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Hexarelin targets neuroinflammatory pathways to preserve cardiac morphology and function in a mouse model of myocardial ischemia-reperfusion



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ABSTRACT

Acute myocardial ischemia and reperfusion injury (IRI) underly the detrimental effects of coronary heart disease on the myocardium. Despite the ongoing advances in reperfusion therapies, there remains a lack of effective therapeutic strategies for preventing IRI. Growth hormone secretagogues (GHS) have been demonstrated to improve cardiac function, attenuate inflammation and modulate the autonomic nervous system (ANS) in models of cardiovascular disease. Recently, we demonstrated a reduction in infarct size after administration of hexarelin (HEX), in a murine model of myocardial infarction. In the present study we employed a *reperfused* ischemic (IR) model, to determine whether HEX would continue to have a cardioprotective influence in a model of higher clinical relevance.

Myocardial ischemia was induced by transient ligation of the left descending coronary artery (tLAD) in C57BL/6J mice followed by HEX (0.3 mg/kg/day; n = 20) or vehicle (VEH) (n = 18) administration for 21 days, first administered immediately prior-to reperfusion. IR-injured and sham mice were subjected to high-field magnetic resonance imaging to assess left ventricular (LV) function, with HEX-treated mice demonstrating a significant improvement in LV function compared with VEH-treated mice. A significant decrease in interstitial collagen, TGF- β 1 expression and myofibroblast differentiation was also seen in the HEX-treated mice after 21 days. HEX treatment shifted the ANS balance towards a parasympathetic predominance; combined with a significant decrease in cardiac troponin-I and TNF- α levels, these findings were suggestive of an anti-inflammatory action on the myocardium mediated via HEX.

In this model of IR, HEX appeared to rebalance the deregulated ANS and activate vagal anti-inflammatory pathways to prevent adverse remodelling and LV dysfunction. There are limited interventions focusing on IRI that have been successful in improving clinical outcome in acute myocardial infarction (AMI) patients; this study provides compelling evidence towards the translational potential of HEX where all others have largely failed.

Abbreviations: AMI, acute myocardial infarction; ANS, autonomic nervous system; CAP, cholinergic anti-inflammatory pathway; cMRI, cardiac magnetic resonance imaging; CSNA, cardiac sympathetic nerve activation; CTnI, cardiac troponin I; CV, cardiovascular; EDV, end diastolic volume; EF, ejection fraction; ESV, end systolic volume; Gd-DTPA, gadopententate dimeglumine; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; HEX, hexarelin; HF, heart failure; HF_r, high-frequency; HR, heart rate; HRV, heart rate variability; HW, heart weight; IC, interstitial collagen; IL, interleukin; IR, ischemia reperfusion; IRI, myocardial ischemia reperfusion injury; LAD, left descending coronary artery; LF, low-frequency; LGE, late gadolinium enhancement; LV, left ventricle; MI, myocardial infarction; nHF_r, normalized high frequency power; nLF, normalized low frequency power; NTS, nucleus tractus solitaries; PPCI, percutaneous coronary intervention; SMA, smooth muscle actin; SNS, sympathetic nervous system; SV, stroke volume; TL, tibial length; tLAD, transient ligation of the left descending coronary artery; TNF, tumour necrosis factor; VEH, vehicle; VLF, very-low-frequency; VN, vagus nerve; VNS, vagal nerve stimulation

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1. Introduction

Ischemic heart disease is a major cause of death and disability worldwide [1,2]. Myocardial ischemia occurs when coronary blood flow and oxygen availability are insufficient to meet the requirements of the heart [1]. The consequences of this are dependent on the nature and severity of the ischemic episode and the elapsed time to subsequent re-establishment of coronary blood flow [1].

During AMI, occlusion of a coronary artery territory leads to myocyte necrosis. Without reperfusion or collateral blood supply, complete necrosis ensues. In the absence of reperfusion, very few interventions are able to limit infarct development [3,4]. The most effective and well established therapeutic strategy for reducing myocardial ischemic injury and infarct size is timely myocardial reperfusion through thrombolytic therapy or percutaneous coronary intervention (PPCI) [1]. Although necessary for tissue salvage, reperfusion may lead to further complications including diminished cardiac contractile function, arrhythmias and irreversible cell injury leading to necrosis and apoptosis [5]. The introduction of early reperfusion therapies have substantially reduced mortality and morbidity amongst AMI patients, however, there remains no effective therapy for the prevention of myocardial IRI and its sequelae [1]. Early arrhythmias can often prove fatal and chronic LV remodelling and heart failure (HF) continue to be major determinants of prognosis after AMI [6].

Activation of the sympathetic nervous system (SNS) in patients with AMI has been shown to contribute significantly to disease progression and prognosis [7]. Autonomic imbalance, characterized by vagal withdrawal and sympathetic predominance has been linked to impaired cardiovascular (CV) function and elevated mortality in patients with HF, ventricular arrhythmias, IRI and hypertension [8,9]. Traditionally, the CV function of the vagus nerve (VN) has entailed regulating sinus rhythm and atrioventricular node conduction. However, recent evidence indicates that the VN can have profound effects on CV function, remodelling, arrhythmias, IRI and mortality [10]. Reduced vagal tone, characterized by depressed heart rate variability (HRV) and blunted baroreflex sensitivity is closely related to disease progression and poor clinical outcomes in AMI and HF patients [11–13].

Inflammation is an important component of myocardial ischemic injury and HF [14]. It has been well established that enhancing parasympathetic tone decreases inflammation in various models of physiologic insult, such as cecal ligation and puncture, lipopolysaccharide injection, severe burn injury, traumatic brain injury and myocardial IRI [15–18]. Traditionally, CV drugs have had little influence on cytokines or the inflammatory reflex present in HF, thus, modulating the inflammatory response may represent a potential therapeutic strategy to protect against myocardial IRI and improve recovery of cardiac function [19].

Growth hormone secretagogues (GHS), a class of synthetic peptides stimulating growth hormone release through binding of the G-protein coupled receptor (GHS-R) have been demonstrated to have CV actions [20,21]. A number of studies have demonstrated the protective effects of the synthetic hexapeptide, hexarelin (HEX) in AMI, IR injury and cardiac fibrosis [20,22–24]. There are accumulating studies that describe the application of GHS in CV disease clinical trials with promising results [25,26], however there remains a paucity of data detailing the mechanisms underlying these effects. In preclinical disease models, there is evidence supporting the indirect cardioprotective action of GHS through central and peripheral modulation of the ANS [20,21,24,27].

The present study was designed to examine the effects of HEX on LV function and tissue characteristics in a mouse model of myocardial IR. A key event in the progression to HF is the pathological remodelling of the LV secondary to cardiac fibrosis [28]. In a recent publication we demonstrated the ability of HEX therapy to significantly reduce measures of cardiac fibrosis in myocardial-infarct injured mice using a permanent infarct model [24]. Unfortunately, this model is not readily applicable

to the human clinical setting, where early reperfusion therapy is paramount to improving outcome in AMI, thus, there is a clear need for studies employing reperfusion techniques at the preclinical level to allow for translational application. This study represents one of the first *in vivo* myocardial IR studies employing chronic HEX therapy.

2. Materials and methods

All experiments were approved by the Animal Ethics Committee of the University of Queensland and were performed in accordance to national guidelines (Ethics number SBMS/200/13/NHMRC).

2.1. IR surgical protocol

Myocardial IR was induced in 12–14 week old male C57BL/6 JArc inbred mice (n = 38) (JAX stock number: 000664) by transient ligation of the left anterior descending coronary artery (tLAD). Mice were anesthetized by intraperitoneal injection of medetomidine (1 mg/kg) and ketamine (75 mg/kg), intubated and supported by a small animal ventilator (Harvard Apparatus) with tidal volume and respiratory rate calculations described previously [29]. A left-sided thoracotomy was performed and the LAD was ligated 3 mm below the left auricular appendage using a 7–0 Prolene suture threaded through a 2 mm piece of sterile PE10 tubing. The tubing was heat-flared at the end in contact with the heart, creating a blunt “foot-print”, enabling occlusion of the LAD once tension was applied to the free ends of the suture. Successful occlusion was confirmed by visualization of a pallor region distal to the site of ligation along with characteristic electrocardiography changes. The suture was ligated at the distal end of the tubing for the specified 40-minute ischemic time. Sham-operated mice underwent the same procedure excluding ligation of the LAD.

After 40 min of ischemia (or sham procedure) the suture was released and removed with the PE tubing, allowing reperfusion. The chest was closed using 6–0 polydioxanone and the musculature and cutaneous tissues closed using a 5–0 non-absorbable suture.

On recovery, the mice were administered atipamezole (1 mg/kg) and a subcutaneous (SC) injection of carprofen (5 mg/kg) and 0.5 ml saline SC. The mice were recovered in an oxygen- and heat-supplemented environment and subsequently moved to their standard housing environment where they remained for 21 days whilst the experimental data was collected.

2.2. Treatment administration

HEX (0.3 mg/kg/day) or VEH was administered SC to each mouse immediately prior to reperfusion. Similarly, mice undergoing the sham procedure also received either VEH or HEX treatment. This dose was chosen based on previous studies demonstrating a cardioprotective effect [30]. All mice then received their respective treatments once daily throughout the 21-day study period.

2.3. Assessment of myocardial injury, inflammation and remodelling

2.3.1. Cytokine and Cardiac troponin (CnT)-I determination

Blood samples were collected 24 h and 21 days post tLAD ligation or sham procedure. The blood was allowed to clot and samples were centrifuged. The serum was immediately removed and stored at -80°C until assayed. The serum concentrations of CnT-I, interleukin (IL)-1 β , IL-6 and tumor necrosis factor (TNF)- α were measured at 24 h and 21 days post-operatively using a MILLIPLEX[®] map Assay according to the manufacturer's instructions (Merck Millipore).

2.3.2. HRV analysis

HRV analysis was performed 21 days post tLAD ligation or sham procedure. ECG signals were recorded using a physiological analyzing system (Bio Amp, AD Instruments, CA, USA). Mice were anaesthetized

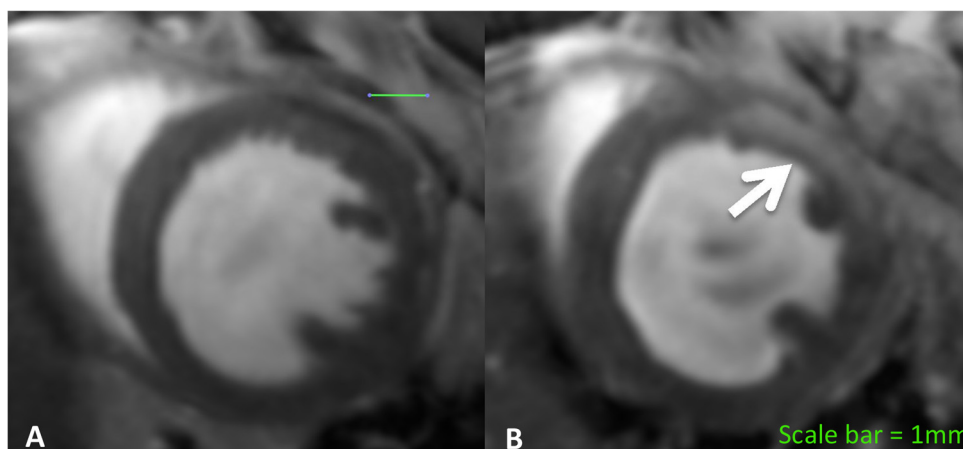


Fig. 1. Representative cMRI images with LGE. Representative LGE images acquired using a T_1 -weighted INTRAGATE gradient-echo sequence 24 h post-transient LAD ligation. The hyperintense region represents the infarcted tissue (indicated by arrow). (A). Sham; (B). tLAD.

cMRI, cardiac magnetic resonance imaging; LGE, late gadolinium enhancement; tLAD, transient ligation of the left descending coronary artery.

with isoflurane and ECG signals were recorded for a minimum of 20 min once the heart rate (HR) had stabilized. ANS function was examined by power spectral analysis of HRV (LabChart Pro 7.0, ADInstruments, Australia) where HR was used to generate a power spectral density curve using a fast Fourier transformation [31,32]. The area under the curve was calculated for the very-low-frequency (VLF: 0–0.15 Hz), low-frequency (LF: 0.15–1.5 Hz), and high-frequency (HF: 1.5–5 Hz) bands, with ranges based on previous studies [31]. The parameters: LF, HF, normalized LF power (nLF), normalized HF power (nHF), and the ratio of LF to HF power (LF/HF) were calculated as described in [32].

2.3.3. Histology and morphometric analysis

Serial transverse paraffin-embedded LV sections from each group of animals studied were stained with 0.1 % picosirius red to detect interstitial collagen deposition. Additional serial transverse LV sections from each corresponding group of animals studied were immunohistochemically stained for selected markers associated with collagen turnover; utilizing a polyclonal antibodies to transforming growth factor (TGF)- β 1 (sc-146; 1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and matrix metalloproteinase (MMP)-13 (the predominant collagenase in mice; 1:100 dilution; ab75606; Abcam; Redfern, NSW, Australia); or a monoclonal antibody to α -smooth muscle actin (α -SMA; a marker of myofibroblast differentiation; M0851; 1:250 dilution; DAKO Antibodies, Carpinteria, CA, USA). Detection of primary antibody staining was detected using DAKO Envision anti-rabbit or anti-mouse kits, respectively, and 3,3-diaminobenzidine. Morphometric analysis of picosirius red- and immunohistochemically-stained sections was performed using Aperio software (Leica Biosystems, North Ryde, NSW, Australia) on whole tissue sections (corrected for the area of tissue stained) per animal. In each case, the percentage staining of each marker analyzed per section was derived and expressed as the fold changes relative to the SHAM-VEH group, which was expressed as 1.

2.4. Assessment of myocardial function

2.4.1. High-field cardiac MRI

Mice were imaged using a 30cm-diameter horizontal bore Bruker Biospec 9.4 T (T) small animal MRI scanner equipped with a BGA 12S HP 660 m T/m gradient set. MRI data was acquired with a 86 mm i.d. quadrature transmit coil and a 2×2 phase array receive coil, running Paravision 5.1. (Bruker Biospin, Ettlingen, Germany).

2.4.2. Animal preparation

Anesthesia was induced using 5 % isoflurane in 100 % medical grade oxygen with a flow rate of 1 L/minute into an induction chamber. The mouse was positioned in a purpose-built cradle (Bruker, Germany)

and maintained with 1.5–2 % isoflurane in 1–2 L/min oxygen via a nose cone. Core temperature was monitored using a rectal probe and maintained with a warm water circulation system incorporated into the animal bed. A SAI Monitoring system (Small Animal Instruments, NY, USA) was used to record the electrocardiogram, using a 3-lead system with surface Ag-AgCl electrodes. Respiration was monitored with a pressure-transducer, from which a respiratory gating signal could be derived.

2.4.3. Protocol

Gadopentate dimeglumine (Gd-DTPA) (0.3 mmol/kg Magnevist, Bayer, Germany) was administered by intravenous (IV) infusion into the lateral tail vein of the mouse.

Following standard pre-scan calibration, 2- and 4-chamber view scout scans were acquired, from which a single mid-cavity short-axis slice was planned. Cine imaging was performed with a retrospectively-triggered (self-gated) INTRAGATE gradient-echo sequence [33], with the following parameters: TR = 5.6 ms, TE = 2.6 ms, flip angle = 10 degrees, number of movie frames = 20, slice thickness = 1 mm, matrix = 512×512 , field-of-view (FOV) = 4×4 cm [2]. This resulted in 78×78 μ m in-plane resolution, with ~ 5 min. acquisition per slice. Seven to nine short-axis slices with no slice gap were acquired to cover the heart from apex to base. Late gadolinium enhancement (LGE) images in the slice locations described above were acquired 10–15 min after IV Gd-DTPA (Fig. 1A/B).

2.4.4. Functional analysis

cMRI images, in DICOM format, were processed with Osirix [34] software. The end-diastolic and end-systolic phases were identified on a slice-by-slice basis and both the endocardial and epicardial borders were traced. The LV end systolic volume (ESV), end diastolic volume (EDV), stroke volume (SV) and ejection fraction (EF) were computed from the traced borders and LV mass was obtained by multiplying the volume by the specific gravity of 1.05 g/cm [3,35].

2.5. Statistical analysis

The data was expressed as mean \pm SEM or relative mean \pm SEM. Statistical analysis was performed using GraphPad Prism 7. Differences between groups were analysed by one-way ANOVA using a Newman Keuls post-hoc test to allow for multiple comparisons between groups. Unpaired *t*-test was applied to comparisons between two groups. $P < 0.05$ was considered statistically significant.

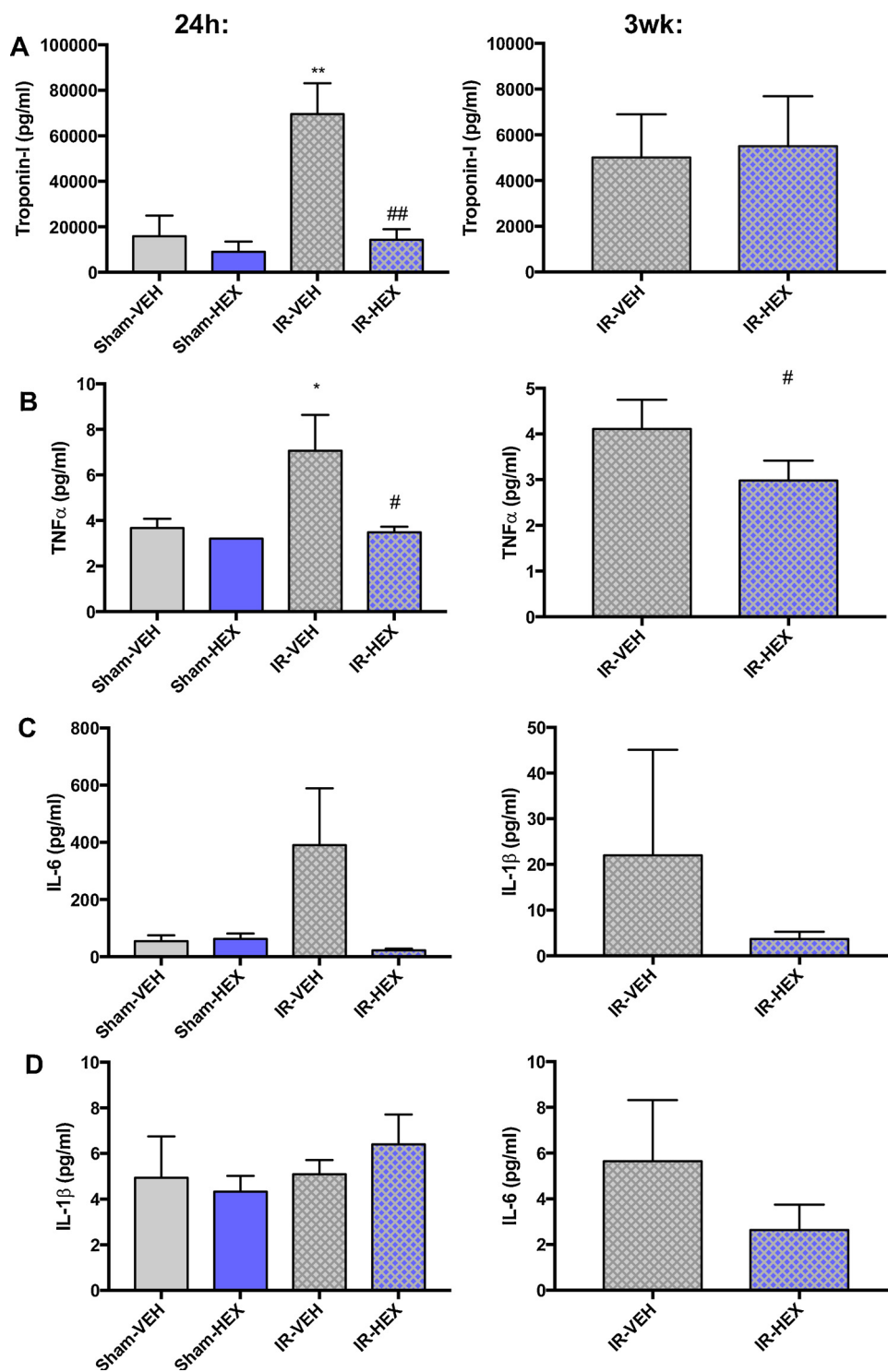


Fig. 2. Serum assessment of: (A). Troponin-I (pg/mL); (B). TNF-α (pg/mL); (C). IL-6 (pg/mL); (D). IL-1β (pg/mL). Serum levels assessed 24 h (left column) and 21 days (right column) post-procedure in sham-injured mice and mice undergoing IR procedure with or without HEX treatment. Data expressed as mean ± SEM; *p < 0.05, **p < 0.01, versus Sham-VEH group; #p < 0.05, ##p < 0.01, versus IR-VEH group. HEX, hexarelin; IR, ischemia reperfusion; LAD, left descending coronary artery; VEH, vehicle.

3. Results

3.1. Assessment of myocardial injury, inflammation and remodelling

3.1.1. CTn-I

In excess of a 4-fold increase in cTn-I was observed 24 h post tLAD

ligation in the IR-VEH compared with the IR-HEX group (Fig. 2A, left column). Thus, by protecting against cardiomyocyte injury after IR, HEX treatment appeared to significantly prevent an acute rise in cTnI. There was no significant difference in cTnI between the IR-VEH and IR-HEX group after 21 days (Fig. 2A, right column).

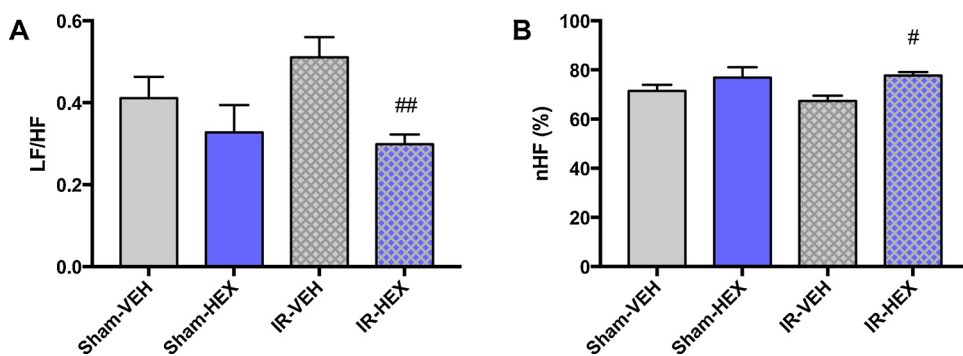


Fig. 3. Heart rate variability analysis. Electrocardiographic data recorded 21 days after sham surgery or transient LAD occlusion with or without HEX treatment. (A): Sympathetic nerve activity represented by low-frequency power (LF/HF); (B): Parasympathetic nerve activity represented by high-frequency power (nHF). Data expressed as mean \pm SEM; [#] $p < 0.05$, ^{##} $p < 0.01$, versus IR-VEH group. HEX, hexarelin; HF, high frequency; IR, ischemia reperfusion; LAD, left descending coronary artery; LF, low frequency; nHF, normalized high frequency power; VEH, vehicle.

3.1.2. Cytokines

Serum IL-1 β , IL-6 and TNF α levels were determined 24 h and 21 days post-sham operation or tLAD occlusion.

HEX treatment was found to prevent the significant rise in TNF α seen in the IR-VEH group (Fig. 2B). There were no significant differences noted in IL-6 or IL-1 β levels between the groups at either time point, although there was a trend towards reduction in each of the respective inflammatory cytokines' within the IR-HEX group (Fig. 2C/D).

3.1.3. HRV

HRV was examined in mice 21 days post-procedure. HRV analysis is considered an indirect measure of cardiac autonomic tone [31,36]. LF/HF is used to represent SNS activity, whereas nHF represents parasympathetic nervous activity [31].

The IR-HEX group demonstrated a parasympathetic predominance and sympathetic down-regulation based on a significantly lower LF/HF ratio and higher nHF compared with the IR-VEH group (Fig. 3A, B respectively).

3.1.4. LV mass and heart weight (HW)

LV mass was determined using cMRI 24 h and 21 days post procedure. There were no significant differences in LV mass at either of these time points (Fig. 4A/B).

To evaluate cardiac hypertrophy, heart weight was measured 21 days post-procedure. Tibial length (TL) was used as an adjustment factor.

HW/TL ratios did not differ between the groups (Fig. 4C), thus our HW/TL data was reflective of our cMRI-determined LV mass results.

3.1.5. Histology and morphometric analysis

Picrosirius red staining of the myocardium was used to detect changes in interstitial collagen (IC) deposition. IC content was significantly increased in the IR-VEH group; the presence of HEX was seen to suppress this aberrant post-IR IC deposition (without affecting basal IC) (Fig. 5A).

TGF- β 1 (Fig. 5B) and α -smooth muscle actin (SMA)-associated myofibroblast, arteriole and artery immunostaining (Fig. 5C) was significantly increased in the IR-VEH group compared to that measured in Sham-VEH counterparts. TGF- β 1 and α -SMA were both significantly suppressed by HEX-treatment post-IR. There was also a trend towards increased MMP-13 levels in the IR-HEX group compared to that measured in the IR-VEH group (Fig. 5D). These results suggest that HEX had a regulatory effect on cardiac remodelling and fibrosis post-IR.

3.2. Analysis of LV function

3.2.1. Twenty-four hours post IR/sham procedure

EF (%) was significantly reduced in the IR-VEH compared to the SHAM-VEH group; HEX treatment was seen to completely prevent this reduction. Both IR-VEH and IR-HEX groups showed a significant

increase in EDV 24-hs post-IR. The significant elevation in ESV seen within the IR-VEH was limited by HEX treatment (Fig. 6A).

3.2.2. Twenty-one days post-procedure

EF% remained significantly reduced in the IR-VEH group, whereas a normalization of EF% was observed in the IR-HEX group. Similarly, the IR-HEX group displayed a normalization in ESV after the 21 days of HEX therapy. There was no significant difference in LV EDV between the groups at this time point (Fig. 6B).

4. Discussion

There is a clear need to determine therapeutic strategies to protect the myocardium from the detrimental effects of IRI. Although improvements in myocardial reperfusion continue to take place with new antiplatelet and thrombolytic agents, there remains to be no effective therapeutic strategy for preventing myocardial IRI [1]. In this study we demonstrated that HEX administration reduced serum inflammatory cytokines profiles and cTn-I, reflecting an overall reduction in cardiomyocyte injury [37]. Downstream, the effects of chronic HEX treatment resulted in significantly reduced measures of cardiac fibrosis and an overall preservation in LV function. Additionally, HEX treatment was seen to shift the balance of ANS activity towards a parasympathetic predominance. These findings provide evidence towards the cumulative effects of simultaneous sympathetic down-regulation, vagal enhancement and activation of anti-inflammatory pathways to prevent adverse LV remodelling and dysfunction.

At the hospital, reduction in AMI ischemic injury requires a treatment protocol that minimizes the door-to-PPCI time. Most preclinical studies in this field consist of a surgical protocol involving permanent ligation of the LAD (without allowing reperfusion of the ischemic tissue) [24,30,38]. Therefore these studies fail to clearly assess the critical interaction of ischemia, reperfusion injury and the subsequent therapeutic intervention. By employing an IR model, the current study closely simulates the critical "door-to-PPCI time" concept; therefore representing a superior model and evidence towards the potential value of HEX in AMI patients.

4.1. Relevance of the autonomic nervous system and inflammation

The high mortality in myocardial infarction has been strongly associated with an acute and sustained increase in cardiac SNS activity [6,7,32]. Although chronic therapeutic interventions have been developed for chronic HF, it is the period immediately after AMI, before autonomic modulation of cardiac function becomes irreversibly activated, that appears to be critical for optimizing short and long-term outcome by therapeutic intervention [6]. Targeting adverse SNS activation and uncontrolled inflammation immediately post-infarction appears to offer a chronic cardioprotective advantage by preventing adverse fibroblast activation, ventricular remodelling and dysfunction. There is accumulating clinical and experimental evidence that indicates

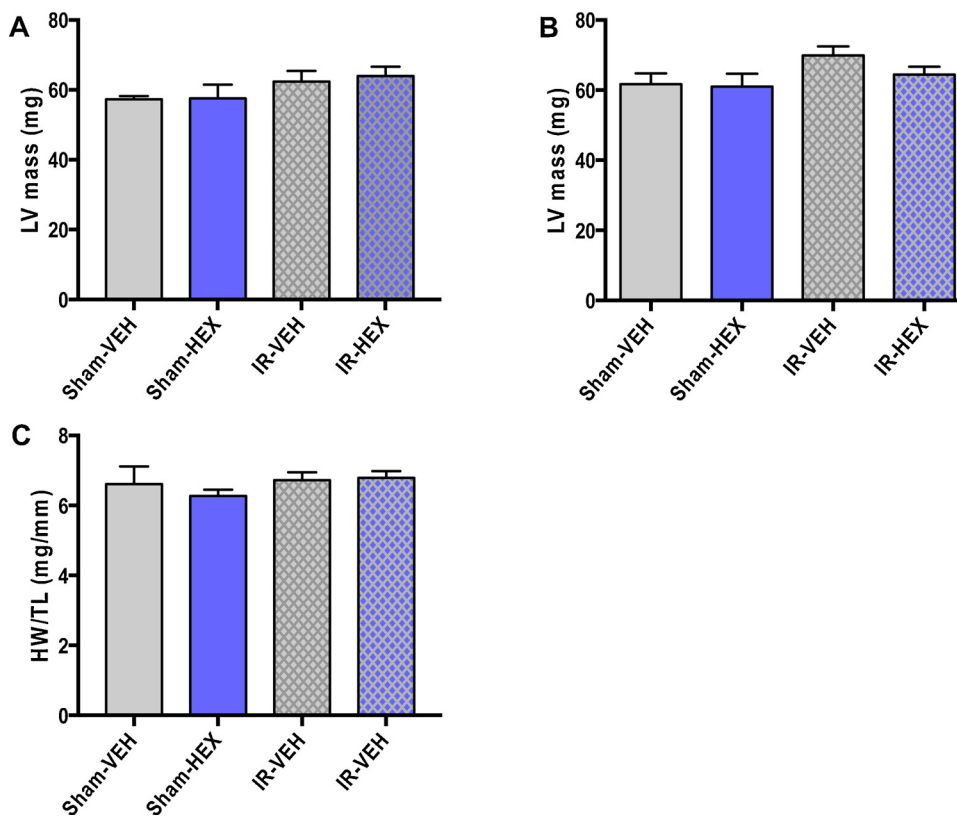


Fig. 4. Assessment of myocardial mass and heart weight.

Myocardial mass (mg) assessed by cMRI after: (A). 24 h post tLAD ligation; (B). 21 days post-procedure in sham-injured mice and mice undergoing IR procedure with or without HEX treatment.

Heart weight (C). measured post-mortem after 21 days. Tibial length (TL) was used as an adjustment factor.

The data is expressed as mean \pm SEM. There were no significant differences in myocardial mass or HW/TL ratio between groups. cMRI, cardiac magnetic resonance imaging; HEX, hexarelin; HW, heart weight; IR, ischemia reperfusion; LAD, left descending coronary artery; LV, left ventricle; TL, tibial length; VEH, vehicle.

inhibition of cardiac sympathetic nerve activation improves survival and mitigates LV remodelling and dysfunction post-MI [6,39].

4.2. GHS and the autonomic nervous system

Early treatment with the endogenous GHS ghrelin has been shown to reduce the incidence of ventricular arrhythmias and improve early survival prognosis by preventing the early increase in cardiac sympathetic nervous system activation (CSNA) in rodent models of MI [39]. CSNA inhibition was maintained for at least 2 weeks after a single bolus of ghrelin, resulting in attenuation of cardiac dysfunction, remodelling and reduced mortality [6]. Comparable findings were demonstrated with chronic ghrelin treatment [40]. Similarly, mice that received one oral dose of HEX treatment within 30 min of permanent LAD ligation were observed to have a higher ejection fraction 14 days after MI with lowered plasma epinephrine and dopamine levels. These results suggest that early GHS therapy preserves chronic cardiac function after AMI [38].

In a recent study, ghrelin knock-out mice were employed to demonstrate the crucial role of endogenous ghrelin in preventing CSNA, reducing the incidence of arrhythmias and improving survival prognosis post-AMI [41]. Catecholamine concentrations were dramatically increased after AMI in ghrelin KO mice, reflective of stronger sympathetic nerve activation in this group. Endogenous ghrelin was demonstrated to prevent adverse ventricular remodelling and preserve myocardial function [41]. Interestingly, the beneficial effect of ghrelin was abolished after blockade of the VN [32]. Furthermore, it has been suggested that in healthy human subjects ghrelin may suppress CSNA and stimulate cardiac PS nerve activity [42], with the sympatho-inhibitory effects of ghrelin not found to be evident in vagotomised humans [43].

4.3. The cholinergic anti-inflammatory pathway and GHS

Excessive immune-mediated inflammatory responses in reaction to

myocardial injury have been shown to have deleterious effects on post-infarction LV remodelling and dysfunction and can influence progression to HF [44]. A timely, well-orchestrated inflammatory response is critical to achieve successful infarct healing in patients surviving an AMI [44]. It has been well established that increasing parasympathetic tone, through vagal nerve stimulation (VNS) can decrease inflammation in various models of physiologic insult, including myocardial IRI, cecal ligation and puncture, LPS injection, severe burn injury and traumatic brain injury [16,17,45,46]. Thus, pharmacological stimulation of the VN may offer a novel approach to anti-inflammatory therapy in AMI-patients.

A multitude of studies highlight the importance of the role of the VN in the physiological functions of GHS [47]. Ghrelin is known to cross the blood-brain barrier and is produced both centrally and within the periphery [48]. The nodose ganglion is a constellation of vagal afferent neurons that synthesize the ghrelin receptor (GHS-R). These receptors are transported to their vagal afferent terminals, such as those located within the digestive tract for regulation of energy metabolism and food intake [49]. Studies have also demonstrated the existence of the GHS-R within the infarcted myocardium [40]. Co-staining with acetylcholinesterase suggests that the GHS-R is localized within the VN terminals in the heart and sends afferent projections to the nucleus tractus solitaries (NTS), a major CV control centre within the brain [50]. The ghrelin receptor has also been localized to the dorsal motor nucleus of the vagus [51,52]. It has been demonstrated that GHS-R mediates the action of HEX and HEX is recognized to be both chemically more stable and functionally more potent than ghrelin [53,54]. Peripherally, GHS have been demonstrated to act on the cardiac vagal afferent nerve, resulting in a reflex decrease in sympathetic activity in rats with MI [39, 40].

Furthermore, it has been well described that activation of efferent VN fibres can modulate local and systemic inflammatory responses, through the 'cholinergic anti-inflammatory pathway' (CAP) [18]. The CAP is mediated through vagal efferent firing leading to an acetylcholine-dependent interaction with the α -7 nicotinic acetylcholine receptor

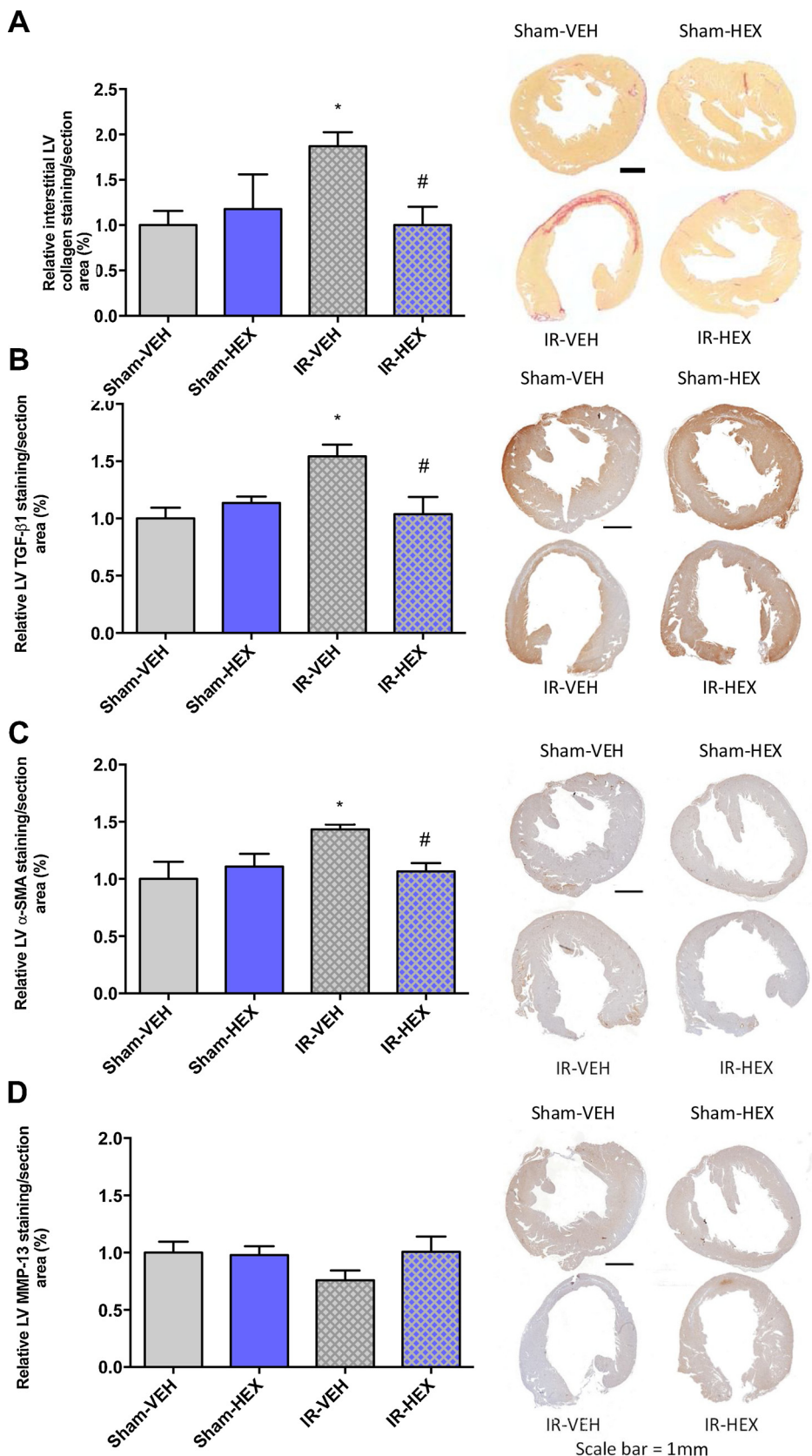


Fig. 5. Histology and morphometric analysis. (A). Interstitial LV collagen content measured by picrosirius red staining; (B). TGF-β1; (C). α-SMA; (D). MMP-13, immunohistochemistry staining measured in sham-injured mice and mice undergoing IR with or without HEX treatment. The right column displays representative low power photomicrograph sections from each treatment group corresponding to the selected markers and methods described above. Low power (whole mount) sections of heart have been selected to demonstrate the transmural staining with regional differences that would not be as clearly observable at high power. Data expressed as mean ± SEM; *p < 0.05, versus Sham-VEH group; #p < 0.05, versus IR-VEH group. HEX, hexarelin; IR, ischemia reperfusion; LV, left ventricle; VEH, vehicle.

(α7nAChR) subunit on monocytes and macrophages and ultimately a reduction in inflammatory cytokine production [18]. Activation of the CAP is possible via VN stimulation or pharmacologically via administration of selective α7nAChR agonists or inhibitors of

acetylcholinesterase [18].

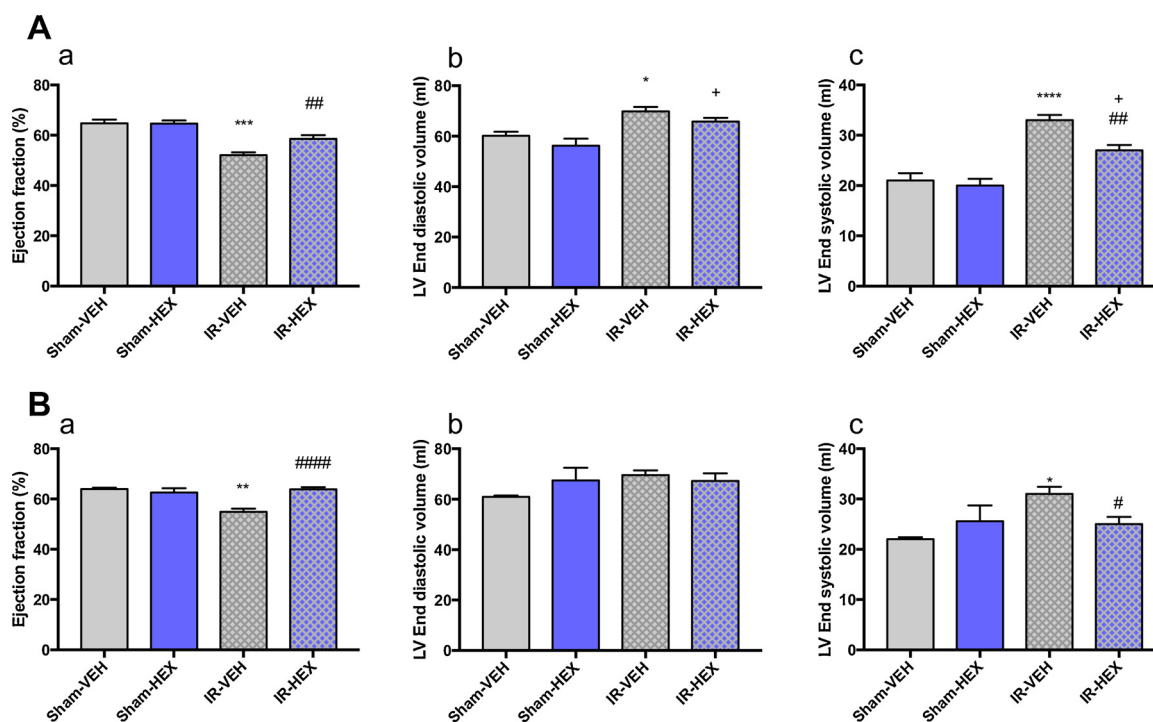


Fig. 6. Cardiac functional parameters measured by cMRI.

(a). Ejection fraction (%), (b). LV end-diastolic volume, (c). LV end-systolic volume measured in sham-injured mice and mice undergoing transient LAD occlusion (IR) with or without HEX treatment.

(A). 24 h post-procedure; (B). 21 days post-procedure.

Data expressed as mean \pm SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, versus Sham-VEH group; # $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$, versus IR-VEH group; + $p < 0.05$, versus Sham-HEX group.

cMRI, cardiac magnetic resonance imaging; HEX, hexarelin; IR, ischemia reperfusion; LAD, left descending coronary artery; LV, left ventricle; VEH, vehicle.

4.4. GHS, inflammation and vagal activation

GHS have been shown to have several anti-inflammatory properties involving vagus activation. Intravenous ghrelin administration in septic mice has been shown to decrease levels of pro-inflammatory cytokines mediated through the VN [55]. Ghrelin administration has been demonstrated to decrease TNF- α and IL-6, increase cardiac output, organ perfusion and reduce mortality in rat models of endotoxemia and polymicrobial sepsis, again mediated via the VN [55–58]. Vagotomy has been demonstrated to completely prevent these effects [55]. Furthermore, ghrelin has been demonstrated to have no direct effect on cytokine release from Kupffer cells or peritoneal macrophages isolated from normal rats, suggesting an indirect anti-inflammatory mechanism [55].

Inflammation has been demonstrated to play a major role in IRI [14]. GHS has been demonstrated to activate the CAP in various IR models, Wu et al. demonstrated that administration of ghrelin after gut IR attenuated excessive inflammation and reduced organ injury through the rapid activation of the CAP [59]. In a rat model of renal IR, ghrelin administration during reperfusion was found to attenuate both systemic and renal-specific inflammatory responses, also mediated through the VN [60,61]. The absence of endogenous ghrelin has been shown to result in severe cardiac hypertrophy and diastolic dysfunction in a mouse model of pressure-overload cardiac hypertrophy. Inhibition of the CAP was thought to be responsible for these adverse processes and administration of ghrelin was shown to reverse these effects [62].

4.5. The cardioprotective action of HEX in AMI

Myocardial infarction leads to progressive ventricular remodelling, increased myocardial wall stress and possible progression to HF [63]. Following ischemia, alterations in the collagen network can result in

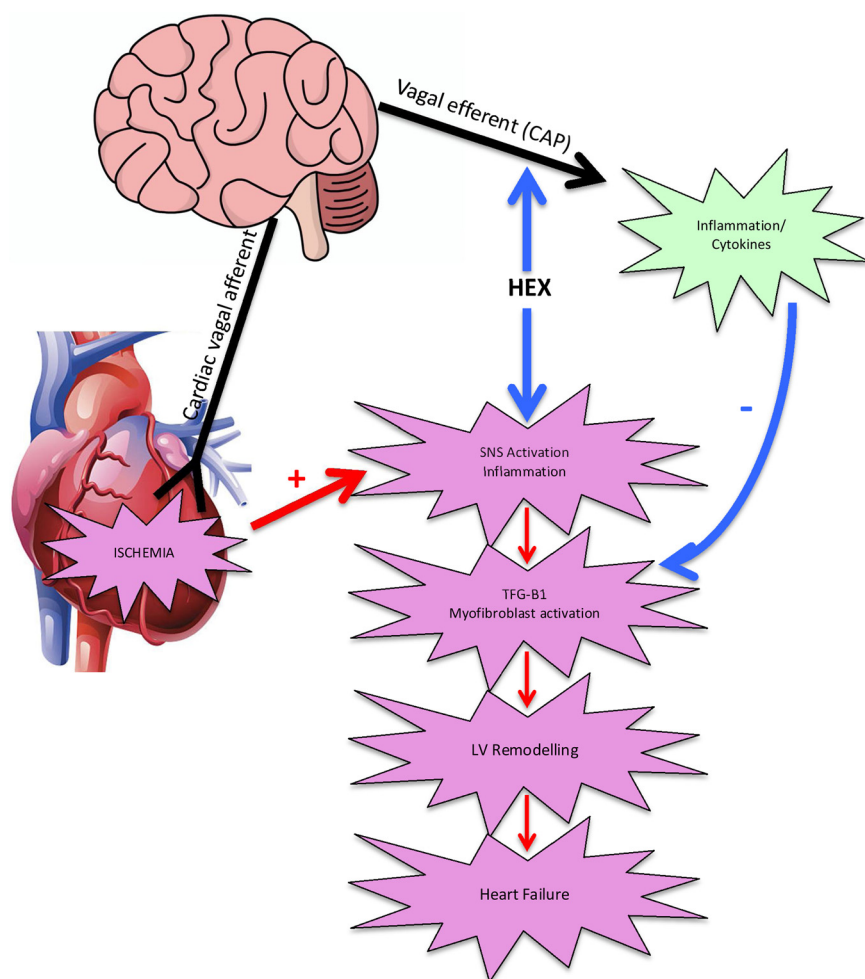
progressive changes in LV morphology and function with an increase in interstitial collagen likely to impair both diastolic and systolic function [64].

Post-infarction repair requires timely suppression of the inflammatory response to prevent catastrophic consequences of uncontrolled inflammation on cardiac geometry and function [63]. Studies suggest a direct involvement of fibroblasts in activation of the post-infarction inflammatory reaction. TGF- β 1 is markedly augmented following infarction, is a potent pro-fibrotic cytokine and has profound effects on fibroblast phenotype and function, including activation of myofibroblasts. Preventing TGF- β 1 activation in a myofibroblast-specific manner holds promise in counteracting cardiac fibrosis [63].

Our recent work demonstrated a reduction in inflammatory cytokines and improvement in LV function in a mouse model of MI, thought to involve activation of the CAP. In this study, HEX influenced post-infarct fibrotic healing, likely by involving the suppression of profibrotic factors and favouring a net reduction in aberrant collagen content [24]. Furthermore, Huang et al. recently demonstrated that HEX protected cardiomyocytes from IR injury partly through modification of the IL-1 signalling pathway via activation of the GHS-R [22].

In this current study, we demonstrated a HEX-mediated decrease in TGF- β 1 expression and myofibroblast differentiation post-myocardial IR. Several studies have shown that TGF- β 1 is a key stimulator of myofibroblast differentiation post-MI [65,66]. MMP-9 and TGF- β 1 also both play key roles in the progression of post-MI remodeling [67,68] and inflammatory cytokines such as IL-1 can regulate fibroblast phenotype. The activation of MMPs may influence changes in extracellular matrix degradation (ECM) and collagen deposition [22,69,70].

Thus, via vagal enhancement and activation of the cholinergic anti-inflammatory pathway, combined with simultaneous suppression of the acute rise in SNS activation, HEX appears to mitigate adverse inflammation. Furthermore, this anti-inflammatory action appears to



downregulate TGF- β 1 expression and myofibroblast differentiation as part of its potential therapeutic effects [24] (Fig. 7).

5. Conclusion

Targeting autonomic imbalance by augmentation of parasympathetic tone has emerged as a promising therapeutic approach for management of ischemic heart disease and HF [14]. The VN is suggested to play a central role in GHS-observed effects and acts as an endogenous mechanism to regulate the immune response and inflammation [20]. In the present study, HEX treatment was demonstrated to modulate the ANS and influence the inflammatory response induced by myocardial IR. Our results demonstrate that HEX may have an important influence on balancing the sympathetic and parasympathetic nervous system and modulating adverse inflammatory pathways in response to AMI. Pharmacological stimulation of the VN by HEX may offer a novel cardioprotective strategy against IRI by preventing myofibroblast activation and LV remodelling in AMI.

5.1. Clinical relevance

The research field of cardio-protection has been plagued by numerous failed attempts to translate promising therapeutic strategies for preventing myocardial ischemic injury discovered in the basic science laboratory into the clinical setting [1]. A major factor underlying this failure entails the inappropriate use of experimental animal models. In our previous study we were able to clearly demonstrate the cardioprotective effect of HEX therapy in a mouse model of permanent MI, however, the translational aspect of this model is limited. HEX as an

Fig. 7. In response to myocardial IR, HEX activates endogenous neural pathways to restrain adverse inflammation and plays a role in inhibiting pathways of pathological LV remodelling.

Myocardial ischemia initiates SNS activation and an acute inflammatory response; inflammatory cytokines exert pro-inflammatory actions on cardiac fibroblasts and can result in LV dysfunction. TGF- β 1, a potent pro-fibrotic cytokine, is increased following MI and can cause excessive ECM production and myofibroblast activation. By suppressing inflammatory pathways and sympathetic activation HEX appears to attenuate these negative effects on cardiac remodelling and dysfunction. Additionally, pharmacological stimulation of vagal pathways by HEX appears to decrease levels of proinflammatory cytokines through activation of the CAP.

emerging therapeutic agent for prevention of myocardial IRI has shown promise in this study using a clinically relevant model, where HEX was administered prior to myocardial reperfusion (removal of the coronary ligature), representing a protocol that could be employed in the reperfusion unit. Therefore, this study signifies a noteworthy contribution to the field of cardio-protection.

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Declaration of Competing Interest

None.

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References

- [1] D.J. Hausenloy, D.M. Yellon, Myocardial ischemia-reperfusion injury: a neglected therapeutic target, *J. Clin. Invest.* 123 (2013) 92–100.

- [2] S. Suvarna, *Cardiac Pathology: a Guide to Current Practice*, Springer Science & Business Media, 2012.
- [3] C. Csonka, K. Kupai, G.F. Kocsis, G. Novak, V. Fekete, P. Bencsik, T. Csont, P. Ferdinandy, Measurement of myocardial infarct size in preclinical studies, *J. Pharmacol. Toxicol. Methods* 61 (2010) 163–170.
- [4] E. Braunwald, R.A. Kloner, Myocardial reperfusion: a double-edged sword? *J. Clin. Invest.* 76 (1985) 1713.
- [5] P. Ferdinandy, R. Schulz, G.F. Baxter, Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning, *Pharmacol. Rev.* 59 (2007) 418–458.
- [6] D.O. Schwenke, T. Tokudome, I. Kishimoto, T. Horio, P.A. Cragg, M. Shirai, K. Kangawa, One dose of ghrelin prevents the acute and sustained increase in cardiac sympathetic tone after myocardial infarction, *Endocrinology* 153 (2012) 2436–2443.
- [7] D.M. Kaye, J. Lefkowitz, G.L. Jennings, P. Bergin, A. Broughton, M.D. Esler, Adverse consequences of high sympathetic nervous activity in the failing human heart, *J. Am. Coll. Cardiol.* 26 (1995) 1257–1263.
- [8] S. Bibeovski, M.E. Dunlap, Evidence for impaired vagus nerve activity in heart failure, *Heart Fail. Rev.* 16 (2011) 129–135.
- [9] F.M. Abboud, S.C. Harwani, M.W. Chapleau, Autonomic neural regulation of the immune system: implications for hypertension and cardiovascular disease, *Hypertension* 59 (2012) 755–762.
- [10] B. Olshansky, H.N. Sabbah, P.J. Hauptman, W.S. Colucci, Parasympathetic nervous system and heart failure: pathophysiology and potential implications for therapy, *Circulation* 118 (2008) 863–871.
- [11] L. Wu, Z. Jiang, C. Li, M. Shu, Prediction of heart rate variability on cardiac sudden death in heart failure patients: a systematic review, *Int. J. Cardiol.* 174 (2014) 857–860.
- [12] M. La Rovere, G. Pinna, R. Maestri, P. Sleight, Clinical value of baroreflex sensitivity, *Netherlands Heart J.* 21 (2013) 61–63.
- [13] P. Schwartz, Vagal stimulation for heart diseases: from animals to men. An example of translational cardiology, *Netherlands Heart J.* 21 (2013) 82–84.
- [14] B. Olshansky, Vagus nerve modulation of inflammation: cardiovascular implications, *Trends Cardiovasc. Med.* (2015).
- [15] L. Calvillo, E. Vanoli, E. Andreoli, A. Besana, E. Omodeo, M. Gnechi, P. Zerbi, G. Vago, P.J. Schwartz, Vagal stimulation, through its nicotinic action, limits infarct size and the inflammatory response to myocardial ischemia and reperfusion, *J. Cardiovasc. Pharmacol.* 58 (2011) 500–507.
- [16] T.W. Costantini, V. Bansal, C.Y. Peterson, W.H. Loomis, J.G. Putnam, F. Rankin, P. Wolf, B.P. Eliceiri, A. Baird, R. Coimbra, Efferent vagal nerve stimulation attenuates gut barrier injury after burn: modulation of intestinal occludin expression, *J. Trauma* 68 (2010) 1349–1354 discussion 1354–1346.
- [17] V. Bansal, T. Costantini, S.Y. Ryu, C. Peterson, W. Loomis, J. Putnam, B. Elicieri, A. Baird, R. Coimbra, Stimulating the central nervous system to prevent intestinal dysfunction after traumatic brain injury, *J. Trauma* 68 (2010) 1059–1064.
- [18] K.J. Tracey, Physiology and immunology of the cholinergic antiinflammatory pathway, *J. Clin. Invest.* 117 (2007) 289–296.
- [19] J. Xiong, Y. Yuan, F. Xue, Q. Wang, S. Li, X. Liao, J. Liu, Y. Chen, R. Li, Combined postconditioning with ischemia and $\alpha 7$ nAChR agonist produces an enhanced protection against rat myocardial ischemia reperfusion injury, *Chin. Med. J.* 125 (2012) 326–331.
- [20] Y. Mao, T. Tokudome, I. Kishimoto, The cardiovascular action of hexarelin, *J. Geriatr. Cardiol.* 11 (2014) 253–258.
- [21] I. Kishimoto, T. Tokudome, D.O. Schwenke, S. Takeshi, H. Hosoda, N. Nagaya, K. Kangawa, Therapeutic potential of ghrelin in cardiac diseases, *Expert Rev. Endocrinol. Metab.* 4 (2009) 283–289.
- [22] J. Huang, Y. Li, J. Zhang, Y. Liu, Q. Lu, The growth hormone secretagogue hexarelin protects rat cardiomyocytes from *in vivo* ischemia/reperfusion injury through Interleukin-1 signaling pathway, *Int. Heart J.* 58 (2017) 257–263.
- [23] J. Berlanga-Acosta, A. Abreu-Cruz, D. García-del Barco Herrera, Y. Mendoza-Marí, A. Rodríguez-Ulloa, A. García-Ojalvo, V. Falcón-Cama, F. Hernández-Bernal, Q. Beichen, G. Guillén-Nieto, Synthetic growth hormone-releasing peptides (GHRPs): a historical appraisal of the evidences supporting their cytoprotective effects, *Clin. Med. Insights Cardiol.* 11 (2017).
- [24] H. McDonald, J. Peart, N. Kurniawan, G. Galloway, S. Royce, C.S. Samuel, C. Chen, Hexarelin treatment preserves myocardial function and reduces cardiac fibrosis in a mouse model of acute myocardial infarction, *Physiol. Rep.* 6 (2018) e13699.
- [25] N. Nagaya, M. Kojima, M. Uematsu, M. Yamagishi, H. Hosoda, H. Oya, Y. Hayashi, K. Kangawa, Hemodynamic and hormonal effects of human ghrelin in healthy volunteers, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 280 (2001) R1483–R1487.
- [26] N. Nagaya, J. Moriya, Y. Yasumura, M. Uematsu, F. Ono, W. Shimizu, K. Ueno, M. Kitakaze, K. Miyatake, K. Kangawa, Effects of ghrelin administration on left ventricular function, exercise capacity, and muscle wasting in patients with chronic heart failure, *Circulation* 110 (2004) 3674–3679.
- [27] M. Shirai, N. Joe, H. Tsuchimochi, T. Sonobe, D.O. Schwenke, Ghrelin suppresses sympathetic hyperexcitation in acute heart failure in male rats: assessing centrally and peripherally mediated pathways, *Endocrinology* 156 (2015) 3309–3316.
- [28] I.M. Dixon, R.H. Cunningham, S.G. Rattan, J.T. Wigle, *Cardiac Fibrosis and Heart Failure—Cause or Effect? Cardiac Fibrosis and Heart Failure: Cause or Effect?* Springer, 2015, pp. 1–4.
- [29] O. Tarnavski, J.R. McMullen, M. Schinke, Q. Nie, S. Kong, S. Izumo, Mouse cardiac surgery: comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies, *Physiol. Genomics* 16 (2004) 349–360.
- [30] Y. Mao, T. Tokudome, I. Kishimoto, K. Otani, H. Hosoda, C. Nagai, N. Minamino, M. Miyazato, K. Kangawa, Hexarelin treatment in male ghrelin knockout mice after myocardial infarction, *Endocrinology* 154 (2013) 3847–3854.
- [31] J. Thireau, B.L. Zhang, D. Poisson, D. Babuty, Heart rate variability in mice: a theoretical and practical guide, *Exp. Physiol.* 93 (2008) 83–94.
- [32] Y. Mao, T. Tokudome, K. Otani, I. Kishimoto, M. Nakanishi, H. Hosoda, M. Miyazato, K. Kangawa, Ghrelin prevents incidence of malignant arrhythmia after acute myocardial infarction through vagal afferent nerves, *Endocrinology* 153 (2012) 3426–3434.
- [33] S.M. Bovens, B. Boekhorst, K. den Ouden, K.W.A. van de Kolk, A. Nauerth, M.G.J. Nederhoff, G. Pasterkamp, M. ten Hove, C.J.A. van Echteld, Evaluation of infarcted murine heart function: comparison of prospectively triggered with self-gated MRI, *NMR Biomed.* 24 (2011) 307–315.
- [34] A. Rosset, L. Spadola, O. Ratib, OsiriX: an open-source software for navigating in multidimensional DICOM images, *J. Digit. Imaging* 17 (2004) 205–216.
- [35] S. Bohl, C.A. Lygate, H. Barnes, D. Medway, L.A. Stork, J. Schulz-Menger, S. Neubauer, J.E. Schneider, Advanced methods for quantification of infarct size in mice using three-dimensional high-field late gadolinium enhancement MRI, *Am. J. Physiol. Heart Circ. Physiol.* 296 (2009) H1200–1208.
- [36] *Cardiology TFOtEsO*, Heart rate variability standards of measurement, physiological interpretation, and clinical use, *Eur. Heart J.* 17 (1996) 354–381.
- [37] A. Frobert, J. Valentin, J.-L. Magnin, E. Riedo, S. Cook, M.-N. Giraud, Prognostic value of troponin I for infarct size to improve preclinical myocardial infarction small animal models, *Front. Physiol.* 6 (2015) 353.
- [38] Y. Mao, T. Tokudome, I. Kishimoto, K. Otani, M. Miyazato, K. Kangawa, One dose of oral hexarelin protects chronic cardiac function after myocardial infarction, *Peptides* 56 (2014) 156–162.
- [39] D.O. Schwenke, T. Tokudome, I. Kishimoto, T. Horio, M. Shirai, P.A. Cragg, K. Kangawa, Early ghrelin treatment after myocardial infarction prevents an increase in cardiac sympathetic tone and reduces mortality, *Endocrinology* 149 (2008) 5172–5176.
- [40] T. Soeki, I. Kishimoto, D.O. Schwenke, T. Tokudome, T. Horio, M. Yoshida, H. Hosoda, K. Kangawa, Ghrelin suppresses cardiac sympathetic activity and prevents early left ventricular remodeling in rats with myocardial infarction, *Am. J. Physiol. Heart Circ. Physiol.* 294 (2008) H426–432.
- [41] Y. Mao, T. Tokudome, K. Otani, I. Kishimoto, M. Miyazato, K. Kangawa, Excessive sympathetic activation and deteriorated heart function after myocardial infarction in male ghrelin knockout mice, *Endocrinology* 154 (2013) 1854–1863.
- [42] T. Soeki, K. Koshiba, T. Niki, K. Kusunose, K. Yamaguchi, H. Yamada, T. Wakatsuki, M. Shimabukuro, K. Minakuchi, I. Kishimoto, K. Kangawa, M. Sata, Effect of ghrelin on autonomic activity in healthy volunteers, *Peptides* 62 (2014) 1–5.
- [43] M.S. Huda, H. Mani, T. Dovey, J.C. Halford, E. Boyland, C. Daoust, J.P. Wilding, J. Pinkney, Ghrelin inhibits autonomic function in healthy controls, but has no effect on obese and vagotomized subjects, *Clin. Endocrinol. (Oxf.)* 73 (2010) 678–685.
- [44] S.C. Latet, V.Y. Hoymans, P.L. Van Herck, C.J. Vrints, The cellular immune system in the post-myocardial infarction repair process, *Int. J. Cardiol.* 179 (2015) 240–247.
- [45] C. Mioni, C. Bazzani, D. Giuliani, D. Altavilla, S. Leone, A. Ferrari, L. Minutoli, A. Bitto, H. Marini, D. Zaffe, A.R. Botticelli, A. Iannone, A. Tomasi, A. Bigiani, A. Bertolini, F. Squadrito, S. Guarini, Activation of an efferent cholinergic pathway produces strong protection against myocardial ischemia/reperfusion injury in rats, *Crit. Care Med.* 33 (2005) 2621–2628.
- [46] V.A. Pavlov, M. Ochani, L.-H. Yang, M. Gallowitsch-Puerta, K. Ochani, X. Lin, J. Levi, W.R. Parrish, M. Rosas-Ballina, C.J. Czura, Selective $\alpha 7$ -nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis, *Crit. Care Med.* 35 (2007) 1139–1144.
- [47] Y. Date, N. Murakami, K. Toshinai, S. Matsukura, A. Nijima, H. Matsuo, K. Kangawa, M. Nakazato, The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats, *Gastroenterology* 123 (2002) 1120–1128.
- [48] C. González-Arancibia, J. Escobar-Luna, C. Barrera-Bugueño, C. Díaz-Zepeda, M.P. González-Toro, L. Olavarría-Ramírez, F. Zanelli-Massai, M. Gotteland, J.A. Bravo, M. Julio-Pieper, What goes around comes around: novel pharmacological targets in the gut–brain axis, *Ther. Adv. Gastroenterol.* 9 (2016) 339–353.
- [49] H. Ueno, M. Nakazato, Mechanistic relationship between the vagal afferent pathway, central nervous system and peripheral organs in appetite regulation, *J. Diabet. Investig.* 7 (2016) 812–818.
- [50] S. Shimizu, T. Akiyama, T. Kawada, T. Sonobe, A. Kamiya, T. Shishido, T. Tokudome, H. Hosoda, M. Shirai, K. Kangawa, M. Sugimachi, Centrally administered ghrelin activates cardiac vagal nerve in anesthetized rabbits, *Auton. Neurosci.* 162 (2011) 60–65.
- [51] W. Zhang, T.R. Lin, Y. Hu, Y. Fan, L. Zhao, E.L. Stuenkel, M.W. Mulholland, Ghrelin stimulates neurogenesis in the dorsal motor nucleus of the vagus, *J. Physiol.* 559 (2004) 729–737.
- [52] Y. Lin, K. Matsumura, M. Fukuhara, S. Kagiya, K. Fujii, M. Iida, Ghrelin acts at the nucleus of the solitary tract to decrease arterial pressure in rats, *Hypertension* 43 (2004) 977–982.
- [53] V. Bodart, M. Febbraio, A. Demers, N. McNicoll, P. Pohankova, A. Perreault, T. Sejlitz, E. Escher, R. Silverstein, D. Lamontagne, CD36 mediates the cardiovascular action of growth hormone-releasing peptides in the heart, *Circ. Res.* 90 (2002) 844–849.
- [54] Q. Sun, W.-J. Zang, C. Chen, Growth hormone secretagogues reduce transient outward K^+ current via phospholipase C/protein kinase C signaling pathway in rat ventricular myocytes, *Endocrinology* 151 (2010) 1228–1235.
- [55] R. Wu, W. Dong, X. Cui, M. Zhou, H.H. Simms, T.S. Ravikumar, P. Wang, Ghrelin down-regulates proinflammatory cytokines in sepsis through activation of the vagus nerve, *Ann. Surg.* 245 (2007) 480–486.

- [56] R. Wu, W. Dong, M. Zhou, F. Zhang, C.P. Marini, T.S. Ravikumar, P. Wang, Ghrelin attenuates sepsis-induced acute lung injury and mortality in rats, *Am. J. Respir. Crit. Care Med.* 176 (2007) 805–813.
- [57] R. Wu, M. Zhou, P. Das, W. Dong, Y. Ji, D. Yang, M. Miksa, F. Zhang, T.S. Ravikumar, P. Wang, Ghrelin inhibits sympathetic nervous activity in sepsis, *Am. J. Physiol. Endocrinol. Metab.* 293 (2007) E1697–E1702.
- [58] L. Chang, J. Zhao, J. Yang, Z. Zhang, J. Du, C. Tang, Therapeutic effects of ghrelin on endotoxic shock in rats, *Eur. J. Pharmacol.* 473 (2003) 171–176.
- [59] R. Wu, W. Dong, Y. Ji, M. Zhou, C.P. Marini, T.S. Ravikumar, P. Wang, Orexigenic hormone ghrelin attenuates local and remote organ injury after intestinal ischemia-reperfusion, *PLoS One* 3 (2008) e2026.
- [60] C.-Y. Chen, A. Asakawa, M. Fujimiya, S.-D. Lee, A. Inui, Ghrelin gene products and the regulation of food intake and gut motility, *Pharmacol. Rev.* 61 (2009) 430–481.
- [61] D. Rajan, R. Wu, K.G. Shah, A. Jacob, G.F. Coppa, P. Wang, Human ghrelin protects animals from renal ischemia-reperfusion injury through the vagus nerve, *Surgery* 151 (2012) 37–47.
- [62] Y. Mao, T. Tokudome, I. Kishimoto, K. Otani, H. Nishimura, O. Yamaguchi, K. Otsu, M. Miyazato, K. Kangawa, Endogenous ghrelin attenuates pressure overload-induced cardiac hypertrophy via a cholinergic anti-inflammatory pathway, *Hypertension* 65 (2015) 1238–1244.
- [63] I.M. Dixon, et al., *Cardiac Fibrosis and Heart Failure- Cause or Effect*, Publishing SI, 2015.
- [64] A.M. Segura, O.H. Frazier, L.M. Buja, Fibrosis and heart failure, *Heart Fail. Rev.* 19 (2014) 173–185.
- [65] C.S. Samuel, S. Cendrawan, X.M. Gao, Z. Ming, C. Zhao, H. Kiriazis, Q. Xu, G.W. Tregear, R.A. Bathgate, X.J. Du, Relaxin remodels fibrotic healing following myocardial infarction, *Lab. Invest.* 91 (2011) 675–690.
- [66] A.V. Shinde, C. Humeres, N.G. Frangogiannis, The role of α -smooth muscle actin in fibroblast-mediated matrix contraction and remodeling, *Biochim. Biophys. Acta (BBA)-Mol. Basis Dis.* 1863 (2017) 298–309.
- [67] T.T.H. Yokota, Y. Mori, T. Kudo, H. Hiraga, N. Suto, T. Higuma, N. Abe, H. Hanada, T. Osanai, K. Okumura, Imidapril and enalapril similarly inhibit plasma matrix metalloproteinase activities and attenuate left ventricular remodeling in patients with acute myocardial infarction, *J. Cardiovasc. Pharmacol.* 63 (2014) 528–532.
- [68] Y. Lu, J.J. Liu, X.Y. Bi, X.J. Yu, S.S. Kong, F.F. Qin, J. Zhou, W.J. Zang, Pyridostigmine ameliorates cardiac remodeling induced by myocardial infarction via inhibition of the transforming growth factor- β 1/TGF- β 1-activated kinase pathway, *J. Cardiovasc. Pharmacol.* 63 (2014) 412–420.
- [69] D.A. Siwik, D.L.-F. Chang, W.S. Colucci, Interleukin-1 β and tumor necrosis factor- α decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro, *Circ. Res.* 86 (2000) 1259–1265.
- [70] M. Bujak, M. Dobaczewski, K. Chatila, L.H. Mendoza, N. Li, A. Reddy, N.G. Frangogiannis, Interleukin-1 receptor type I signaling critically regulates infarct healing and cardiac remodeling, *Am. J. Pathol.* 173 (2008) 57–67.