Invitrogen, Life Technologies Hungary, Budapest, Hungary) solution and cell suspension was centrifuged at 400×g, for 15 min at 4°C. Cell pellet was re-suspended in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Budapest, Hungary) supplemented with L-glutamine (Sigma-Aldrich, Budapest, Hungary), Antibiotic-antimycotic solution (Sigma-Aldrich, Budapest, Hungary) and 10% fetal bovine serum (FBS, Gibco, Life Technologies Hungary Ltd, Budapest, Hungary). Cells were counted in a hemocytometer, and seeded into 48-well plates at a density of 5×10^4 cell/well. After 24 hours, the growth medium was replaced with differentiation medium containing 1% fetal bovine serum. Cardiomyocytes were kept under normoxic conditions (95% air and 5% CO_2 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 [58]. In the simulated ischemic group, the medium of the cultures were replaced with a hypoxic solution [58,64] and plates were kept in a hypoxic chamber (gassed with 95% N_2 and 5% CO_2 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% DMSO. During reoxygenation, cells were covered with differentiation medium (containing 1% fetal bovine serum) and kept in a normoxic incubator for 120 min (Fig.3).

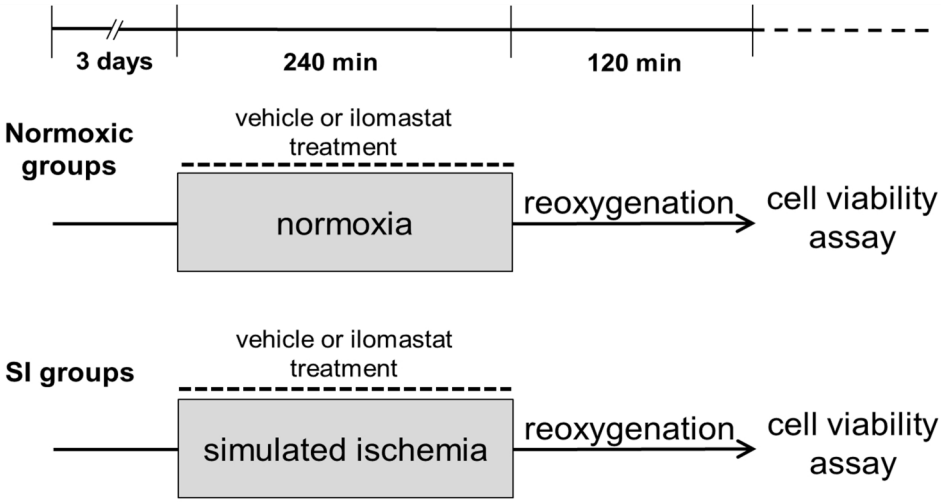

Fig.3: Experimental protocol for testing the direct cardioprotective effect of ilomastat in isolated neonatal rat cardiomyocytes exposed to SI/R injury.

4.1.2.3 Cell viability assay

Cell viability was assessed by a calcein and propidium iodine (PI) assay performed in each group after 2 h simulated reperfusion. Briefly, the growth medium was removed; cells were washed with PBS twice and incubated with calcein (1 μM) for 30 min. Then calcein solution was replaced with fresh PBS and fluorescence intensity of each well was detected by fluorescent plate reader (FluoStar Optima, BMG Labtech, Auro-Science Consulting Ltd, Budapest, Hungary; scan matrix: 10×10; scan diameter: 10 mm; bottom optic; number of flashes/scan point: 3; temp: 37°C; excitation wavelength: 488 nm; emission wavelength: 520 nm). Then to assess total cell count in each well, PI (50 μM) and digitonin (100 μM) (Sigma-Aldrich, St. Louis, MO) were dissolved in PBS and cells were incubated in this solution for 7 minutes. Then PI solution was replaced with fresh PBS and fluorescent intensity was detected with same settings (excitation wavelength: 544 nm; emission wavelength: 610 nm). Calcein data were normalized to total cell count obtained by PI+ digitonin treatment.

4.1.2.4 *In situ* zymography and MMP-2 co-localization

To detect *in situ* MMP-2 activity [65], neonatal rat cardiomyocytes were cultured in 24-well tissue culture plate at the density of 10^5 cells/well for 3 days. The medium of cells was replaced with hypoxic solution supplemented with DQ™ 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 DQ™ gelatin and cells were placed into a normoxic incubator for 120 min. Finally cells were washed with PBS, and fixed in 3.7% paraformaldehyde dissolved in PBS for 15 min. MMP-2 fluorescent immunostaining using anti-proMMP-2 antibody (Chemicon, MAB3308; Merck Ltd, Budapest, Hungary; secondary antibody: rhodamine-labeled goat anti-mouse antibody; Abcam, AB5885, Cambridge, UK) was assessed 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). After the subsequent washing steps, cells were covered with fluorescent mounting medium (Dako, Frank Diagnosztika Ltd, Budapest, Hungary), and fluorescence was detected with 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 fields selected randomly on each coverslip. Four coverslips were analyzed in each group. The number, total area, and area fraction of fluorescent signal, and the analyses of co-localization were quantified on images by ImageJ 1.45 software (National Institutes of Health, Bethesda, MD).

4.1.3 Experimental design of *in vivo* studies

4.1.3.1 Surgical procedure of coronary occlusion

Rats were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital (Euthasol, Produlab Pharma b.v., Raamsdonksveer, The Netherlands). Animals were then intubated and mechanically ventilated (Model 683, Harvard Apparatus, Holliston, MA) with room air in a volume of 6.2 mL/kg and a frequency of 55±5 breath/min according to their body weight. Rats were placed in supine position on a heating pad to maintain body core temperature in physiological range (37.0°C±1.0°C). Right carotid artery was cannulated to measure mean arterial blood pressure by a pressure transducer (Experimetria Inc., Budapest, Hungary). Mean arterial blood pressure and electrocardiogram (ECG) was continuously monitored throughout the experiments (Haemosys, Experimetria Inc., Budapest, Hungary).

Right jugular vein was also cannulated for fluid substitution and drug administration. Left anterior descending coronary artery (LAD) occlusion was induced by left thoracotomy. A 5-0 Prolene suture (Ethicon, Johnson & Johnson, Budapest, Hungary) was placed around LAD artery and a small plastic knob, which was threaded through the ligature and placed onto the heart, was used for making occlusion 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.

4.1.3.2 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. Dimethyl sulfoxide (DMSO), 11.6 w/v% solution diluted with physiological saline) as vehicle or 0.3, 0.75, 1.5, and 3.0 μmol/kg ilomastat were administered intravenously in slow bolus 5 minutes before ischemia (Fig.4, shaded arrow). To maintain serum level of ilomastat, additional 3 boluses of vehicle (5.8% DMSO solution) or ilomastat with half dose (0.15, 0.375, 0.75; and 1.5 μmol/kg, respectively), were given at the 10th, 25th min of ischemia and at the 10th min of reperfusion (Fig.4, open arrows). We estimated the maintaining doses of ilomastat according to its half-life based on pharmacokinetic data described previously in rodents after a single intravenous bolus injection [66].

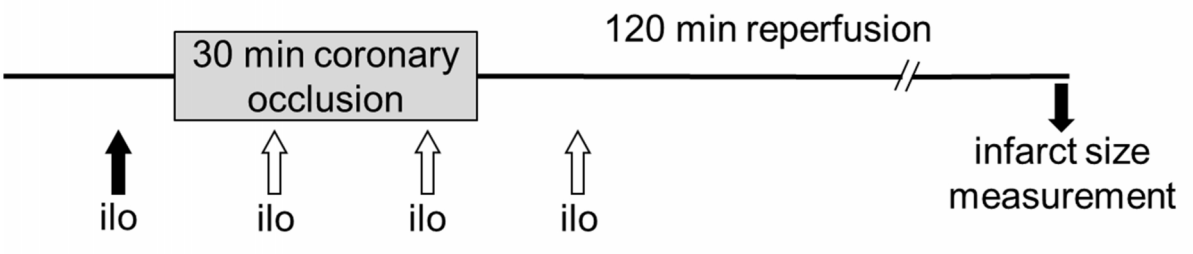

Fig.4: *In vivo* experimental protocol for testing the cardioprotective effect of ilomastat when administered before ischemia. Shaded arrow indicates the loading dose of ilomastat, whereas open arrows show the maintaining doses.

In the second series of *in vivo* experiments DMSO (11.6 m/v% solution) or ilomastat (0.75, 1.5, 3.0, and 6.0 μmol/kg) bolus was injected at the 25th min of ischemia (Fig.5). Maintaining boluses (5.8% DMSO or 0.375, 0.75, 1.5, and 3.0 μmol/kg, respectively) were administered at the 10th, 25th and 40th min of reperfusion (Fig.5) to maintain constant ilomastat concentration in blood during the early phase of reperfusion.

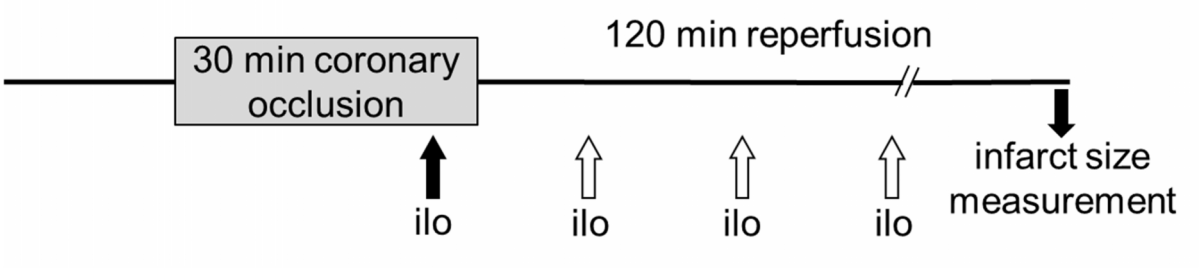

Fig.5: *In vivo* experimental protocol for testing the cardioprotective effect of ilomastat when administered before reperfusion. Shaded arrow indicates the loading dose of ilomastat, whereas open arrows show the maintaining doses.

4.1.3.3 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 [67] to remove blood from the coronary vessels. After 5 min of perfusion, risk area was re-occluded, and hearts were perfused with 4 mL of 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, [68].

4.1.4 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.

4.2 Experimental design of Study 2

4.2.1 Mouse Embryonic Stem Cell Culture

Undifferentiated mouse embryonic stem cells (mESCs) (Nkx2.5/EGFP transgenic C57BL/6 mouse ES cell line; $Tg^{Nkx2.5/EGFP}$ C57BL/6; passages 10-12) were cultured on feeder layers of mitomycin C-inactivated mouse embryonic fibroblasts (MEFs) which were obtained from 13.5 days postcoitus mouse embryos as described earlier [69]. mESCs were maintained in ES medium (DMEM) supplemented with 15% (v/v) FBS (Sera Laboratories International, West Sussex, RH17 5PB, UK), 1000 U/mL mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon International, Budapest, Hungary), 0.1 mM nonessential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME) and 50 U/mL penicillin / 50 µg/mL streptomycin. mESCs were cultured on feeder layers for at least two passages after thawing and subsequently were cultured without feeder cells on 0.1 % gelatin-coated tissue culture plates in the presence of LIF (2000 U/mL). Medium was changed daily for standards maintenance. mESCs were usually passaged every 1-2 days prior reaching 70% confluences.

4.2.2 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 [70]; Briefly, mESCs were resuspended at the density of $4x10^4$ cells/mL in differentiation medium (regular DMEM without LIF) resulting in 800 cells/drop. 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 simulated ischemia/reoxygenation experiments. At this stage, the ratio of Nkx2.5-eGFP positive cells, an early marker for cardiac differentiation, exhibited 52.5±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).

4.2.3 Experimental Groups

For cell SI/R experiments, mESC-derived cardiomyocytes were tested under normoxic condition or were subjected to SI (Fig.6).

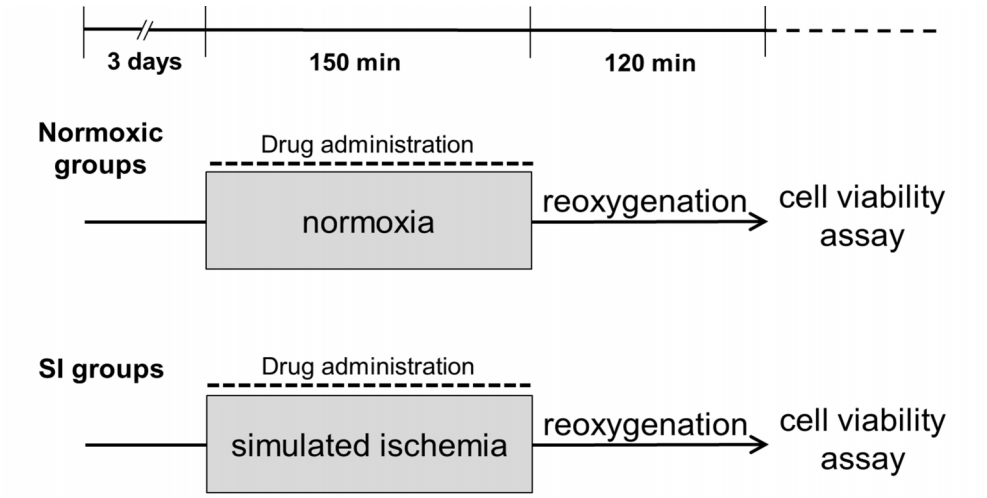

Fig.6: Experimental protocol for testing cardioprotective compounds in a stem-cell-based a drug screening.

The normoxic mESC-derived cardiomyocytes were kept under normoxic conditions; the growth medium was changed to a normoxic solution [58,64] and the cells were incubated under 95% air and 5% CO_2 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 [58,64] and placing the plates in a humidified 37°C hypoxic chamber exposed to a constant flow of a mixture of 95% N_2 and 5% CO_2 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) [58]; (**3**) selective PKG inhibitor KT-5823 (60 nM, an effective concentration that does not affect cell viability alone; Sigma, St. Louis, MO) [58]; (**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) [71]; (**6**) the nitric oxide synthase (NOS) inhibitor N-Nitro-L-arginine (L-NNA; 100 µM, 10 µM; Sigma, St. Louis, MO) [72]; (**7**) the non-selective K_{ATP} channel inhibitor glibenclamide (1 µM, an effective K_{ATP} blocking concentration that does not affect ischemia/reperfuion injury alone; Sigma, St. Louis, MO) [73]; (**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% CO_2 at 37 °C.

4.2.4 Cell Viability Assay

Cell viability was assessed by a PI assay performed in each group after 120 min reoxygenation. PI was chosen, as it stains cells with severely impaired membrane integrity and it does not necessitate dissociation of the cells. Briefly, the growth medium was removed, cells were washed with PBS twice and incubated with PI (50 μM; Sigma, St. Louis, MO) for 7 minutes. Each experiment included a digitonin (10 μM; Sigma, St. Louis, MO) treated positive control well and PI control (mESC-derived cardiomyocytes without treatment and stained for PI for 7 min) (Fig.14). Then PI solution was replaced with fresh PBS and fluorescence intensity of each EB was detected by fluorescent plate reader (FluoStar Optima, BMG Labtech). Fluorescence intensity was measured in well scanning mode (scan matrix: 10x10; scan diameter: 10 mm; bottom optic; number of flashes/scan point: 3; temp: 37°C; excitation wavelength: 544 nm; emission wavelength: 610 nm). PI intensity reflecting the cell death was evaluated on a standard area (21 scan box) in each well placed to the center of EB. The cardiomyocyte-rich region can be found predominantly near the edge of the embryonic body. Therefore, the evaluation of cardiac myocyte rich regions was performed manually on several plates by detecting eGFP expression driven by the promoter of the early cardiac myocyte marker Nkx2.5. The ratio of cardiac myocyte death was the same as the ratio of cell death of all cells found in the embryonic body. Background fluorescence intensity (dye control) was subtracted from the fluorescence intensity of each well after PI staining, and the average intensity of each group was plotted. The cytoprotective effect of different compounds was compared to simulated ischemic control groups.

4.2.5 Statistical Analysis

Results are expressed as mean ± 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 $p<0.05$.

5. RESULTS

5.1 Results of Study 1

5.1.1 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 (Fig.7).

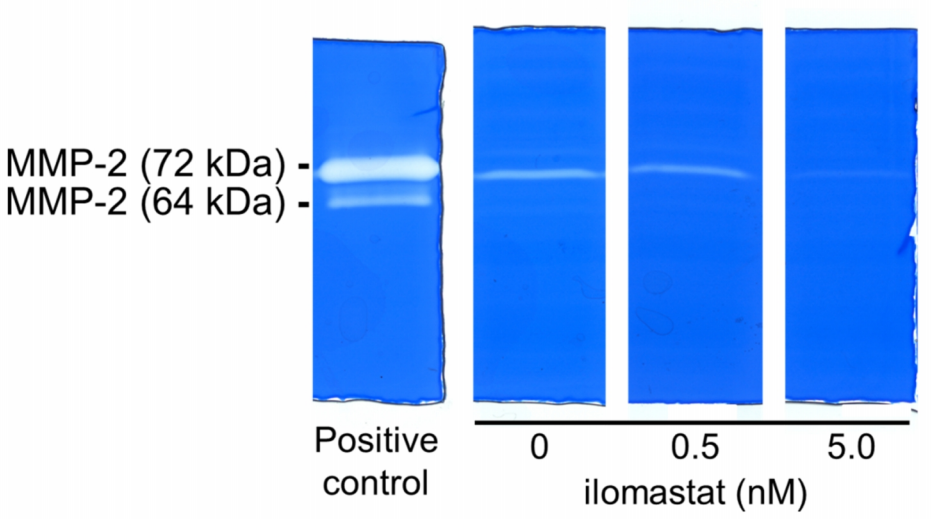

Fig.7: Representative images of MMP-2 activity in rat cardiac tissue homogenate in the presence of 0; 0.5 and 5.0 nM ilomastat.

5.1.2 Effect of ilomastat on cardiomyocytes injured by ischemia/reoxygenation

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 subjected to normoxia or SI/R (Fig.3). Ilomastat at a concentration range of 0.5 nM up to 5 µM did not influence cell viability in normoxic conditions (Table 1). However, ilomastat at 500 nM and 5 µM significantly increased cell viability as compared to vehicle treated group (from 8.6±0.3 to 9.8±0.4 and 9.7±0.2,

respectively, expressed in arbitrary units of fluorescent intensity) in neonatal rat cardiomyocytes exposed to SI/R injury (Fig.8).

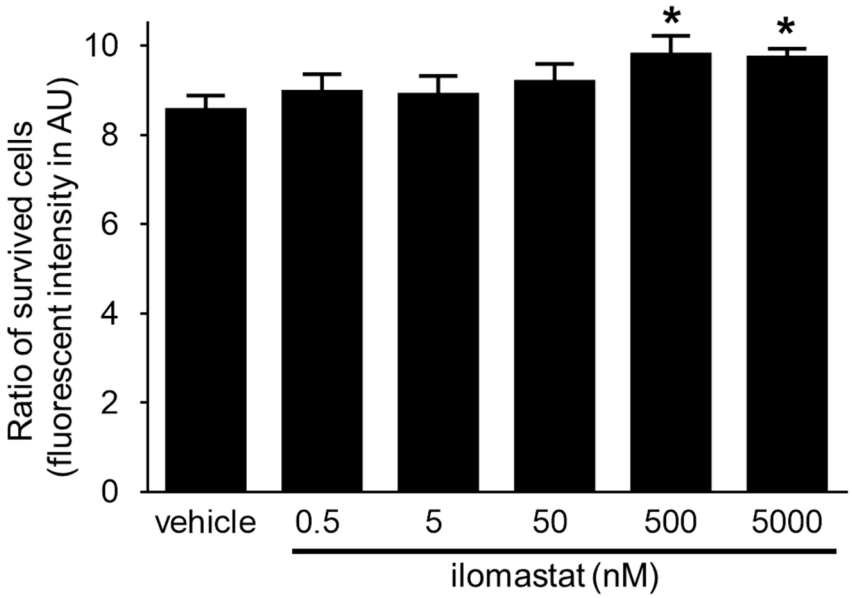

Fig.8: Effect of ilomastat on cell viability of neonatal rat cardiomyocytes after SI/R.
Data are expressed as mean ± SEM; n=6; one-way ANOVA followed by Fischer LSD posthoc test; *p<0.05 compared to vehicle treated group.

Table 1: Effect of ilomastat on cell viability of neonatal rat cardiomyocytes after 240 min normoxia

Cell viability	**(% of vehicle control)**
vehicle	100±4
ilo 0.5 nM	102±6
ilo 5 nM	92±5
ilo 50 nM	104±10
ilo 500 nM	105±9
ilo 5 µM	97±8.3
medium control	115±7

Table 1: Effect of ilomastat on cell viability of neonatal rat cardiomyocytes after 240 min normoxia (suitable for the hypoxic period) followed by 120 min reoxygenation. Data are expressed as mean±SEM; n=5-6; one-way ANOVA followed by Fischer LSD posthoc test.

5.1.3 *In situ* MMP-2 inhibition by ilomastat in cardiomyocytes injured by ischemia/reoxygenation

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 (Fig.9).

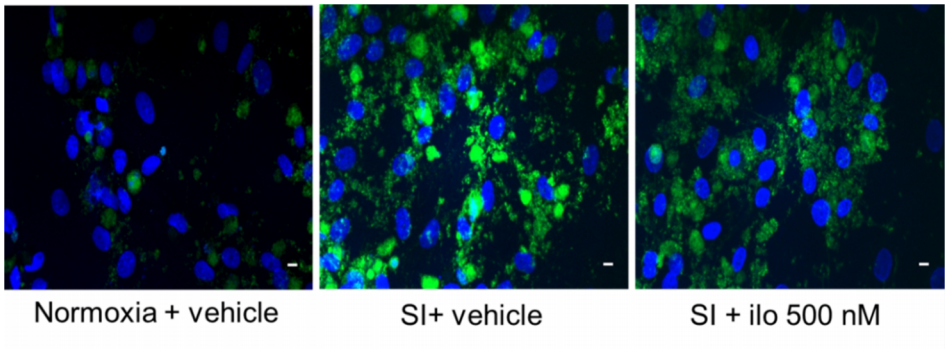

Fig.9: Representative fluorescent confocal images from neonatal rat cardiomyocytes subjected to normoxia, SI, or SI in the presence of 500 nM ilomastat. Green color represents MMP-2 activity as measured by *in situ* zymography FITC signal; blue color shows the nuclei of the cells. Scale bars = 20 µm.

SI/R injury increased gelatinolytic activity significantly from its control value of 0.48±0.04% to 0.93±0.05% of area fraction. The cardiocytoprotective concentration of ilomastat (500 nM, see Fig.8) moderately inhibited the *in situ* gelatinolytic activity approximately by 25%, i.e. from 0.93±0.05% to 0.70±0.04% of area fraction, during SI/R (Fig.10).

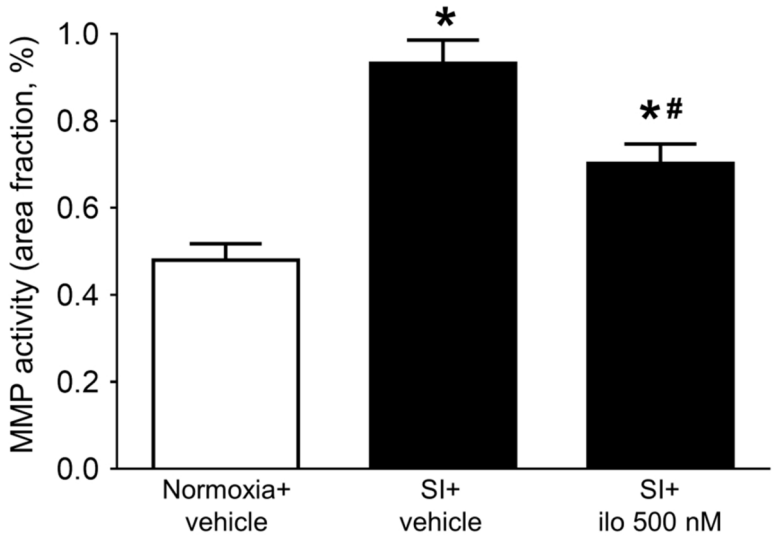

Fig.10: Effect of ilomastat on intracellular gelatinolytic activity in neonatal rat cardiomyocytes after SI/R. Data are expressed as mean ± SEM; n=6; one-way ANOVA followed by Fischer LSD posthoc test; *p<0.001 compared to normoxic vehicle-treated group; #p<0.01 compared to simulated ischemia/reperfusion, vehicle-treated group,

Moreover, we performed separate experiments to show co-localization of MMP-2 with the fluorescent gelatinolytic signal in isolated neonatal rat cardiomyocytes exposed to SI/R injury. MMP-2 showed over 90% co-localization rate with gelatinolytic activity in all groups (Fig.11).

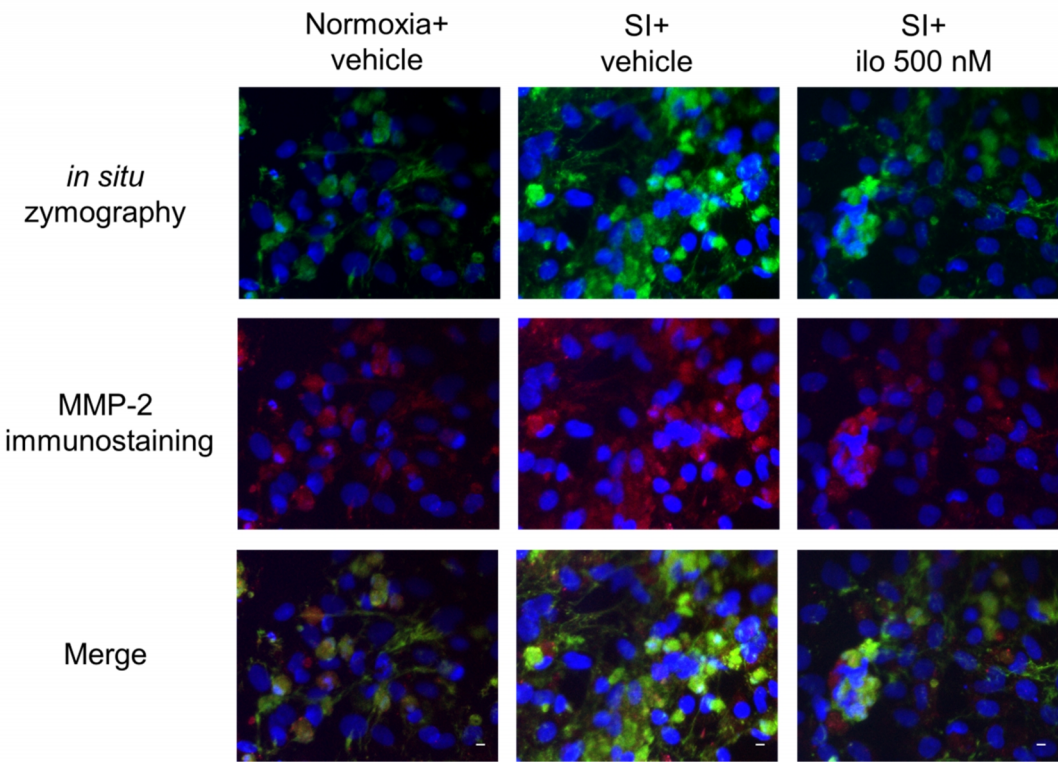

Fig.11: Representative confocal images of cultured neonatal rat cardiomyocytes exposed to normoxia or SI in the presence or absence of ilomastat (500 nM). Green fluorescence: fluorescent signal of gelatin substrate after proteolytic cleavage. Red fluorescence: MMP-2 immunostaining, Blue fluorescence: cell nuclei. Scale bars = 20 μm

5.1.4 Effect of ilomastat on infarct size *in vivo*

The cardioprotective effect of ilomastat administered before the onset of ischemia (Fig.4) or before the onset of reperfusion (Fig.5) 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 μmol/kg doses reduced infarct size significantly as compared to vehicle-treated group (from 66.1±4.6% to 45.3±7.0% and 46.7±5.5% of area at risk, respectively) showing a U-shaped dose-response relationship (Fig.12). When administered before the onset of reperfusion, ilomastat at 6.0 μmol/kg reduced infarct size significantly (from 65.4±2.5% to 52.8±3.7% of area at risk), however, lower doses were

ineffective (Fig.13). There were no significant differences in the area at risk among the groups (data not shown). There were no significant differences in the mean arterial blood pressure and heart rate among the groups (Table 2 and Table 3) as well.

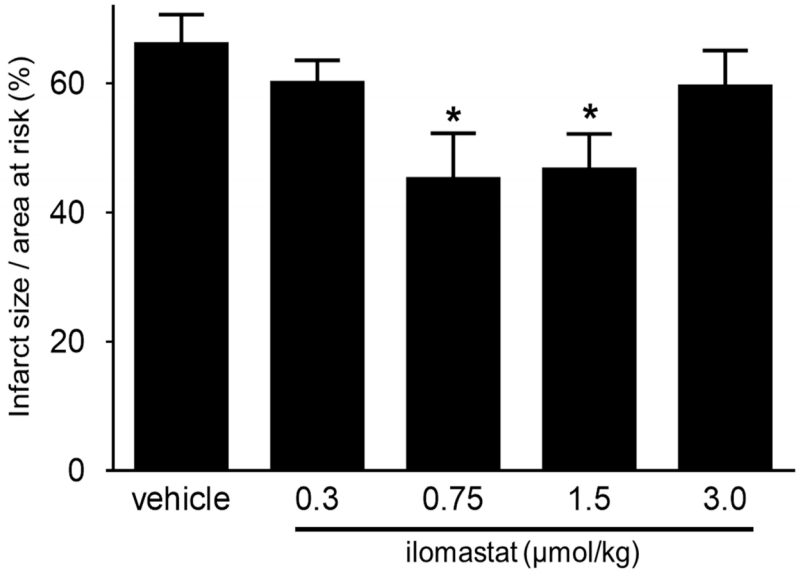

Fig.12: Effect of ilomastat treatment on infarct size when administered before ischemia.
Data are shown as mean ± SEM; n=7-8; one-way ANOVA followed by Fischer LSD posthoc test; *p<0.05 compared to vehicle-treated group.

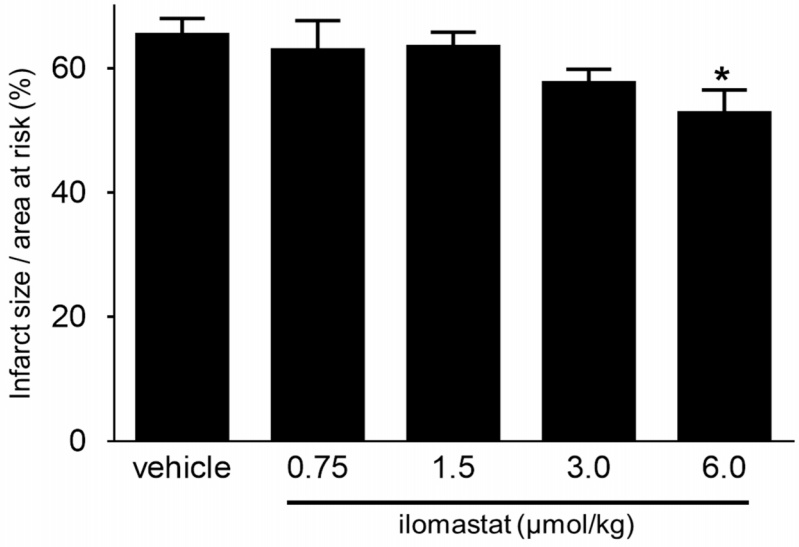

Fig.13: Effect of ilomastat treatment on infarct size when administered before reperfusion.
Data are shown as mean ± SEM; n=7-8; one-way ANOVA followed by Fischer LSD posthoc test; *p<0.05 compared to vehicle-treated group.

Table 2: Effects of ilomastat treatment on mean arterial blood pressure and heart rate when administered before the onset of ischemia.

Mean arterial blood pressure (mmHg)	***Baseline***	***30th min of ischemia***	***120th min of reperfusion***
vehicle	121.1±6.7	82.4±6.6	73.1±1.7
ilo 0.3 µmol/kg	118.6±4.4	76.5±6.1	73.6±4.8
ilo 0.75 µmol/kg	117.5±2,3	90.5±3.0	77.6±7.1
ilo 1.5 µmol/kg	118.1±5.9	75.9±3.7	58.3±5.3
ilo 3.0 µmol/kg	120.0±4.7	80.5±8.5	71.2±6.0
Heart Rate (beats/min)			
vehicle	408±7	392±16	373±9
ilo 0.3 µmol/kg	398±10	374±21	378±18
ilo 0.75 µmol/kg	441±20	422±11	390±16
ilo 1.5 µmol/kg	400±14	388±24	410±15
ilo 3.0 µmol/kg	449±20	400±17	401±27

Table 2: Effects of ilomastat treatment on mean arterial blood pressure and heart rate when administered before the onset of ischemia. Data are expressed as mean ± SEM; n=7-8; one-way ANOVA followed by Fischer LSD posthoc test.

Table 3: Effects of ilomastat treatment on mean arterial blood pressure and heart rate when administered before the onset of reperfusion

Mean arterial blood pressure (mmHg)	***Baseline***	***30th min of ischemia***	***120th min of reperfusion***
vehicle	114.1±4.3	87.3±5.1	77.7±5.2
ilo 0.75 µmol/kg	127.5±3.6	91.3±7.4	83.1±5.3
ilo 1.5 µmol/kg	111.8±5.4	86.8±6.5	81.3±5.3
ilo 3.0 µmol/kg	112.6±4.3	97.4±6.7	74.4±6.8
ilo 6.0 µmol/kg	104.1±6.7	80.2±9.0	83.6±6.3

Heart Rate (beats/min)			
vehicle	408±14	424±10	406±11
ilo 0.75 µmol/kg	429±15	424±14	392±15
ilo 1.5 µmol/kg	402±15	387±10	396±11
ilo 3.0 µmol/kg	423±15	412±14	392±15
ilo 6.0 µmol/kg	444±13	417±17	411±13

Table 3: Effects of ilomastat treatment on mean arterial blood pressure and heart rate when administered before the onset of reperfusion. Data are expressed as mean ± SEM; n=10-18; one-way ANOVA followed by Fischer LSD posthoc test.

5.2 Results of Study 2

5.2.1 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 (Fig.14).

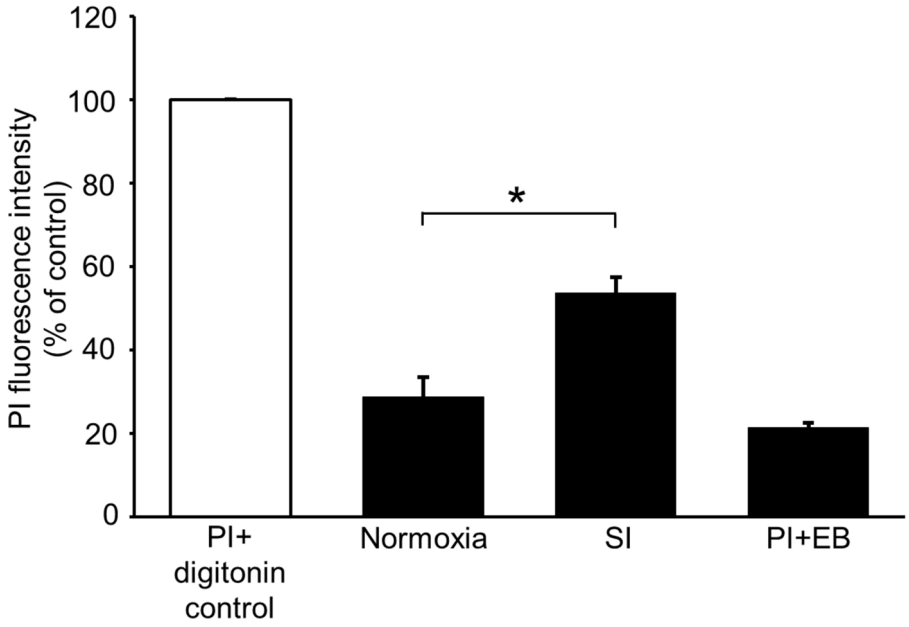

Fig.14: Cell viability of mESC-derived cardiomyocytes subjected to normoxia or SI. Background fluorescence intensity is represented by using non-treated EB + PI in three wells. PI+ digitonin control alone was applied in one well. Data are expressed as mean ± SEM; n=5-6; unpaired t-test; * $p<0.05$ compared to normoxic group

Simulated ischemia killed roughly 20-40% of cells in embryonic body. The cytoprotective action 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, *vs.* vehicle control, $p<0.05$) when applied during SI period (Fig.15). 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 (Fig.16).

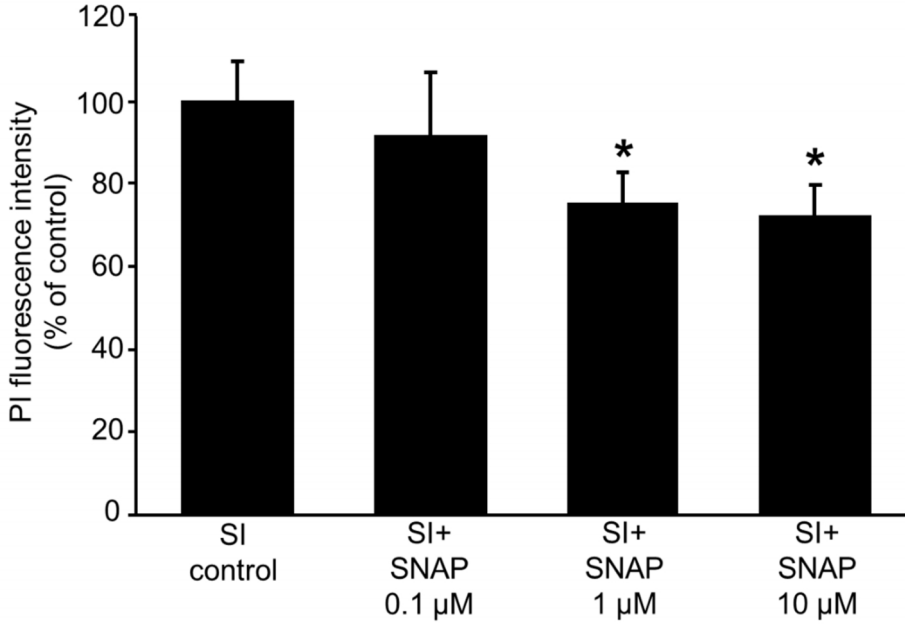

Fig.15: Effect of SNAP on cell viability of mESC-derived cardiomyocytes.
Data are expressed as mean ± SEM; n=10-12; one-way ANOVA followed by Fischer LSD posthoc test;
* $p<0.05$ compared to SI control.

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 (Fig.17).

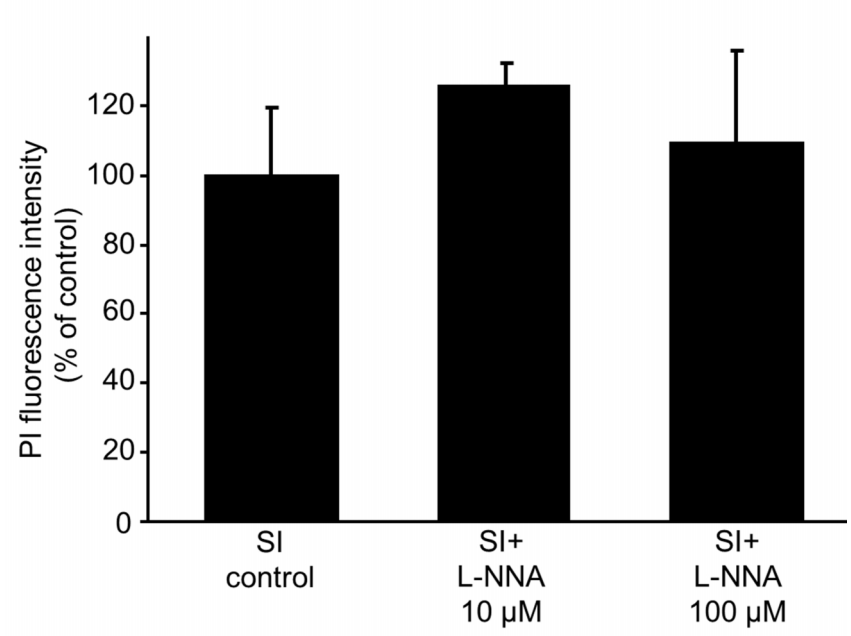

Fig.16: Effect of L-NNA on cell viability of mESC-derived cardiomyocytes.
Data are expressed as mean ± SEM; n=10-12

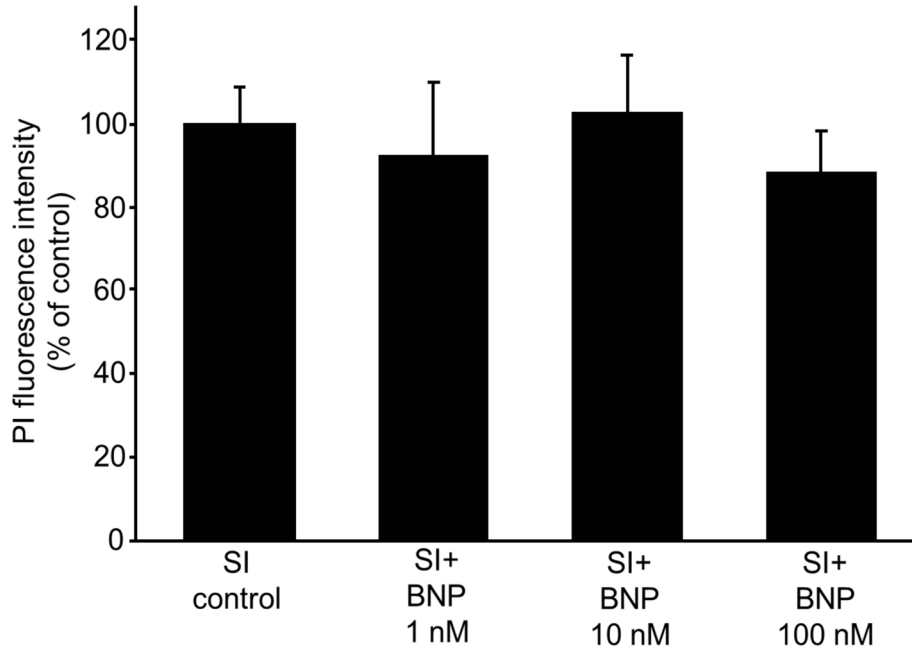

Fig.17: Effect of BNP on cell viability of mESC-derived cardiomyocytes.
Data are expressed as mean ± SEM; n=10-12

In separate experiments, the downstream pathways of SNAP-induced protection of mESC-derived cardiomyocytes were studied (Fig.18).

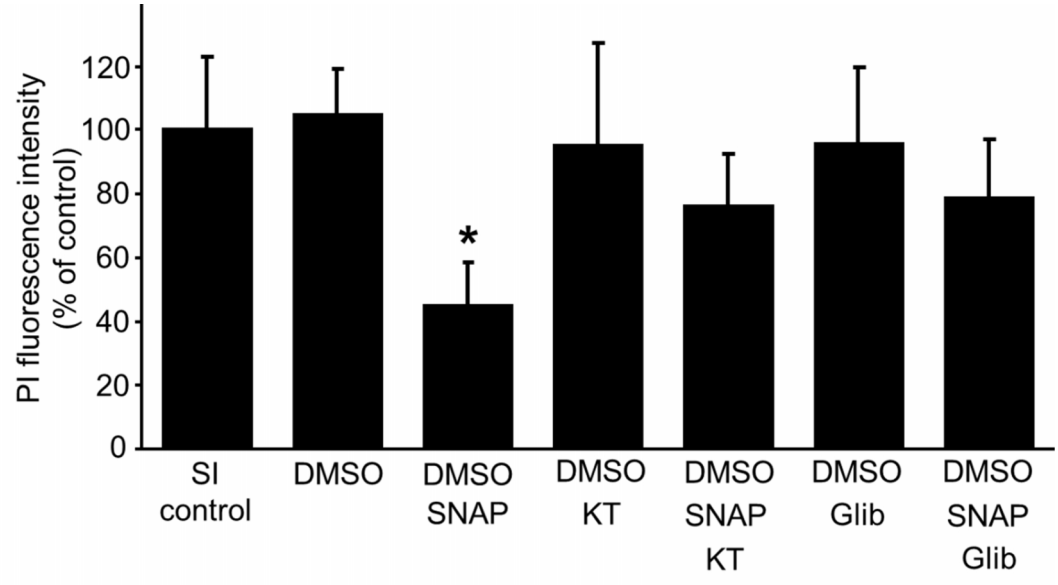

Fig.18: Effect of PKG (KT) and K_{ATP} (Glib) inhibitor on cell viability of mESC-derived cardiomyocytes.
Data are expressed as mean ± SEM; n=10-12; one-way ANOVA followed by Fischer LSD posthoc test;
* $p<0.05$ compared to SI control.

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 (Fig. 18).

In time-matched normoxic control groups, none of the above treatment influenced cell viability significantly (data not shown).

6. DISCUSSION

6.1 Study 1: MMP-2 inhibition by ilomasat 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.

Endogenous cardioprotection by early and late ischemic preconditioning as well as postconditioning might act via MMP-2 inhibition [34–36,74]; Bell *et al*. reported that ilomastat protects the heart against reperfusion injury independently from the well-known cardioprotective cellular RISK/mPTP modulating pathways [37]. 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 reported by Kupai *et al.* [31].

Recently, a large number of studies focused on the intracellular actions of MMP-2, that can degrade several newly identified intracellular targets including cardiac troponin I, myosin light chain-1, α-actinin (for review see ref. 28). The degradation of myocardial contractile proteins is an early event in myocardial infarction; which may contribute to the induction of proapoptotic cascades in cardiomyocytes and thus leads to cell death and contractile dysfunction [75]. Therefore, here we tested the direct cytoprotective effect of ilomastat in a previously established drug screening platform using isolated neonatal rat cardiomyocytes exposed to SI/R injury [58]. 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. Additionally, we have previously shown that MMP-2 activity can be

detected in both intact and in ischemia/reperfused rat ventricular samples [35]. Thus, MMP-2 is suspected to be the only MMP isoform with gelatinolytic activity in the rat myocardium. This is in accordance with previous findings by others, who have shown that MMP-2 possesses a predominant expression in both animal and human cardiomyocytes and cardiac tissue [27,76]. 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%. Previously we have demonstrated that cardioprotection by late ischemic preconditioning reduced MMP-2 activity by approximately 20% [36]. 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 [39,77], 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 myocardial infarction. 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 μmol/kg, however, higher doses of ilomastat were not significantly effective. Nevertheless, when ilomastat was administered before the onset of reperfusion, 6 μmol/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*. Our present results are supported by previous studies describing that Zn^{2+}-binding type MMP inhibitors, such as doxycycline and o-phenantroline, improved cardiac mechanical function after ischemia/reperfusion injury via the inhibition of MMP-2 in isolated rat hearts [25,78,79]. We have recently shown that the intravenously injected ilomasat (at 1.5 μmol/kg, administered before the onset of ischemia) decreased infarct size and had a comparable cardioprotective effect to ischemic late preconditioning [36]. More recently, Bell and colleagues reported cardioprotection in an *in vivo* mouse heart model of ischemia/reperfusion when ilomasat was administered intravenously at 6 μmol/kg upon releasing coronary occlusion [37]. However, the percentage of *in situ* MMP-2 inhibition was

not determined in the abovementioned studies and in the latter study, authors did not examine directly the MMP inhibitory effect of ilomastat.

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.

6.2 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. Either a selective inhibitor of PKG or a non-selective inhibitor of K_{ATP} channels interfered with this protection. 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 using primary neonatal cardiomyocytes have limitations for screening cardioprotective agents, including variability introduced by the isolation procedure and limited proliferation [80]. Adult cardiomyocytes are suitable to study individual cells, especially their electrophysiological properties. However, extracellular matrix proteins are required for their maintenance which may influence viability during SI [81]. The H9c2 cardiomyoblast cell line is a widely used model for in vitro drug screening as well. However, H9c2 cells differ from primary cardiomyocytes, because of lacking spontaneous electric activity and clearly developed sarcomeric structures [82]. 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. It exerts both early and late preconditioning-like cardioprotective effect in various models [83,84] and attenuates apoptosis in neonatal cardiomyocytes [85]. Accordingly, 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 [86].

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 ref. 87). We have recently shown that SNAP induces cytoprotection via the activation of soluble guanylate cyclase in neonatal cardiomyocytes [58]. However, in our previous studies the efficacy of SNAP-induced cytoprotection was more pronounced in neonatal cardiomyocytes than shown here in mESC-derived cardiomyocytes. This difference is probably due to the low expression level of soluble guanylate cyclase and nitric oxide synthase (NOS) at 6-8 days old stage of mESC-derived cardiomyocytes [88]. The latter is in accordance with our present results that 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 cardiocytoprotection.

To test if activation of particulate guanylate cyclase can increase cell viability similar to SNAP, the effect of BNP was tested. BNP is a potent cardioprotective peptide, as it is able to reduce infarct size in rat heart preparations [71] and to protect neonatal rat cardiomyocytes against SI/R injury [58]. 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 [89].

We further identified cardioprotective signaling pathways downstream of cGMP in mESC-derived cardiomyocytes. In the cardiomyocytes, at least three classes of protein targets are activated by cGMP, including cGMP-dependent PKG, cGMP-regulated phosphodiesterases, and cyclic nucleotide-gated ion channels [60], 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. Our present findings in mESC-derived cardiomyocytes are consistent with our previous results obtained in neonatal rat cardiomyocytes, in which the PKG inhibitor abolished the protective effect of SNAP [58]. However, it is of interest that Mobley *et al*. showed that PKG was down-regulated during cardiomyocyte differentiation and inhibition of PKG facilitated cardiac-committed differentiation of mESCs [90].

Xu *et al*. demonstrated that exogenous NO mediates the production of reactive oxygen species and may act via the activation of cGMP/PKG signaling, triggering cardiocytoprotection by mitochondrial K_{ATP} channel opening or by opening the mitochondrial permeability transition pore in adult rat cardiomyocytes K_{ATP} channels have a prominent role in the electrical excitability of cardiac-committed mESC-derived cells [91]. Therefore, in the present study, 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 [92,93].

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. The major advantages of ESC-based screening platforms over other cellular assays are the well reproducible production of beating myocardial cells and that it does not require to sacrifice a number of animals. 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.

7. CONCLUSIONS AND FURTHER 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 firs 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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10. ANNEX

I.

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Moderate inhibition of myocardial matrix metalloproteinase-2 by ilomastat is cardioprotective

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ABSTRACT

Pharmacological inhibition of matrix metalloproteinase-2 (MMP-2) is a promising target for acute cardioprotection against ischemia/reperfusion injury. Therefore, here we investigated if the MMP inhibitor ilomastat administered either before ischemia or before reperfusion is able to reduce infarct size via inhibition of MMP-2, the most abundant MMP in the rat heart.

Infarct-size limiting effect of ilomastat (0.3–6.0 μmol/kg) was tested in an in vivo rat model of myocardial infarction induced by 30 min coronary occlusion/120 min reperfusion. Ilomastat at 0.75 and 1.5 μmol/kg decreased infarct size significantly as compared to the vehicle-treated (dimethyl sulfoxide) group (from 66.1 ± 4.6% to 45.3 ± 7.0% and 46.7 ± 5.5% of area at risk, $p < 0.0.5$, respectively), when administered 5 min before the onset of ischemia. Ilomastat at 6.0 μmol/kg significantly reduced infarct size from its control value of 65.4 ± 2.5% to 52.8 ± 3.7% of area at risk ($p < 0.05$), when administered 5 min before the onset of reperfusion. Area at risk was not significantly affected by ilomastat treatments. To further assess the cytoprotective effect of ilomastat, primary cardiomyocytes isolated from neonatal rats were subjected to 240 min simulated ischemia followed by 120 min simulated reperfusion in the presence of ilomastat (5 nM–5 μM). Ilomastat at 500 nM and 5 μM significantly increased cell viability when compared to vehicle treated group. To assess the in situ MMP-2 inhibitory effect of ilomastat, in separate experiments in situ zymography was performed in cardiomyocytes. The cytoprotective concentration of ilomastat (500 nM) showed a moderate (approximately 25%) inhibition of intracellular MMP-2 in ischemic/reperfused cardiomyocytes. In these cells, MMP-2 immunostaining showed a 90% colocalization with the in situ gelatinolytic activity.

We conclude that the MMP inhibitor ilomastat reduces infarct size when administered either before the onset of ischemia or before the onset of reperfusion in vivo. Furthermore, this is the first demonstration that a moderate inhibition of intracellular MMP-2 is sufficient to confer cardiocytoprotection.

1. Introduction

Acute myocardial infarction and its complications are the leading cause of death in the industrialized countries. The treatment of acute ischemic heart disease has entered a new era through early reperfusion therapy, however, irreversible cell injury leading to apoptosis and necrosis may be precipitated by reperfusion, which may contribute to the development of infarction [1,2]. Therefore, to protect the heart from acute ischemic and reperfusion injury, i.e. to reduce infarct size is of great clinical relevance.

The pathomechanism of myocardial ischemia and reperfusion injury is not completely revealed. Since the original observation by the research group of Richard Schulz, the involvement of matrix metalloproteinases (MMP) in acute myocardial ischemia/reperfusion injury has been well-established [3–9]. MMPs are zinc dependent, neutral endopeptidases involved in several physiological processes, such as embryogenesis, angiogenesis and re-building of extracellular matrix (ECM). Gelatinase types of MMPs, MMP-2 and -9, are implicated in numerous cardiovascular diseases including ischemia/reperfusion injury [10]. Recently, the presence of MMP-2 has been shown in the cytosol of intact

Abbreviations: DMSO, dimethyl sulfoxide; ILO, ilomastat; LAD, left anterior descending coronary artery; MMP, matrix metalloproteinase; PBS, phosphate buffer solution; PI, propidium iodine.

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cardiomyocytes [11]. Moreover, several cardiac contractile proteins, such as titin and troponins, were shown to be potential targets of acute intracellular MMP-2 activation during ischemia/reperfusion [12]. Therefore, MMP-2 became a major target for drug development in acute cardiovascular pathologies including myocardial infarction [13,14].

We have previously reported that MMP-2 activity was moderately decreased during ischemic preconditioning [6] and that exogenous inhibition of MMPs by ilomastat, a non-selective MMP inhibitor, diminished ischemia-induced MMP-2 activity in isolated rat hearts [5]. Furthermore, we have described that the activities of MMP-2 and MMP-9 were decreased significantly in an in vivo rat model of ischemic late preconditioning [3]. Moreover, we and others have shown that ilomastat reduces infarct size in rats and mice ([3,15], for review see Refs. [10,16]). Nevertheless, the dose–response relationships of ilomastat administered before the onset of ischemia as well as before the onset of reperfusion are still unknown. Moreover, there is no proof if ilomastat-induced cardioprotection is due to MMP-2 inhibition. Furthermore, it is also not known, what extent of intracellular MMP-2 inhibition is needed for effective cardioprotection.

Therefore, in the present study, we aimed to investigate the dose–response relationships 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. Furthermore, to test if 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 cardiomyocytes subjected to simulated ischemia/reperfusion.

2. Materials and methods

2.1. Animals

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.

Male Wistar rats (Charles-River, Germany) weighing 280–370 g 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.

2.2. In vivo studies

2.2.1. Surgical procedure of coronary occlusion

Rats were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital (Euthasol, Produlab Pharma b.v., Raamsdonksveer, The Netherlands). Animals were mechanically ventilated (Model 683, Harvard Apparatus, Holliston, MA) with room air in a volume of 6.2 ml/kg and a frequency of 55 ± 5 breath/min according to body weight. Rats were placed in supine position on a heating pad to maintain body core temperature in physiological range (37.0 ± 1.0 °C). Right carotid artery was cannulated to measure mean arterial blood pressure by a pressure transducer (Experimetria Inc., Budapest, Hungary). Mean arterial blood pressure and body surface electrocardiogram (ECG) was monitored throughout the experiments (Haemosys, Experimetria Inc., Budapest, Hungary). Right jugular vein was also cannulated for fluid substitution and drug administration. Left anterior descending coronary artery (LAD) occlusion was induced by left thoracotomy. A 5-0 Prolene suture (Ethicon, Johnson & Johnson, Budapest, Hungary) was placed around LAD artery and a small plastic knob, which was threaded through the ligature and placed in contact with the heart, was used for making occlusion for 30 min. 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.

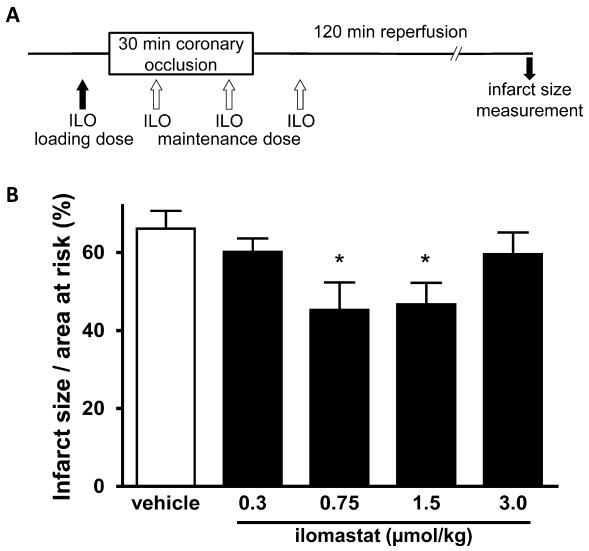

Fig. 1. *Panel A*: In vivo experimental protocol: rats were subjected to 30 min ischemia/120 min reperfusion to measure infarct size. Ilomastat at 0.3, 0.75, 1.5 and 3.0 μmol/kg or vehicle (DMSO) was administered intravenously (upward closed arrow) at 5 min before the onset of ischemia. To maintain serum level of ilomastat, repeated boluses with half dose of the first bolus were administered in every 15 min, three times: at the 10th and 25th min of ischemia and at the 10th min of reperfusion (upward open arrows). *Panel B*: Effect of ilomastat treatment on infarct size when administered before ischemia. $^{*}p < 0.05$ compared to vehicle-treated group, $n = 7–8$, data are shown as mean ± S.E.M.

2.2.2. 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. Dimethyl sulfoxide (DMSO; 11.6 w/v% solution diluted with physiological saline) as vehicle or 0.3, 0.75, 1.5, and 3.0 μmol/kg ilomastat were administered intravenously in slow bolus 5 min before ischemia (Fig. 1A). To maintain serum level of ilomastat, additional 3 boluses of vehicle (5.8 w/v% DMSO solution) or ilomastat with half dose (0.15, 0.375, 0.75; and 1.5 μmol/kg, respectively), were given at the 10th, 25th min of ischemia and at the 10th min of reperfusion. We estimated the maintaining doses of ilomastat according to its half-life based on pharmacokinetic data described previously in rodents after a single intravenous bolus injection [17].

In the second series of in vivo experiments DMSO (11.6 w/v% solution) or ilomastat (0.75, 1.5, 3.0, and 6.0 μmol/kg) bolus was injected at the 25th min of ischemia. Maintaining boluses (5.8 w/v% DMSO or 0.375, 0.75, 1.5, and 3.0 μmol/kg, respectively) were administered at the 10th, 25th and 40th min of reperfusion (Fig. 2A) to maintain constant ilomastat concentration in blood during the early phase of reperfusion.

2.2.3. Determination of infarct size

After 120 min of reperfusion hearts were isolated for infarct size measurements. Hearts were perfused in Langendorff perfusion system with 37 °C Krebs–Henseleit buffer (composition given in Ref. [18]) to remove blood from the coronary vessels. After 5 min of perfusion, risk area was re-occluded, and hearts were perfused with 4 ml of 0.1% Evans blue dye through the ascending aorta.

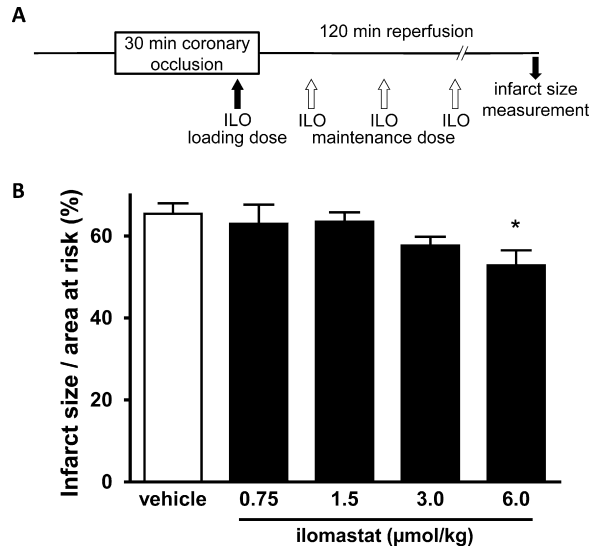

Fig. 2. *Panel A*: In vivo experimental protocol: rats were subjected to 30 min ischemia/120 min reperfusion to measure infarct size. Ilomastat at 0.75, 1.5, 3.0, and 6.0 μmol/kg or vehicle (DMSO) was administered intravenously (upward closed arrow) at 5 min before the onset of reperfusion. To maintain serum level of ilomastat, repeated boluses with half dose of the first bolus were administered in every 15 min, three times: at the 10th, 25th and the 40th min of reperfusion (upward open arrows). *Panel B*: Effect of ilomastat treatment on infarct size when administered before reperfusion. $^{*}p < 0.05$ compared to vehicle-treated group, $n = 7–8$, data are shown as mean ± S.E.M.

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, (Pharmahungary, Szeged, Hungary; [19]).

2.3. In vitro studies

2.3.1. Inhibition of gelatinase activity by ilomastat

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 [20]. Briefly, 8% polyacrylamide gels were copolymerized with gelatin (2 mg/ml, type A from porcine skin, Sigma–Aldrich, Budapest, Hungary), and 50 μg of protein per lane was loaded. After electrophoresis (90 V, 1.5 h), gels were washed firstly with renaturation buffer (Bio-Rad, Hercules, CA; containing 2.5% Triton X-100) for 3× 15 min then incubated in development buffer (Bio-Rad, Hercules, CA) for 20 min to eliminate Triton-X-100. Gels were sliced according to the lanes and the slices were incubated separately for 20 h at 37 °C at pH 7.4 in development buffer in the presence of vehicle and/or different concentrations of ilomastat (0.5 nM and 5.0 nM). Recombinant, human MMP-2 was used as positive control. Gels were then stained with 0.05% Coomassie brilliant blue (G-250, Sigma–Aldrich, Budapest, Hungary). 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.

2.3.2. Simulated ischemia/reperfusion in cardiomyocytes

Neonatal rat cardiomyocytes were cultured in 48-well plates. Neonatal cardiomyocytes were chosen for the present study, as they can be harvested on culture dishes without coating. The lack of coating is important in the present study, as coating materials (e.g. laminin, collagen, etc.) could influence the action of matrix metalloproteinases [21,22]. Cell isolation and culturing method was described previously in detail [23,24]. Briefly, neonatal rats were sacrificed by cervical dislocation and hearts were placed into ice cold PBS solution. Ventricles were digested in 0.25% trypsin (Invitrogen, Life Technologies Hungary Ltd., Budapest, Hungary) solution and cell suspension was centrifuged at 400 × *g*, for 15 min at 4 °C. Cell pellet was re-suspended in Dulbecco's Modified Eagle Medium (Sigma–Aldrich, Budapest, Hungary) supplemented with L-glutamine (Sigma–Aldrich, Budapest, Hungary), Antibiotic–antimycotic solution (Sigma–Aldrich, Budapest, Hungary) and 10% fetal bovine serum (Gibco, Life Technologies Hungary Ltd., Budapest, Hungary). Cells were counted in a hemocytometer, and seeded into 48-well plates at a density of 5×10^4 cell/well. After 24 h, the growth medium was replaced with differentiation medium containing 1% fetal bovine serum. Cardiomyocytes were kept under normoxic conditions (37 °C, in 95% air and 5% CO_2 gas mixture) for three days prior to simulated ischemia/reperfusion experiments. We used a combination of hypoxic chamber and hypoxic solution to simulate tissue ischemia. In the simulated ischemic group, the medium of the cultures were replaced with a hypoxic solution (composition given in Ref. [25]) and plates were kept in a hypoxic chamber (gassed with 95% N_2 and 5% CO_2 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 with 0.2% DMSO. During simulated reperfusion cells were covered with differentiation medium (containing 1% fetal bovine serum) and kept in a normoxic incubator for 120 min.

2.3.3. Cell viability assay

Cell viability was assessed by a calcein and propidium iodine (PI) assay performed in each group after 2 h simulated reperfusion [26]. Briefly, the growth medium was removed, cells were washed with PBS twice and incubated with calcein (1 μM) for 30 min. Then calcein solution was replaced with fresh PBS and fluorescence intensity of each well was detected by fluorescent plate reader (FluoStar Optima, BMG Labtech, Auro-Science Consulting Ltd., Budapest, Hungary). Then PI (50 μM) and digitonin (100 μM) (Sigma–Aldrich, St. Louis, MO) were added to PBS and cells were incubated for 7 min. Then PI solution was replaced with fresh PBS and fluorescent intensity was detected with same settings.

2.3.4. 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 at the density of 10^5 cells/well for 3 days. The medium of cells was replaced with hypoxic solution supplemented with DQ™ 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 DQ gelatin and cells were placed into a normoxic incubator for 120 min. Finally cells were washed with PBS, and fixed in 3.7% paraformaldehyde dissolved in PBS for 15 min. MMP-2 fluorescent immunostaining using anti-proMMP-2 antibody (Chemicon, MAB3308; Merck Ltd., Budapest, Hungary; secondary antibody: rhodamine-labeled goat anti-mouse antibody; Abcam, AB5885, Cambridge, UK) was assessed 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). After the subsequent washing steps, cells were covered with fluorescent mounting medium (Dako, Frank Diagnosztika Ltd., Budapest, Hungary), and fluorescence was

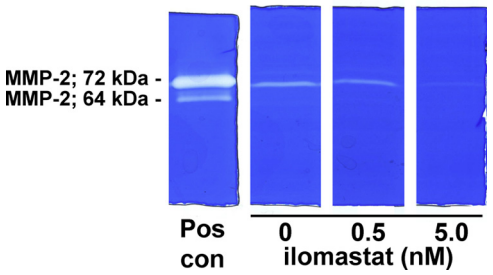

Fig. 3. Representative images of MMP-2 activity in gelatin zymograms in the presence of 0, 0.5 and 5.0 nM ilomastat.

detected with 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 fields selected randomly on each coverslip. Four coverslips were analyzed in each group. The number, total area, and area fraction of fluorescent signal, and the analyses of co-localization were quantified on images by ImageJ 1.45 software (National Institutes of Health, Bethesda, MD).

2.4. Statistical analysis

Statistical analysis was performed using Sigmaplot 11.0 software. All data were given as mean ± standard error of the mean (S.E.M.). One-way analysis of variance 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. Results

3.1. Effect of ilomastat on infarct size in vivo

The cardioprotective effect of ilomastat administered before the onset of ischemia (Fig. 1) or before the onset of reperfusion (Fig. 2) 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 μmol/kg doses reduced infarct size significantly as compared to vehicle-treated group (from 66.1 ± 4.6% to 45.3 ± 7.0% and 46.7 ± 5.5% of area at risk, respectively) showing a U-shaped dose–response relationship (Fig. 1B). When administered before the onset of reperfusion, ilomastat at 6.0 μmol/kg reduced infarct size significantly (from 65.4 ± 2.5% to 52.8 ± 3.7% of area at risk), however, lower doses were ineffective (Fig. 2B). There was no significant difference in the area at risk among the groups (data not shown). There was no significant difference in the mean arterial blood pressure and heart rate among the groups (Tables 1 and 2 are shown in data supplement).

3.2. 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 IC50 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 (Fig. 3).

3.3. Effect of ilomastat on ischemia/reperfused cardiomyocytes

In order to test if a direct cardiocytoprotection by MMP-2 inhibition of ilomastat is involved in its cardioprotective

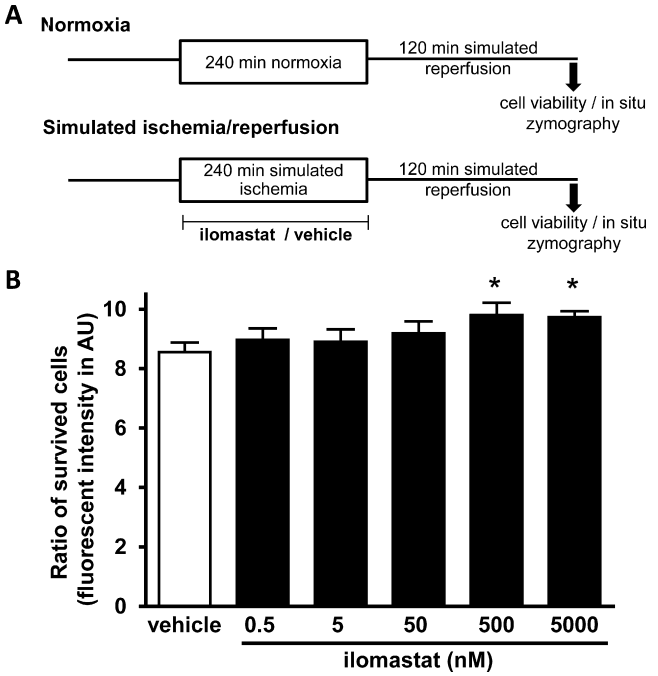

Fig. 4. *Panel A*: Experimental protocol of cell culture studies. Simulated ischemia/reperfusion was induced in the presence of vehicle (DMSO) or ilomastat. Viability assays and in situ zymography were performed after the end of simulated reperfusion. Normoxic time-matched control groups were kept under normoxic solution in normoxic conditions. *Panel B*: Effect of ilomastat on cell viability in neonatal rat cardiomyocytes after simulated ischemia/reperfusion. $^*p < 0.05$ compared to vehicle treated group, $n = 6$; data are shown as mean ± S.E.M.

effect, we examined ilomastat-induced cytoprotection in isolated neonatal rat cardiomyocytes subjected to normoxia or simulated ischemia/reperfusion (Fig. 4A). Ilomastat at a dose range of 0.5 nM up to 5 μM did not influence cell viability in normoxic conditions (Table 3, supplementary material). However, ilomastat at 500 nM and 5 μM significantly increased cell viability as compared to vehicle treated group (from 8.6 ± 0.3 to 9.8 ± 0.4 and 9.7 ± 0.2, respectively, expressed in arbitrary units of fluorescent intensity) in cardiomyocytes subjected to simulated ischemia/reperfusion (Fig. 4B).

3.4. In situ MMP-2 inhibition by ilomastat in ischemia/reperfused cardiomyocytes

To test the in situ MMP inhibitory efficacy of the cardiocytoprotective concentration of ilomastat, we performed in situ zymography on isolated rat cardiomyocytes subjected to simulated ischemia/reperfusion (Fig. 5). Simulated ischemia/reperfusion increased gelatinolytic activity significantly from its control value of 0.48 ± 0.04% to 0.93 ± 0.05% of area fraction. The cardiocytoprotective concentration of ilomastat (500 nM, see Fig. 4B) moderately inhibited the in situ gelatinolytic activity approximately by 25%, i.e. from 0.93 ± 0.05% to 0.70 ± 0.04% of area fraction, during simulated ischemia/reperfusion (Fig. 5/D). Moreover, we performed separate experiments to show co-localization of MMP-2 with the fluorescent gelatinolytic signal in isolated rat cardiomyocytes subjected to simulated ischemia/reperfusion. MMP-2 showed over 90% co-localization rate with gelatinolytic activity in all groups (Fig. 6).

4. Discussion

Here we have demonstrated that ilomastat, a non-selective MMP inhibitor, reduced infarct size when administered either before the onset of ischemia or before the onset of reperfusion

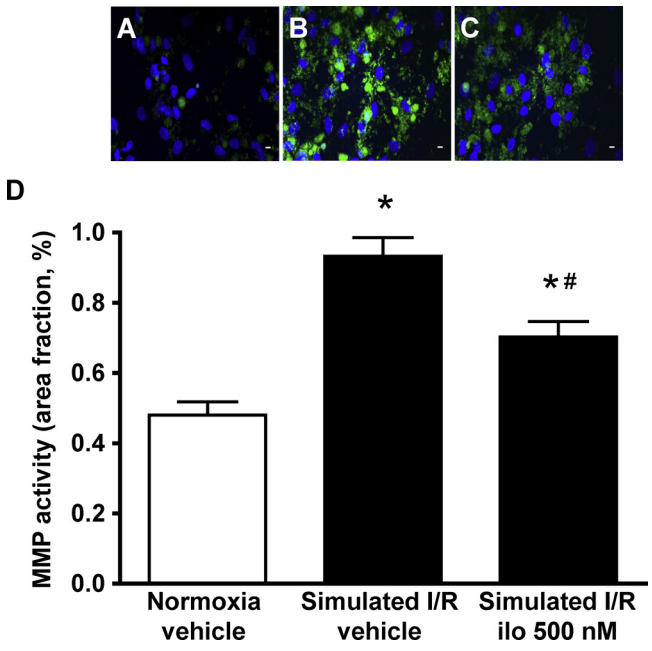

Fig. 5. Effect of the cardiocytoprotective concentration of ilomastat (500 nM) on in situ MMP-2 activity in neonatal rat cardiomyocytes subjected to simulated ischemia/reperfusion. *Panel A–C*: Representative fluorescent confocal images from neonatal rat cardiomyocytes subjected to normoxia (A), simulated ischemia/reperfusion (B), or simulated ischemia/reperfusion in the presence of 500 nM ilomastat (C). Green color represents MMP-2 activity as measured by in situ zymography FITC signal; blue color represents the nuclei of the cells. Scale bars = 20 μm. *Panel D* shows area fraction of fluorescent images. $^{*}p < 0.001$ compared to normoxic vehicle-treated group, $^{\#}p < 0.01$ compared to simulated ischemia/reperfusion, vehicle-treated group, $n = 6$; data are shown as mean ± S.E.M. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in vivo and revealed its cardioprotective dose–response relationship. Moreover, we have shown that an approximately 25% inhibition of intracellular MMP-2 activity by ilomastat confers significant cardiocytoprotection. This is the first demonstration that the cardioprotective effect of ilomastat may involve a cardiocytoprotective mechanism due to a moderate inhibition of MMP-2.

In our present study, ilomastat reduced infarct size dose-dependently when administered either before ischemia or before reperfusion. We have found a slightly different dose range between the 2 administration patterns. When administered before the onset of ischemia, the effective doses of ilomastat were 0.75 and 1.5 μmol/kg, however, higher doses of ilomastat were not significantly effective. Nevertheless, when ilomastat was administered before the onset of reperfusion, 6 μmol/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. Our present results are supported by previous studies describing that Zn^{2+}-binding type MMP inhibitors, such as doxycycline and o-phenantroline, improved cardiac mechanical function after ischemia/reperfusion injury via the inhibition of MMP-2 in isolated rat hearts [4,27,28]. We have previously shown that iv. injection of 1.5 μmol/kg ilomastat before a 30-min ischemia decreased infarct size comparable to ischemic late preconditioning in an in vivo rat model of coronary occlusion [3]. Recently, Bell and colleagues reported cardioprotection in a mouse model of ischemia/reperfusion when administered iv. 6 μmol/kg ilomastat at the release of coronary occlusion [15]. However, in the above-mentioned studies the percentage of in situ MMP-2 inhibition was not determined and in the latter study, authors did not examine directly the MMP inhibitory effect of ilomastat.

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 detectable according to the molecular weights of the two active isoforms of MMP-2. Bands of other molecular weights were not present on the zymogram. Furthermore, here we proved that gelatinolytic activity was co-localized with MMP-2 protein in cardiomyocytes. These results show that gelatinolytic activity in the heart is derived solely from MMP-2

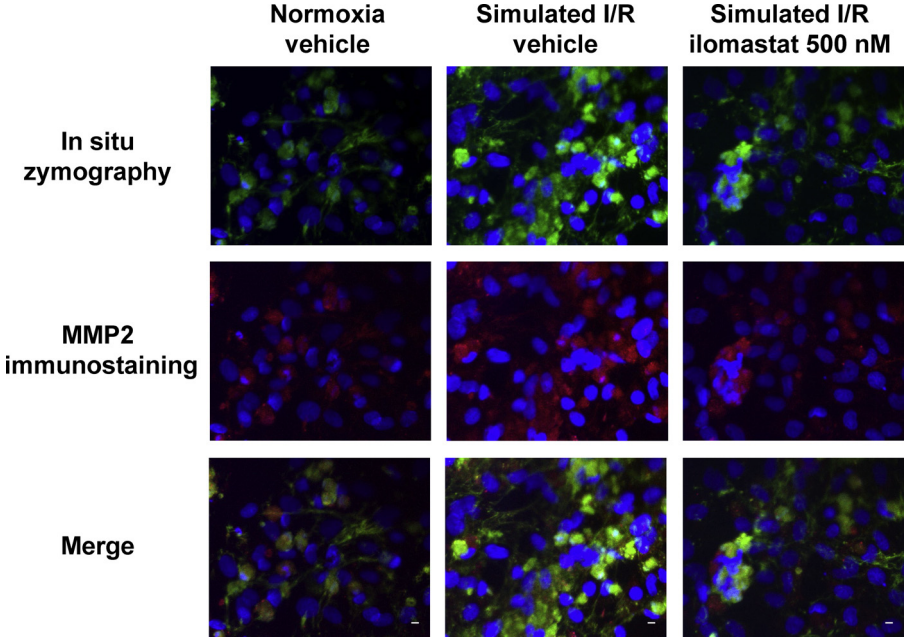

Fig. 6. Representative immunofluorescent confocal images from cultured cardiomyocytes subjected to normoxia or simulated ischemia/reperfusion in the presence or absence of ilomastat, respectively. Green fluorescence: fluorescent signal of gelatin substrate after proteolytic cleavage. Red fluorescence: MMP-2 immunostaining, blue fluorescence: cell nuclei. Scale bars = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activity. Accordingly, we have previously shown that MMP-2 activity can be detected both in intact and in ischemia/reperfused rat ventricular samples [5], thus, MMP-2 is suspected to be the only MMP isoform with gelatinolytic activity in the rat myocardium. This is in accordance with previous findings by others, who have shown that MMP-2 possesses a predominant expression in both animal and human cardiomyocytes and cardiac tissue [29,30]. Nevertheless, it cannot be excluded that inhibition of other non-gelatinolytic proteases may be involved in the cardiocytoprotective effect of ilomastat.

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 previous findings showing that cardioprotection by late ischemic preconditioning reduced MMP-2 activity by approximately 20% strongly supports our present results [3]. 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 (for review see Ref. [14,31]), 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.

The cardioprotective cellular mechanism in which MMP-2 inhibition might be involved is not known and has not been investigated in the present study. Although endogenous cardioprotection by early and late ischemic preconditioning as well as postconditioning involve an MMP-2 inhibition-dependent mechanism [3,5,6,32] the exact mechanism by which MMP inhibition results in cardioprotection is not known. Bell et al. reported that ilomastat protects the heart against reperfusion injury independently from the well-known cardioprotective Reperfusion Injury Salvage Kinase/mitochondrial permeability transition pore opening pathways [15]. Recently, a large number of studies focused on the intracellular actions of MMP-2, which can degrade several newly identified intracellular targets including troponin I, myosin light chain-1, α-actinin (for review see Ref. [11]) and titin [12]. The degradation of myocardial contractile proteins may contribute to the induction of proapoptotic signals in cardiomyocytes and thus leads to cell death and contractile dysfunction (for review see Ref. [33]).

We conclude that 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.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2013.12.007.

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RESEARCH

Cytoprotection by the NO-Donor SNAP Against Ischemia/Reoxygenation Injury in Mouse Embryonic Stem Cell-Derived Cardiomyocytes

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Abstract Embryonic stem cell (ESC)-derived cardiomyocytes are a promising cell source for the screening for potential cytoprotective molecules against ischemia/reperfusion injury, however, little is known on their behavior in hypoxia/reoxygenation conditions. Here we tested the cytoprotective effect of the NO-donor SNAP and its downstream cellular pathway. Mouse ESC-derived cardiomyocytes were subjected to 150-min simulated ischemia (SI) followed by 120-min reoxygenation or corresponding non-ischemic conditions. The following treatments were applied during SI or normoxia: the NO-donor *S*-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP), the protein kinase G (PKG) inhibitor, the K_{ATP} channel blocker glibenclamide, the particulate guanylate cyclase activator brain type natriuretic peptide (BNP), and a non-specific NO synthase inhibitor (*N*-Nitro-L-arginine, L-NNA) alone or in different combinations. Viability of cells was assayed by propidium iodide staining. SNAP attenuated SI-induced cell death in a concentration-dependent manner, and this protection was attenuated by inhibition of either PKG or K_{ATP} channels. However, SI-induced cell death was not

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affected by BNP or by L-NNA. We conclude that SNAP protects mESC-derived cardiomyocytes against SI/R injury and that soluble guanylate-cyclase, PKG, and K_{ATP} channels play a role in the downstream pathway of SNAP-induced cytoprotection. The present mESC-derived cardiomyocyte-based screening platform is a useful tool for discovery of cytoprotective molecules.

Keywords Stem cell · Nitric oxide · Ischemia/reoxygenation · Signal-transduction · Cardioprotection

Introduction

Ischemic heart disease is the leading cause of death in the industrialized world, therefore, development of cardioprotective therapies are of great importance.

In vitro cardiac myocyte-based drug screening platforms at the early stage of the development of cardioprotective agents are widely used. However, currently used assays based on cardiomyoblast cell lines (H9c2) as well as primary neonatal and adult cardiac myocytes [1] have limitations including limited proliferation capacity, uncontrolled stress during cell isolation and dissociation of cultured cells, low-throughput nature, and poor predictability of the assays toward in vivo efficacy [2]. Embryonic stem cells (ESC) are capable of unlimited proliferation in vitro and differentiation into cardiac myocytes [3], therefore, ESCs provide a promising source of cardiac myocytes for in vitro drug screening [4, 5]. ESCs may also become tools for regeneration therapy [6], however, several limitations were reported including ethical, immunological, and tumorigenicity problems, which restrict their clinical application [7]. Moreover, transplanted cells undergo a significant rate of cell death shortly after transplantation, approaching 90 % within the first 24-h after transplantation [8]. One reason is may be the unfavorable microenvironment grafted cells face when injected into host cardiac muscle. In most studies, well-nourished and oxygenated stem cells are transplanted into poorly perfused tissue, where they are exposed to increased oxidative stress and local inflammation [9]. Characterization of these cells in a high throughput ischemia/reoxygenation test system would be important, since little is known about the ischemic tolerance and signal transduction pathways involved in protection of ESC-derived cardiomyocytes.

It has been previously shown that nitric oxide (NO) has a direct cytoprotective effect in case of simulated ischemia (SI) in cardiomyocytes [10]. Furthermore, administration of the NO-donor *S*-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP, 2 mM) has been shown to mimic preconditioning protection in mouse hearts [11]. NO-mediated cytoprotection may act via different signaling pathways. Intracellular elevation of cyclic guanosine monophosphate (cGMP) by NO or natriuretic peptides has been proposed to influence cellular responses to ischemia and to contribute to cardioprotection. However, the contribution of NO and its downstream signaling pathway in the protection of ESC-derived cardiomyocytes has not been studied yet. Opening of sarcolemmal and mitochondrial ATP-sensitive potassium (K_{ATP}) channels can be activated by exogenous NO and these channels have been demonstrated to mediate cardioprotection [12]. However, the importance of this pathway in mouse ESC (mESC)-derived cardiomyocytes is not known.

The aim of the present study was to test an mESC-derived cardiomyocyte-based drug screening platform by investigating whether the cytoprotective NO-donor SNAP is able to protect these cells against SI/reoxygenation injury. We also investigated the downstream pathways of the protection in this platform.

Methods

Mouse ESC Culture

Undifferentiated mESCs (Nkx2.5/EGFP transgenic C57BL/6 mouse ES cell line; $Tg^{Nkx2.5/EGFP}$ C57BL/6; passages 10–12) were cultured on feeder layers of mitomycin C-inactivated mouse embryonic fibroblasts (MEFs) which were obtained from 13.5 days postcoitus mouse embryos, as described earlier by Belteki [13]. mESCs were maintained in ES medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 15 % (v/v) fetal bovine serum (FBS, Sera Laboratories International, West Sussex, RH17 5PB, UK) supplemented with 1,000 U/mL mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon International, Budapest, Hungary), 0.1 mM non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME), and 50 U/mL penicillin/50 μg/mL streptomycin. mESCs were cultured on feeder layers for at least two passages after thawing and subsequently were cultured without feeder cells on 0.1 % gelatin-coated tissue culture plates in the presence of LIF (2,000 U/mL). Medium was changed daily for standards maintenance. mESCs were usually passaged every 1–2 days prior reaching 70 % confluences.

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 the hanging drop (HD) method [14]; in brief, mESCs were seeded as 4×10^4 cells/mL (resulting in 800 cells/drop) suspension in differentiation medium (regular DMEM without LIF). 2 days later, the EBs were transferred and plated into 24-well plates on gelatin-coated coverslips. 0.1 mg/mL ascorbic acid was supplemented to induce cardiac differentiation. mESC-derived cardiomyocytes were used at 6–8-day-old stage for SI/reoxygenation

experiments. At this stage, the ratio of Nkx2.5-eGFP positive cells, an early marker for cardiac differentiation, exhibited 52.5 ± 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).

Experimental Groups

For cell viability experiments, mESC-derived cardiomyocytes were tested under normoxic condition or were subjected to SI (Fig. 1). The normoxic mESC-derived cardiomyocytes were kept under normoxic conditions, i.e., the growth medium was changed to a normoxic solution (in mM: NaCl 125, KCl 5.4, NaH_2PO_4 1.2, $MgCl_2$ 0.5, HEPES 20, glucose 15, taurine 5, $CaCl_2$ 1, creatine 2.5, BSA 0.1 %, pH 7.4, 310 mOsm/l) [15] and the cells were incubated under 95 % air and 5 % CO_2 at 37 °C for 2.5 h. In the second series of experiments, mESC-derived cardiomyocytes were subjected to SI by incubating the cells in hypoxic solution (in mM: NaCl 119, KCl 5.4, $MgSO_4$ 1.3, NaH_2PO_4 1.2, HEPES 5, $MgCl_2$ 0.5, $CaCl_2$ 0.9, Na-lactate 20, BSA 0.1 %, 310 mOsm/l, pH = 6.4) [15] and placing the plates in a humidified 37 °C hypoxic chamber exposed to a constant flow of a mixture of 95 % N_2 and 5 % CO_2 for 2.5 h. The cells were then subjected to the following treatments during SI or normoxic protocol: (1) untreated control; (2) SNAP (10^{-7}, 10^{-6}, 10^{-5} M) (10) (Sigma, St. Louis, MO, USA); (3) selective protein kinase G (PKG) inhibitor KT-5823 (6×10^{-8} M), an effective concentration that does not affect cell viability alone (10) (Sigma, St. Louis, MO, USA); (4) SNAP (10^{-6} M, a concentration found here protective) in combination with KT-5823 (6×10^{-8} M); (5) brain type natriuretic peptide-32 (BNP, 10^{-9}, 10^{-8}, 10^{-7} M) [16] (American Peptides, Sunnyvale, CA, USA); (6) nitric oxide synthase (NOS) inhibitor *N*-Nitro-L-arginine (L-NNA, 10^{-4}, 10^{-5} M) [17] (Sigma, St. Louis, MO, USA); (7) non-selective K_{ATP} channel inhibitor glibenclamide (10^{-6} M, an effective K_{ATP} blocking concentration that does not affect ischemia/reperfusion injury alone) [18] (Sigma, St. Louis, MO, USA); (8) SNAP (10^{-6} M) and glibenclamide (10^{-6} M); and dimethyl-sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) control groups.

Either normoxic or SI treatments were followed by 2 h reoxygenation with growth medium without ascorbic acid and superfusion with 95 % air and 5 % CO_2 at 37 °C.

Cell Viability Assay

Cell viability was assessed by a propidium iodide (PI) assay performed in each group after 2 h reoxygenation. PI (Sigma, St. Louis, MO, USA) was chosen, as it stains cells with severely impaired membrane integrity and it does not necessitate dissociation of the cells. Briefly, the growth medium was removed, cells were washed with PBS twice and incubated with PI (50 μM) for 7 min. Each experiment included a digitonin (10^{-4} M) (Sigma, St. Louis, MO, USA) treated positive control well and PI control (mESC-derived cardiomyocytes without treatment and stained for PI for 7 min) (Fig. 2). Then PI solution was replaced with fresh PBS and fluorescence intensity of each EB was detected by fluorescent plate reader (FluoStar Optima, BMG Labtech). Fluorescence intensity was measured in well scanning mode (scan matrix: 10 × 10; scan diameter: 10 mm; bottom optic; no of flashes/scan point: 3; temp. 37 °C; excitation wavelength: 544 nm; emission wavelength: 610 nm). PI intensity reflecting the cell death was evaluated on a standard area (21 scan box) in each well placed to the center of EB. The cardiac myocyte-rich region can be found predominantly near the edge of the embryonic body. Therefore, the evaluation of cardiac myocyte rich regions was performed manually on several plates by detecting GFP expression driven by the promoter of the early cardiac myocyte marker Nkx2.5. The ratio of cardiac myocyte death was

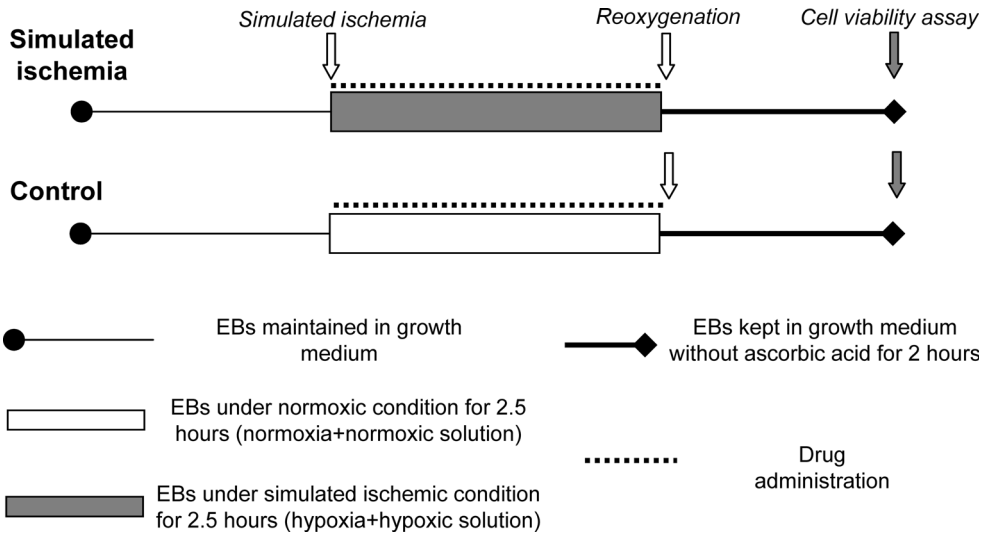

Fig. 1 Experimental protocol of SI and reoxygenation in mESC-derived cardiomyocytes

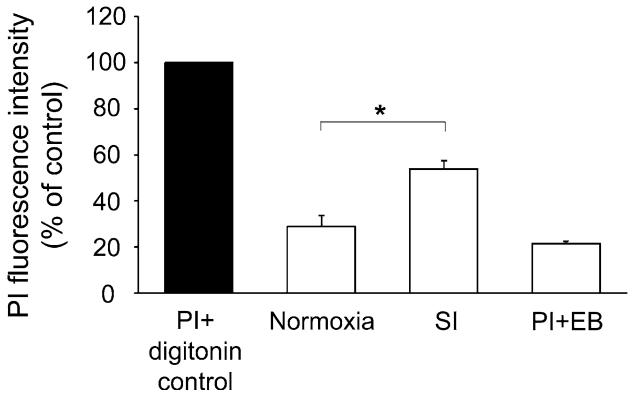

Fig. 2 Cell viability of mESC-derived cardiomyocytes subjected to normoxia or SI: representative results obtained from one plate. Background fluorescence intensity is represented by using non-treated EB+PI in three wells. Pi+digitonin control alone was applied in one well. Data are mean ± SEM. $*p < 0.05$ normoxia versus SI; t test, $n = 5$–6 in both groups

the same as the ratio of cell death of all cells found in the embryonic body. Background fluorescence intensity (dye control) was subtracted from the fluorescence intensity of each well after PI staining, and the average intensity of each group was plotted. The cytoprotective effect of different compounds was compared to simulated ischemic control groups.

Statistical Analysis

Results are expressed as mean ± 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 $p < 0.05$.

Results

Cell Viability After SI/Reoxygenation

We applied SI/reoxygenation to mimic ischemia/reperfusion injury in mESC-derived cardiomyocytes. Two and half hours of SI followed by reoxygenation caused significantly higher cell death in mESC-derived cardiomyocytes than time-matched controls kept under normoxic conditions (Fig. 2). SI killed roughly 20–40 % of cells in embryonic body.

The cytoprotective action 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 (10^{-6} and 10^{-5} M, $p < 0.05$) when applied during SI period (Figs. 3, 4). 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^{-5} and 10^{-4} M concentration. The presence of L-NNA did not influence cell death after SI (Fig. 5).

BNP, an activator of particulate GC, was also tested under SI condition at 10^{-9}, 10^{-8}, and 10^{-7} M concentrations. However, BNP did not influence cell death significantly (Fig. 6).

In separate experiments, the downstream pathways of SNAP-induced protection of mESC-derived cardiomyocytes were studied. The cytoprotective effect of SNAP (at 10^{-6} M)

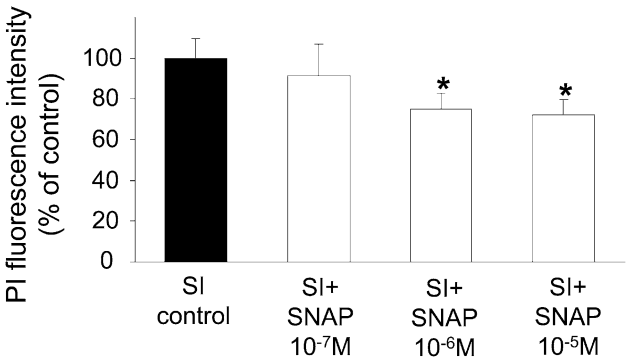

Fig. 4 Effect of SNAP on cell viability of mESC-derived cardiomyocytes. Cell viability of mESC-derived cardiomyocytes subjected to SI. SNAP administration was applied during SI. Data are mean ± SEM. $*p < 0.05$ versus SI control; one-way ANOVA followed by Fischer LSD post-hoc test, $n = 10$–12 in each group

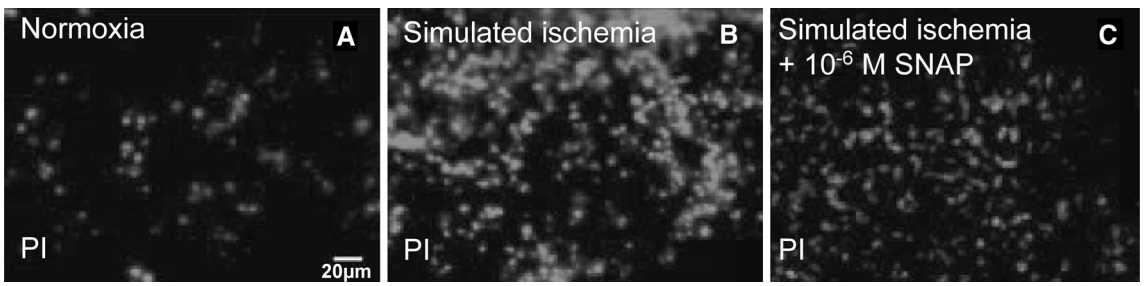

Fig. 3 Cell viability indicated by PI staining on mESC-derived cardiomyocytes. Representative fluorescent images of normoxic (**a**), SI (**b**), and SI+SNAP (10^{-6} M) (**c**) treated groups showing the amount of dead cells (increased nuclear fluorescence) in mESC-derived cardiomyocytes

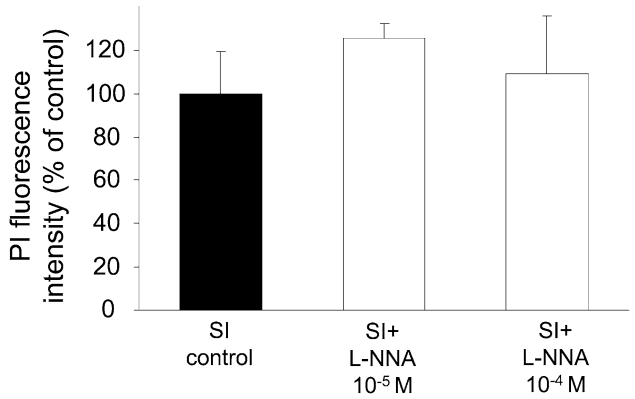

Fig. 5 Effect of L-NNA on cell viability of mESC-derived cardiomyocytes. Cell viability of mESC-derived cardiomyocytes subjected to SI. L-NNA was applied during SI. Data are mean ± SEM; $n = 10$–12 in each groups

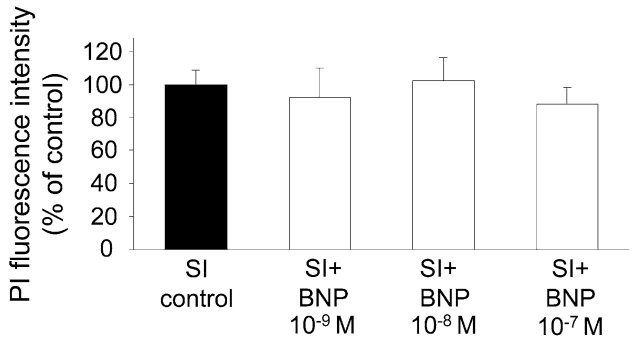

Fig. 6 Effect of BNP on cell viability of mESC-derived cardiomyocytes. Cell viability of mESC-derived cardiomyocytes subjected to SI. BNP was applied during SI. Data are mean ± SEM, $n = 10$–12 in each groups

was attenuated either by simultaneous administration of the selective PKG inhibitor KT-5823 (6×10^{-8} M) or by simultaneous administration of K_{ATP} channel inhibitor glibenclamide (10^{-6} M). Inhibitors administered alone, or their vehicle DMSO did not influence cell viability (Fig. 7).

In time-matched normoxic control groups, none of the above treatment influenced cell viability significantly (data not shown).

Discussion

In the present study we established an ESC-derived cardiac myocyte-based in vitro drug screening system and showed that the NO-donor SNAP was protective against SI/reoxygenation-induced cell death. Either a selective inhibitor of PKG or a non-selective inhibitor of K_{ATP} channels interfered with this protection. In contrast to

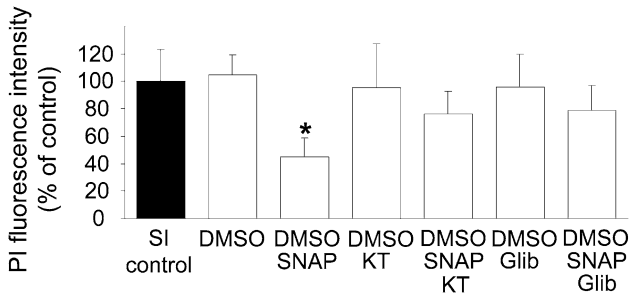

Fig. 7 Effect of PKG (KT) and K_{ATP} (Glib) inhibitor on cell viability of mESC-derived cardiomyocytes. Cell viability of mESC-derived cardiomyocytes subjected to SI. Drugs were applied during SI. Data are mean ± SEM; $*p < 0.05$ versus SI control; one-way ANOVA followed by Fischer LSD post-hoc test, $n = 10$–12 in each groups

SNAP, the particulate guanylyl 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 ischemia/reperfusion injury.

Currently used cell-based assays based on primary neonatal cells have limitations for screening cardioprotective agents, including variability introduced by the isolation procedure and limited proliferation [19]. Adult cardiomyocytes are suitable to study individual cells, especially their electrophysiological properties. In addition, extracellular matrix proteins are required for their maintenance which may influence viability during SI [2]. The cardiomyoblast cell line (H9c2) is widely used for in vitro drug screening. However, H9c2 cells differ from primary cardiomyocytes, e.g., they are lacking spontaneous electric activity and clearly developed sarcomeric structures [20]. Therefore, advantages of ESC-based assays are the well reproducible production of contracting myocardial cells and that they do not require sacrificing a 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. It exerts both early and late preconditioning-like cardioprotective effect in various models [21, 22] and attenuates apoptosis in neonatal cardiomyocytes [23]. Accordingly, in the present study, SNAP showed a concentration-dependent increase in viability of mESC-derived cardiomyocytes after SI/reoxygenation. This finding indicates that mESC-derived cardiac myocytes are useful tools for testing cardioprotective agents and suggests that NO donors may also be cytoprotective for stem cells implanted into ischemic areas of the myocardium. It is of interest that NO has been also shown to promote ESC differentiation and cardiomyogenesis in mESCs [24].

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 (see for a review [25]). We have recently shown that SNAP induces cytoprotection via the activation of soluble guanylate cyclase in neonatal cardiomyocytes [10]. However, in our previous studies the efficacy of SNAP-induced cytoprotection was more pronounced in neonatal cardiomyocytes than shown here in mESC-derived cardiomyocytes. This difference is probably due to the low expression level of soluble guanylyl cyclase and NOS at 6–8-days-old stage of mESC-derived cardiomyocytes [26]. The latter is in line with our present results that the NOS inhibitor L-NNA did not affect cell viability after SI/reoxygenation injury of mESC-derived cardiomyocytes, showing that endogenous NO is not involved in cardiocytoprotection.

To test if activation of particulate guanylate cyclase can increase cell viability similar to SNAP, the effect of BNP was tested. BNP is a potent cardioprotective peptide, as it is able to reduce infarct size in rat hearts [16] and to protect neonatal rat cardiomyocytes against SI/reoxygenation injury [10]. 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 [27].

We further identified cardioprotective signaling pathways downstream of cGMP in mESC-derived cardiomyocytes. In the cardiovascular system, at least three classes of protein targets are activated by cGMP, i.e., cGMP-dependent PKG, cGMP-regulated phosphodiesterases, and cyclic nucleotide-gated ion channel. 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 mESC-derived cardiomyocyte viability, but interfered with the cytoprotective effect of SNAP, which suggests that the mechanism of SNAP-induced protection involves PKG. Our present findings in mESC-derived cardiomyocytes are consistent with our previous results obtained in neonatal rat cardiomyocytes, in which the PKG inhibitor abolished the protective effect of SNAP [10]. However, it is of interest that Mobley et al. [28] showed that PKG was down-regulated during cardiomyocyte differentiation and inhibition of PKG produced significantly more differentiated mESC-derived cardiomyocytes.

Xu et al. [29] demonstrated that exogenous NO mediates the production of reactive oxygen species and may act via activation cGMP/PKG signaling, triggering cardiocytoprotection by mitochondrial K_{ATP} channel opening or by opening mitochondrial permeability transition pores in adult rat cardiomyocytes K_{ATP} channels have a prominent role in the electrical excitability of early stage of mESC-derived cardiomyocytes. Therefore, in the present study, we investigated the involvement of K_{ATP} channels in SNAP-induced cytoprotection of 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 [30, 31].

Conclusions

Although the genotypic and phenotypic features of primary and ESC-derived cardiac myocytes are very similar [31], here we have shown that these cell types show differences under different test conditions, such as, e.g., hypoxia and reoxygenation. These findings emphasize the necessity for 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 an organ.

Our present study is the first demonstration that mESC-derived cardiomyocytes subjected to SI/reoxygenation injury are a useful alternative tool for in vitro screening for potential cardioprotective agents and to study their downstream cellular signaling pathways. The major advantages of ESC-based screening platforms over other cellular assays are the well reproducible production of beating myocardial cells and that it does not require sacrificing a number of animals.

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Conflict of interest None.

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