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Original Paper

H3 Relaxin Protects Against Myocardial Injury in Experimental Diabetic Cardiomyopathy by Inhibiting Myocardial Apoptosis, Fibrosis and Inflammation

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Key Words
H3 relaxin • Diabetic cardiomyopathy • NLRP3 inflammasome • Apoptosis • Cardiac fibrosis •

Abstract
Background/Aims: Apoptosis, fibrosis and NLRP3 inflammasome activation are involved in the development of diabetic cardiomyopathy (DCM). Human recombinant relaxin-3 (H3 relaxin) is a novel bioactive peptide that inhibits cardiac injury; however, whether H3 relaxin prevents cardiac injury in rats with DCM and the underlying mechanisms are unknown. ***Methods:*** To investigate the effect of H3 relaxin on DCM, we performed a study using H3 relaxin treatment in male Sprague-Dawley (SD) rats with streptozotocin (STZ)-induced diabetes (DM). We measured apoptosis, fibrosis and NLRP3 inflammasome markers in the rat hearts four and eight weeks after the rats were injected with STZ (65 mg/kg) by western blot analysis. Subsequently, 2 or 6 weeks after the STZ treatment, the rats were treated with H3 relaxin [2 μg/kg/d (A group) or 0.2 μg/kg/d (B group)] for 2 weeks. Cardiac function was evaluated by echocardiography to determine the extent of myocardial injury in the DM rats. The protein levels of apoptosis, fibrosis and NLRP3 inflammasome markers were used to assess myocardial injury. In addition, we determined the plasma levels of IL-1β and IL-18 using a Milliplex MAP Rat Cytokine/Chemokine Magnetic Bead Panel kit. ***Results:*** The protein expression of cleaved caspase-8, caspase-9 and caspase-3 as well as fibrosis markers increased at 4 and 8 weeks in the STZ-induced diabetic hearts compared with the levels in the control group. Furthermore, the NLRP3 inflammasome was substantially activated in STZ-induced diabetic hearts, leading to increased IL-1β and IL-18 levels. Compared with the DM group, the A group exhibited substantially better cardiac function. The protein levels of apoptosis markers were attenuated by H3 relaxin, indicating that H3 relaxin inhibited myocardial apoptosis in the hearts of diabetic rats. The protein expression of fibrosis markers was inhibited by H3 relaxin. Additionally, the

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protein expression and activation of the NLRP3 inflammasome were also effectively attenuated by H3 relaxin. **Conclusions:** This study is the first to demonstrate that H3 relaxin plays an anti-apoptotic, anti-fibrotic and anti-inflammatory role in DCM.

Introduction

Diabetic cardiomyopathy (DCM) is a specific cardiomyopathy that develops in diabetic patients independently of coronary heart disease and hypertension [1, 2]. Despite the importance of DCM, the underlying mechanisms are not fully understood. Diabetes mellitus (DM) is characterised by myocardial inflammation, oxidative stress, apoptosis and myocardial fibrosis [3, 4]. Hyperglycaemia-induced reactive oxygen species (ROS) generation is considered responsible for the progression and development of DCM [5-10]. Increased ROS can induce multiple cytokines and inflammatory factors, such as nuclear factor-κB (NF-κB) and thioredoxin interacting/inhibiting protein (TXNIP), and subsequent activation of inflammasomes [11-13]. Although inflammasomes have been shown to be involved in the pathogenic mechanisms of DM and related complications [14, 15], the potential role and regulatory mechanism of inflammasomes in DCM have remained largely unexplored. A preponderance of evidence from recent clinical and experimental studies supports the hypothesis that the NLRP3 inflammasome is involved in the mechanism of DCM. However, NLRP3 gene silencing was also shown to exert a protective effect against DCM [16]. The NLRP3 inflammasome is a complex of intracellular interacting proteins that recognises damage-associated molecular pattern molecules and triggers the maturation of proinflammatory cytokines to initiate and amplify the inflammatory response. The NLRP3 inflammasome is composed of an NOD (nucleotide binding oligomerisation domain)-like receptor, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and pro-caspase-1 [17]. The activated NLRP3 inflammasome cleaves pro-caspase-1 into cleaved caspase-1. In turn, cleaved caspase-1 activates the IL-1 family proinflammatory cytokines IL-1β and IL-18 by cleaving pro-IL-1β and pro-IL-18 into their active forms. Thus, the NLRP3 inflammasome is a powerful mediator of the immune response via the caspase-1 activation of IL-1β and IL-18. IL-1β- and IL-18-activated inflammation mediates apoptotic and fibrotic processes [18].

Recently, relaxin-3 was shown to be predominantly expressed in the brain and was found to play a role in regulating arousal, feeding, learning, memory and central responses to physiological stressors [19-21]. Rat relaxin-3 mRNA expression has been clearly identified in atrial and ventricular cells; however, rat relaxin-3 mRNA is up-regulated in the myocardium during isoproterenol-induced myocardial ischaemia injury. The administration of exogenous H3 relaxin effectively attenuated isoproterenol-induced myocardial injury, potentiated cardiac function and ameliorated cardiac fibrosis [22]. Recent findings have demonstrated that H3 relaxin exerts anti-fibrotic effects similar to those of H2 relaxin via relaxin family peptide receptor 1 (RXFP1) and inhibits cardiac fibrosis during cardiac-restricted transgenic overexpression of β2-AR [23]. H3 relaxin administration has been reported to limit the apoptosis of neonatal rat ventricular myocytes induced by high concentrations of glucose [24]. However, whether H3 relaxin exerts similar cardioprotective effects in DCM remains unclear.

This study was designed to investigate whether H3 relaxin attenuates the damage in experimental DCM by ameliorating myocardial apoptosis and fibrosis and regulating the expression and activation of the NLRP3 inflammasome.

Materials and Methods

Animals and reagents

One hundred and ten Sprague-Dawley (SD) rats (weighing 200-250 g) were obtained from the Experimental Animal Centre at the Second Affiliated Hospital of Harbin Medical University. All animal care

and experimental protocols complied with the Animal Management Rule of the Ministry of Health, People's Republic of China (Document No. 55, 2001) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Synthetic H3 relaxin was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA), and STZ was obtained from Sigma (St. Louis, MO, USA).

Antibodies against cleaved caspase-1, α-SMA, MMP-2 and MMP-9 were purchased from Abcam (Cambridge, UK); antibodies against cleaved caspase-8, cleaved caspase-3 and cleaved caspase-9 were purchased from Cell Signaling Technologies (Beverly, MA, USA). Antibodies against ASC were purchased from Santa Cruz Biotech (CA, USA). Antibodies against type I collagen, type III collagen and NLRP3 were purchased from Bioss (Beijing, China). Antibodies against IL-1β and IL-18 were purchased from Novus Biologicals (CO, USA). Other chemicals and reagents were of analytical grade.

Induction of experimental diabetes in rats and grouping

DM was induced in rats by a single injection of STZ (65 mg/kg, intraperitoneal) dissolved in freshly prepared ice-cold citrate buffer (pH 4.5). After 1 week of administration, animals that had serum glucose greater than 240 mg/dl in a random test were considered diabetic rats. In experiment 1, indicators were evaluated at 4 and 8 weeks after STZ injection. We surveyed the cardiac injury in diabetic rats (at least 8 rats in each group). In experiment 2, the rats were divided into 4 groups: control, DM, A group (DM+2 μg/kg/d H3 relaxin) and B group (DM+0.2 μg/kg/d H3 relaxin) (at least 8 rats in each group). In the B and A groups, 2 or 6 weeks after STZ treatment, the rats were treated with subcutaneous H3 relaxin [2 μg/kg/d (A group) or 0.2 μg/kg/d (B group)] for 2 weeks. We surveyed the cardiac injury in the rats (at least 8 rats in each group). The left ventricle weight (LVW), including the interventricular septum weight, was noted and expressed as milligrams per gram of body weight.

Cardiac function analysis using echocardiography

For analysis of cardiac function of the rats, 8 weeks after STZ treatment, echocardiographic images were recorded from anaesthetised rats using a Philips Sonos 7500 ultrasound system. We detected the left atrial diameter (LA, mm), left ventricular end-diastolic diameter (LVEDD, mm), left ventricular ejection fraction (LVEF), left ventricular end-diastolic volume (LVEDV, ml), left ventricular end-systolic volume (LVESV, ml) and heart rates (HR). All measurements were performed in triplicate during 3 consecutive cardiac cycles by the same investigator. Average values were used for our analyses.

Cardiac structure analysis using transmission electron microscopy

Tissues were obtained from the left ventricle and fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer for 2 h. The samples were then fixed in cacodylate-buffered 2% osmium tetroxide and rinsed in phosphate-buffered saline. The tissues were dehydrated in a graded ethanol series followed by acetone and then embedded in Epon 812. Subsequently, superthin slices were prepared and stained with uranyl acetate and lead citrate. Finally, the specimens were studied by transmission electron microscopy.

Western immunoblot analyses

Frozen hearts were homogenised in ice-cold lysis buffer in the presence of a proteinase inhibitor cocktail. Homogenates were centrifuged at 12, 000 g for 10 min at 4°C. The protein contents of the supernatant were determined using a BCA-200 protein assay kit (Beyotime, China). Equal amounts of proteins (20 μg) were loaded and separated using 12% SDS-PAGE and transferred to a PVDF membrane. The non-specific proteins were blocked by incubating the membrane with 5% non-fat dry milk for 1 h at room temperature with agitation. The membrane was then incubated overnight at 4°C with the following primary antibodies: anti-β-actin (1:1000), anti-NLRP3 (1:1000), anti-ASC (1:1000), anti-cleaved caspase-1 (1:1000), anti-IL-1β (1:1000), anti-IL-18 (1:1000), anti-cleaved caspase-9 (1:1000), anti-cleaved caspase-8 (1:1000), anti-cleaved caspase-3 (1:1000), anti-I-collagen (1:1000), anti-III-collagen (1:1000), anti-α-SMA (1:1000), anti-MMP-2 (1:1000) and anti-MMP-9 (1:1000). The membrane was then washed three times for 10 min each and subsequently incubated with the appropriate fluorescently labelled secondary IgG antibody. Antigen-antibody complexes were visualised using an Odyssey Infrared Imaging System and Odyssey v3.0 software. The protein levels were normalised to the level of β-actin.

Detection of cytokines in plasma

The plasma was obtained from SD rat blood that was centrifuged for 10 min at 1000 g. IL-1β and IL-18 were detected using a Milliplex MAP Rat Cytokine/Chemokine Magnetic Bead Panel kit (Cat. RECYTMAG-65K, EMD Millipore, Darmstadt, Germany).

Statistical analyses

Each experiment was repeated a minimum of six times, each group contained eight rats, and the results are expressed as the mean ± SD. GraphPad Prism 5.0 software was used for data analyses. For more than two groups, we used one-way ANOVA, followed by a Newman-Keuls multiple comparison test. $P < 0.05$ was considered significant.

Results

Expression of apoptosis-related proteins in diabetic rat hearts

To determine whether apoptosis is involved in the pathophysiology of DCM, we measured cleaved caspase-3 in the hearts of diabetic rats 4 and 8 weeks after the onset of diabetes by western blot analysis. We further determined whether apoptosis occurred through the extrinsic, intrinsic or both pathways of apoptosis. We observed up-regulation of cleaved caspase-8, cleaved caspase-9 and cleaved caspase-3 protein expression (Fig. 1).

Protein expression of fibrosis markers in diabetic rat hearts

The concentrations of types I and III collagen increased at 4 and 8 weeks after STZ treatment. The expression levels of MMP-2 and MMP-9 were higher in the DM group than in the control group. Additionally, diabetes induced interstitial fibrosis and increased the expression of α-smooth muscle actin (α-SMA), which is a reliable marker of myofibroblast phenotypes (Fig. 2).

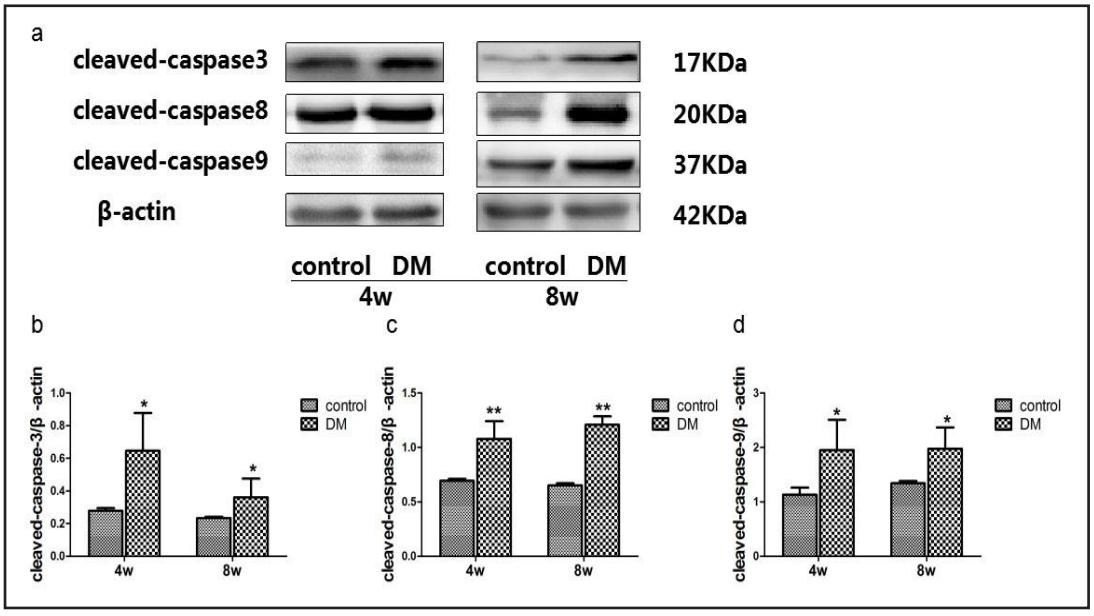

Fig. 1. The protein expression of apoptosis in heart of diabetic rats. (a) The protein expression of apoptosis markers (cleaved caspase-3, 8, 9) at 4 and 8 weeks in diabetic rat hearts. (b) The protein levels of the cleaved caspase-3 were normalized to β-actin (cleaved caspase-3/β-actin). (c) The protein levels of the cleaved caspase-8 were normalized to β-actin (cleaved caspase-8/β-actin). (d) The protein levels of the cleaved caspase-9 were normalized to β-actin (cleaved caspase-9/β-actin). Data are the means ± SD, and each measurement carried out six times. *P<0.05 vs. control, **P<0.01 vs. control.

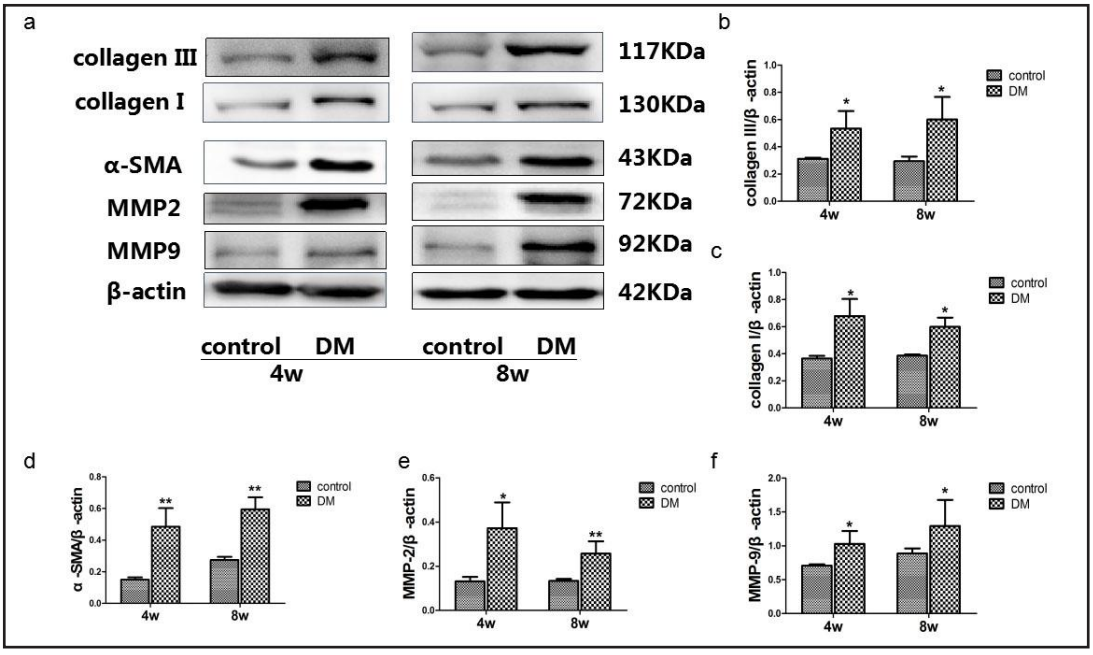

Fig. 2. The protein expression of fibrosis in heart of diabetic rats. (a) The protein expression of fibrosis markers (collagen types III and I, α-SMA, MMP-2, MMP-9) at 4 and 8 weeks in heart of diabetic rats. (b)The protein levels of collagen type III were normalized to β-actin (collagen type III/β-actin). (c) The protein levels of collagen type I were normalized to β-actin (collagen type I/β-actin). (d) The protein levels of α-SMA were normalized to β-actin (α-SMA /β-actin). (e) The protein levels of MMP-2 were normalized to β-actin (MMP-2/β-actin). (f) The protein levels of MMP-9 were normalized to β-actin (MMP-9/β-actin). Data are the means ± SD, and each measurement carried out six times. *P<0.05 vs. control, **P<0.01 vs. control.

Activation and expression of the NLRP3 inflammasome in diabetic rat hearts

Previous studies have shown that IL-1β and IL-18 are important proinflammatory cytokines in the development of DCM [25]. IL-1β activation is primarily mediated by the cysteine protease caspase-1, which in turn is activated by the NLRP3 inflammasome [26]. To assess whether the NLRP3 inflammasome was activated in hearts during DCM, we used western blot analyses to measure the protein levels of NLRP3, ASC, cleaved caspase-1, IL-1β and IL-18 after 4 and 8 weeks of DM. We discovered that diabetic rats exhibited rapid accumulation of the NLRP3, ASC, cleaved caspase-1, IL-1β and IL-18 proteins (Fig. 3).

H3 relaxin ameliorated the disordered cardiac structure and impaired cardiac function in diabetic rats

A significant decrease in body weight was observed in the STZ-treated rats. Treatment with H3 relaxin significantly increased the body weight of diabetic rats. Additionally, a significant increase in the LVW/BW ratio was observed in diabetic rats. Treatment with H3 relaxin significantly decreased the LVW/BW ratio in diabetic rats. Interestingly, H3 relaxin significantly decreased the glucose level in diabetic rats (Table 1).

To test our hypothesis that cell death, one of the major early cellular events of the heart in response to diabetes, is critically involved in the development of late-stage cardiomyopathy, we examined the cardiac ultrastructure in diabetic rats 4 and 8 weeks after STZ treatment. As shown in Fig. 4, the hearts of diabetic rats showed extensive structural abnormalities, including reduced and disarranged muscular fibroses with fewer or irregular Z-lines. These changes were rarely observed in the hearts of control rats. H3 relaxin alleviated these STZ-induced changes (Fig. 4).

Transthoracic echocardiography was used to evaluate the protective effect of H3 relaxin in diabetic rats using various cardiac functional parameters, including LA, LVEDD, LVEDV,

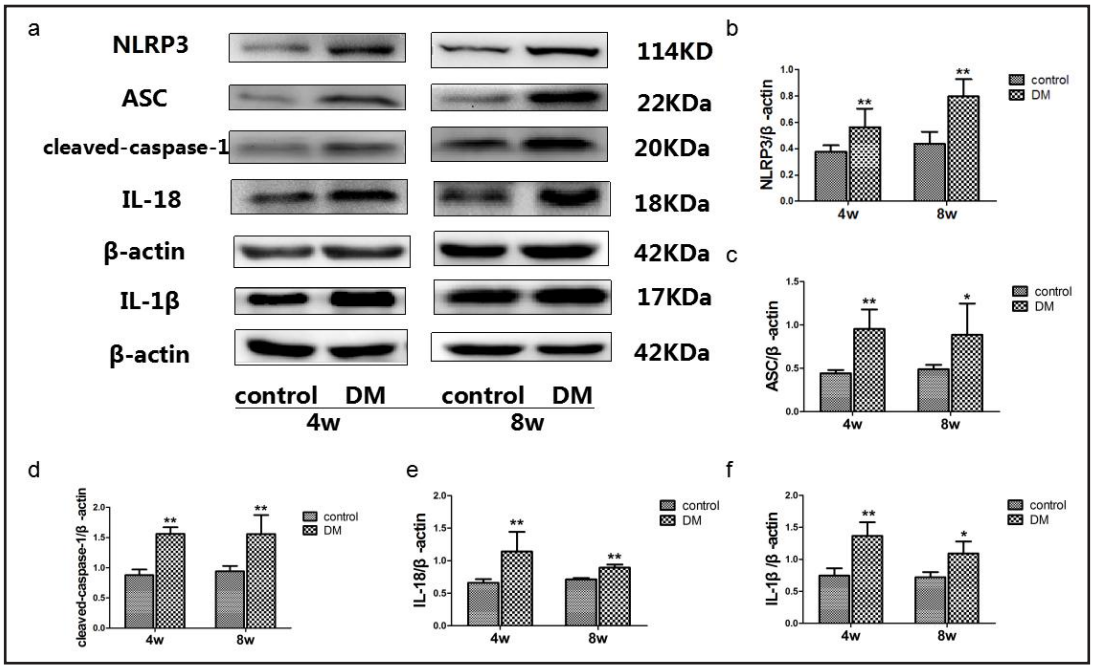

Fig. 3. The activation and expression of NLRP3 inflammasome in hearts of diabetic rats. (a) The protein expression of NLRP3 inflammasome markers (NLRP3, ASC, cleaved caspase-1, IL-1β, IL-18) at 4 and 8 weeks in hearts of diabetic rats. (b) The protein levels of NLRP3 were normalized to β-actin (NLRP3/β-actin). (c) The protein levels of ASC were normalized to β-actin (ASC/β-actin). (d) The protein levels of cleaved caspase-1 were normalized to β-actin (cleaved caspase-1/β-actin). (e) The protein levels of IL-18 were normalized to β-actin (IL-18/β-actin). (f)The protein levels of IL-1β were normalized to β-actin (IL-1β/β-actin). Data are the means ± SD, and each measurement carried out six times. *P<0.05 vs. control, **P<0.01 vs. control.

LVESV, LVEF and HR. Compared with the control group, the DM group exhibited significantly greater LA, LVEDD, LVEDV, and LVESV and decreased HR. There was no significant difference between the control and DM groups in LVEF, although LVEF decreased in the rats with DM. Compared to the DM group, the A group exhibited decreased LVEDD, LVEDV and LVESV. The B group showed no significant differences compared to the DM group (Table 2).

Table 1. H3 relaxin improved left ventricle weight/body weight and serum glucose in diabetic rats. Data are mean ± SD, each carried out in eight. *P < 0.05 vs. control, **P < 0.01 vs. control, #P < 0.05 vs. DM, ##P < 0.01 vs. DM

	4 week				8 week			
	BW (g)	LVW (mg)	LVW/BW(mg/g)	Glucose(mg/dl)	BW (g)	LVW (mg)	LVW/BW(mg/g)	Glucose(mg/dl)
Control	335.00±23.45	1.36±0.84	4.07±2.18	101.07±3.24	422.50±12.07	1.37±0.33	3.27±0.00	145.38±18.91
DM	121.00±8.42*	0.68±0.06**	5.65±0.11**	563.63±42.10**	243.30±6.43**	1.20±0.31	5.63±0.00**	540.83±45.58**
B	170.00±20.83##	0.75±0.11	4.50±0.00##	486.01±49.00##	229.5±66.70	0.88±0.86	4.10±0.00#	331.49±104.49
A	191.00±20.04##	0.78±0.08#	4.07±0.00##	232.40±73.1##	265.30±39.70	1.18±0.07	3.06±0.00#	376.53±194.57

Effect of H3 relaxin treatment on apoptosis in the diabetic heart

To determine whether apoptosis participated in the H3 relaxin cardioprotection against DM, we evaluated the levels of apoptosis markers, including cleaved caspase-3, cleaved cas-

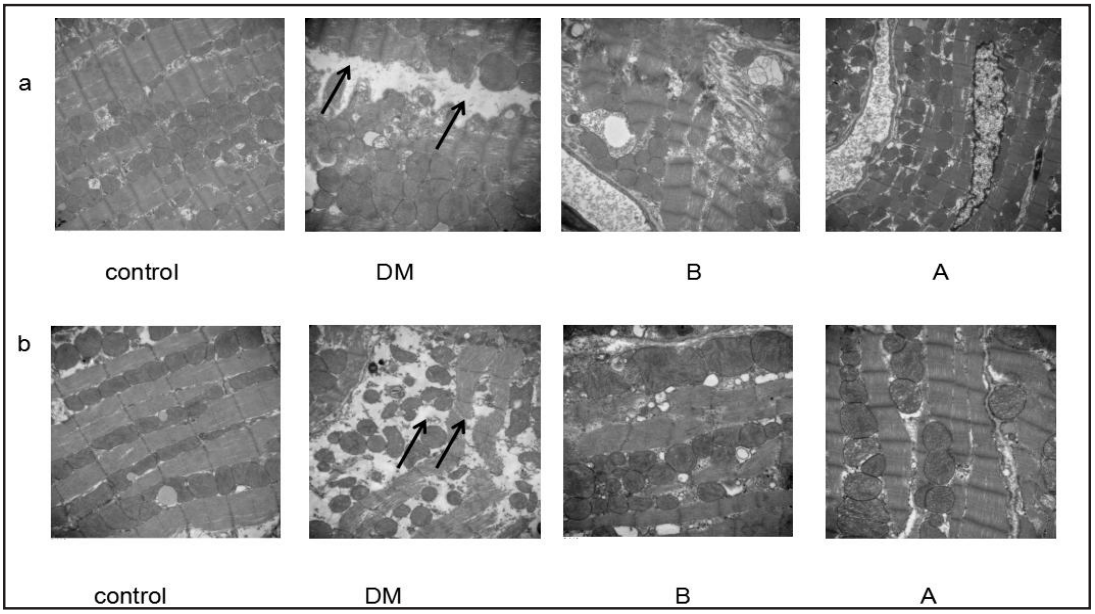

Fig. 4. Cardiac tissue was observed by transmission electron microscopy (original magnification, ×10000). Ultrastructural evaluation was performed by electron microscopy: (a) for the hearts of diabetic rats 4 weeks after STZ treatment; Diabetic rats at 4weeks showed extensive structural abnormalities, including reduced and disarranged muscular fibroses with fewer or irregular Z-lines; These changes were rarely observed in the hearts of H3 relaxin administered diabetic rats. (b) for the hearts of diabetic rats 8 weeks after STZ treatment. Diabetic rats at 8 weeks showed more extensive structural abnormalities than that at 4 weeks, including reduced and destructive muscular fibroses with fewer or irregular Z-lines. These changes were rarely observed in the hearts of H3 relaxin administered diabetic rats. Arrows showed disarranged muscular fibroses with fewer or irregular Z-lines.

pase-8 and cleaved caspase-9. The results shown in Fig. 5 indicated that the expression of cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 significantly increased in the DM group. Furthermore, the H3 relaxin treatment attenuated the expression of cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 (Fig. 5).

Table 2. H3 relaxin improved cardiac function in diabetic rats. Left atrial diameter (LA,mm), left ventricular end-diastolic diameter (LVEDD, mm), left ventricular ejection fraction (LVEF), left ventricular end-systolic volume (LVESV, ml), left ventricular end-diastolic volume (LVEDV, ml), heart rates (HR). *P < 0.05 vs. control, #P < 0.05 vs. DM

	LA(mm)	LVEDD(mm)	LVEF(%)	LVEDV(ml)	LVESV (ml)	HR(bpm)
Control	2.87±0.004	4.57±0.221	82.33±4.64	0.44±0.15	0.08±0.03	349.33±8.08
DM	4.47±0.010*	7.86±0.125*	77.33±2.49	0.78±0.10*	0.17±0.019*	331.00±3.60*
B	4.03±0.303	7.19±0.332	77.00±4.97	0.68±0.15	0.16±0.08	326.67±10.97
A	3.71±0.997	6.49±0.333#	80.00±0.81	0.53±0.07#	0.10±0.01#	331.00±4.58

Effect of H3 relaxin treatment on cardiac fibrosis in the diabetic heart

Compared with the DM group, the A group had significantly lower levels of types I and III collagen (Fig. 6). Fibroblast activation in diabetic hearts results in cardiac fibrosis upon fibroblast conversion to hypersynthetic cardiac myofibroblasts. Cardiac fibroblasts are relatively quiescent cells that contribute little to matrix remodelling or wound healing in normal hearts, whereas phenoconverted myofibroblasts contribute to excessive extracellular matrix deposition in the hearts of diabetic rats [27-31]. The STZ-treated diabetic rats showed

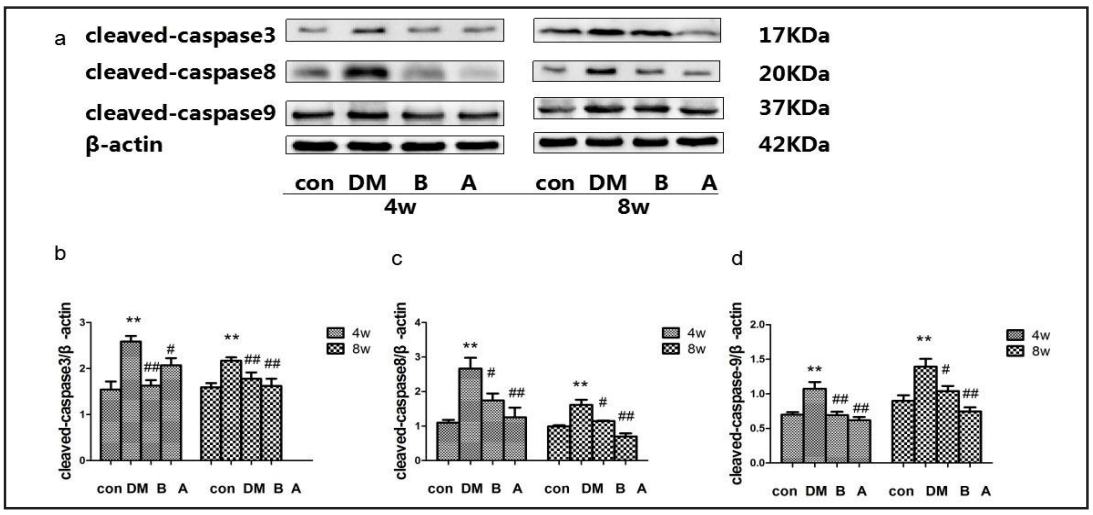

Fig. 5. Effect of H3 relaxin treatment on apoptosis in the heart of diabetic rats. (a) Cleaved caspase-3, 8, 9 protein expressions were analyzed by western blot. (b) The protein levels of cleaved caspase-3 were normalized to β-actin (cleaved caspase-3/β-actin). (c) The protein levels of cleaved caspase-8 were normalized to β-actin (cleaved caspase-8/β-actin). (d) The protein levels of cleaved caspase-9 were normalized to β-actin (cleaved caspase-9/β-actin). Data are the means ± SD, and each measurement carried out six times. *P<0.05 vs. control, **P<0.01 vs. control, #P<0.05 vs. DM, ##P<0.01 vs. DM.

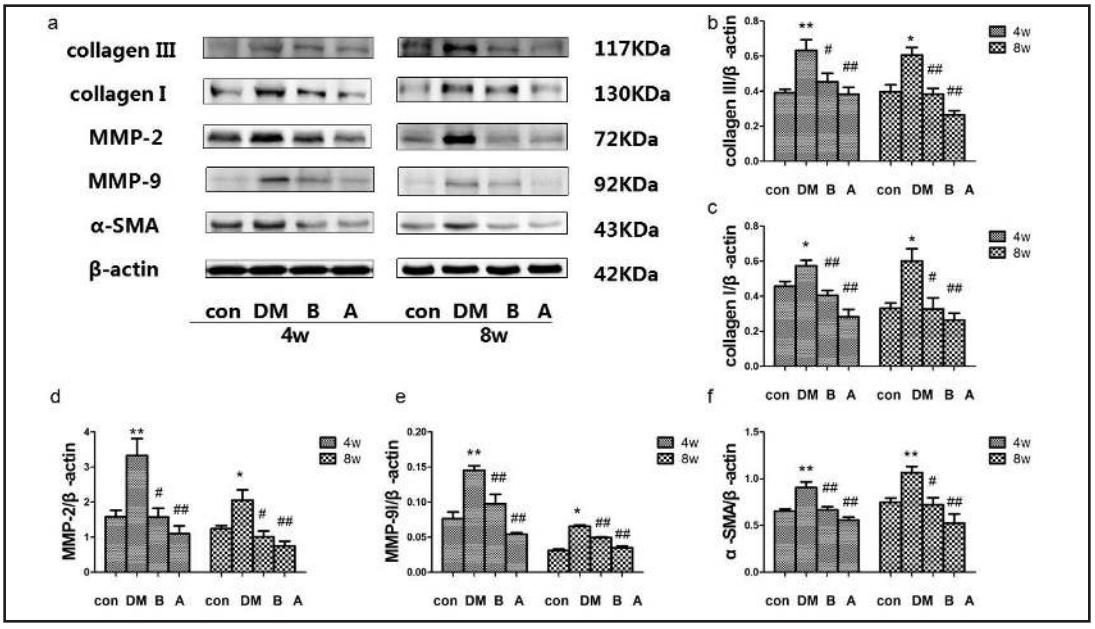

Fig. 6. Effect of H3 relaxin treatment on cardiac fibrosis in the heart of diabetic rats. (a) The protein expression of fibrosis markers was analyzed by western blot. (b) The protein levels of collagen type III were normalized to β-actin (collagen type III/β-actin). (c) The protein levels of collagen type I were normalized to β-actin (collagen type I/β-actin). (d) The protein levels of MMP-2 were normalized to β-actin (MMP-2/β-actin). (e) The protein levels of MMP-9 were normalized to β-actin (MMP-9/β-actin). (f) The protein levels of α-SMA were normalized to β-actin (α-SMA /β-actin). Data are the means ± SD, and each measurement carried out six times. *P<0.05 vs. control, **P<0.01 vs. control, #P<0.05 vs. DM, ##P<0.01 vs. DM.

increased expression of α-SMA, consistent with the observed increases in cardiac collagen concentration. H3 relaxin decreased the expression of α-SMA in diabetic rats (Fig. 6).

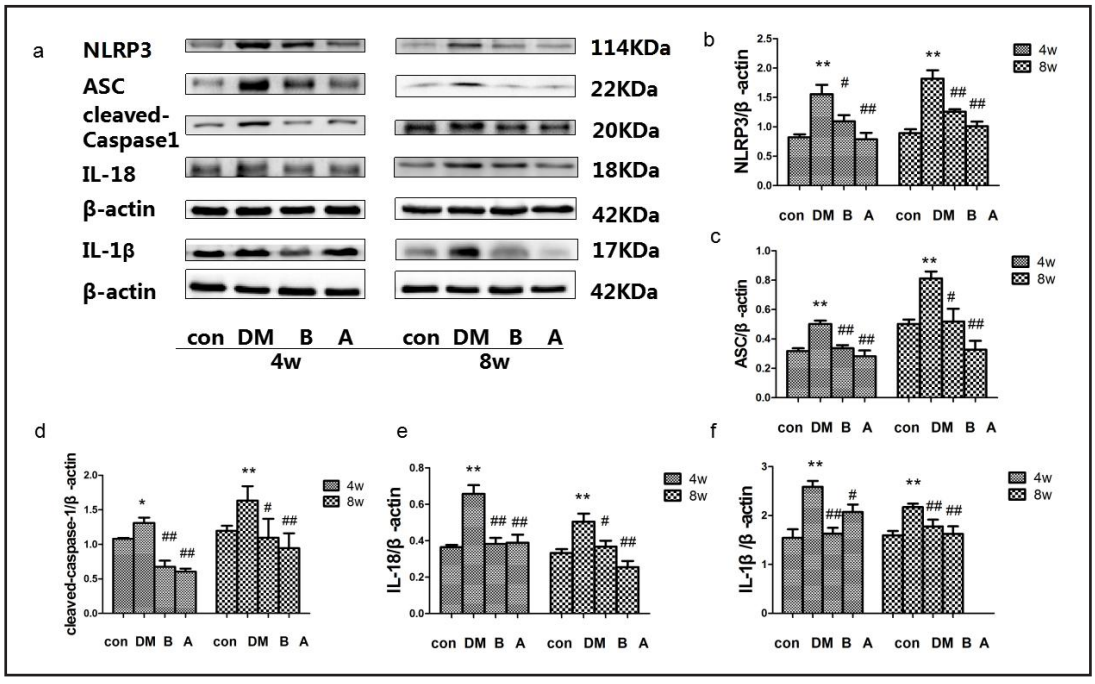

Fig. 7. H3 relaxin protects myocardium by inhibiting NLRP3 inflammasome activation. (a) The protein expression of NLRP3 inflammasome markers (NLRP3, ASC, cleaved caspase-1, IL-1β, IL-18) at 4 and 8 weeks in heart of diabetic rats. (b) The protein levels of NLRP3 were normalized to β-actin (NLRP3/β-actin). (c) The protein levels of ASC were normalized to β-actin (ASC/β-actin). (d) The protein levels of cleaved caspase-1 were normalized to β-actin (cleaved caspase-1/β-actin). (e) The protein levels of IL-18 were normalized to β-actin (IL-18/β-actin). (f) The protein levels of IL-1β were normalized to β-actin (IL-1β/β-actin). Data are the means ± SD, and each measurement carried out six times. *P<0.05 vs. control, **P<0.01 vs. control, #P<0.05 vs. DM, ##P<0.01 vs. DM.

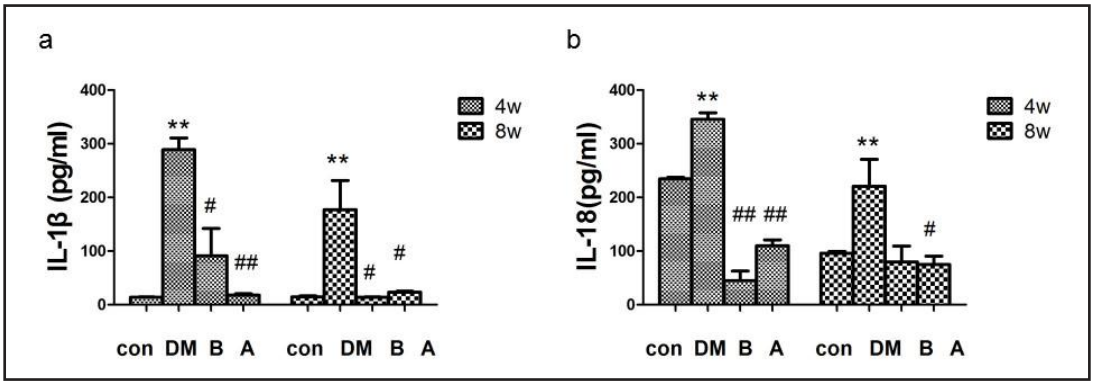

Fig. 8. H3 relaxin inhibited plasma IL-1β and IL-18 expression in diabetic rats. The protein expression of IL-1β at 4 and 8 weeks in plasma of diabetic rats. (b) The protein expression of IL-18 at 4 and 8 weeks in plasma of diabetic rats. Data are the means ± SD, and each measurement carried out eight times. *P<0.05 vs. control, **P<0.01 vs. control, #P<0.05 vs. DM, ##P<0.01 vs. DM.

Under normal conditions, the MMP/TIMP system remains in a dynamic balance. Under pathological conditions, MMPs are increased, and TIMPs are down-regulated. Diabetic rats have increased levels of MMP-2 and MMP-9. H3 relaxin administration over a 2-week period decreased the expression of MMP-2 and MMP-9 (Fig. 6), consistent with the observed changes in cardiac collagen concentration.

H3 relaxin protects the myocardium from NLRP3 inflammasome activation

Considerable evidence has shown that the NLRP3 inflammasome contributes to apoptosis and fibrosis in DCM. NLRP3 gene silencing may exert a protective effect on DCM [12]. H3 relaxin has been shown to limit proinflammatory cytokine production in astrocytes [32]. In our work, the expression of NLRP3, ASC, and cleaved caspase-1 in the hearts of diabetic rats increased. Furthermore, H3 relaxin abolished the activation of NLRP3, ASC, and cleaved caspase-1. By measuring the plasma levels of IL-1β and IL-18, we found that H3 relaxin inhibited IL-1β and IL-18 production (Fig. 7) and subsequent IL-1β and IL-18 release (Fig. 8).

Discussion

Accumulating evidence suggests that increased oxidative/nitrative stress coupled to the activation of various downstream proinflammatory and cell death pathways plays pivotal roles in the development of complex biochemical, mechanical and structural alterations that are associated with DCM [33, 34]. However, despite this accumulated knowledge, treatments for DCM remain poor.

In our study, we found that diastolic-centred cardiac dysfunction developed at 8 weeks in rats with DM. As reported previously, diastolic dysfunction preceded systolic dysfunction in our experiments, similar to the pattern observed in humans, beginning 2 to 3 months after induction of DM. The ejection fraction was used to investigate systolic function. The average time from the DM induction to the development of heart failure, defined by both systolic and diastolic dysfunction, was 4 months [35].

H3 relaxin, a member of the relaxin peptide family, has been shown to exert anti-inflammatory and anti-fibrosis effects both *in vitro* and in various models of cardiac fibrosis via RXFP1 [20, 21]. Furthermore, H3 relaxin was recently reported to lower high glucose-induced neonatal rat ventricular myocyte apoptosis by inhibiting the extrinsic and intrinsic pathways of apoptosis and endoplasmic reticulum stress [22]. In a recent study, we reported no difference in the plasma relaxin-3 concentrations between controls and patients with DM, i.e., the relaxin-3 levels are not related to the component traits of DM [36]. In the present study, we evaluated the effects of H3 relaxin treatment (administered for 2 weeks at 2 and 6 weeks after the development of type 1 DM) on the signalling pathways related to both cell apoptosis and fibrosis using a rat model of type 1 DCM. Significant cardiac dysfunction began to develop in this model after 4 weeks of established DM, with gradually increasing fibroses thereafter (peaking at approximately 8 weeks of established DM). Consequently, for the first treatment protocol (2 weeks after STZ injection), we aimed to study whether H3 relaxin treatment could prevent the development of characteristic alterations of type 1 DCM. Then, in the second treatment protocol (6 weeks after STZ injection), we sought to determine whether these changes were reversible. We found that H3 relaxin inhibited apoptosis and fibrosis in the hearts of diabetic rats. Interestingly, we found that H3 relaxin dramatically improved the glucose level in diabetic rats; the underlying mechanism is poorly understood. In a separate study, human relaxin-3 was administered for 14 days via osmotic minipumps, which produced a significant increase in food intake and body weight, a significant increase in blood leptin and insulin levels and increased epididymal fat mass [37]. In addition, chronic administration of the RXFP3 agonist R3/I5 for 2 weeks increased the plasma insulin and leptin levels [38]. Lipids are energy substrates for cardiomyocytes, components of cell membranes, precursors of signalling molecules, and ligands for nuclear transcription factors. Their myocardial metabolism is disturbed in type 1 diabetes because of insulin deficiency [39]. In summary, we hypothesized that H3 relaxin increased feed intake and insulin levels and thus increased body weight and decreased glucose levels. However, whether H3 relaxin inhibits cardiac injury in diabetic rats by increasing feed intake and decreasing glucose will be studied in the future by our group.

The NLRP3 inflammasome is a complex of intracellular interacting proteins and is composed of a NOD-like receptor, ASC, and pro-caspase-1. The activated inflammasome cleaves pro-caspase-1 into the active enzyme caspase-1. In turn, caspase-1 activates the IL-1 family proinflammatory cytokines IL-1β and IL-18 by cleaving pro-IL-1β and pro-IL-18 into their active forms. Subsequently, IL-1β and IL-18 activate inflammation-mediated apoptosis and fibrosis. The NLRP3 inflammasome can also induce pyroptosis in a caspase-1-dependent manner [40]. The loss of cardiomyocytes via pyroptosis reduces contractile reserves and leads to heart failure [41]. Additionally, as cytosolic components are released with pyroptosis, extracellular ASC becomes a danger signal and initiates inflammasome formation. Extracellular ASC continues to activate pro-caspase-1, thus propagating the inflammatory cascade. IL-1β is a cytokine with major roles in inflammation, the innate immune response and fibrosis. IL-1β is produced by activated monocytes, macrophages, dendritic cells and fibroblasts and induces the production of cytokines, such as TNF-α and IL-6, or proteases, such as MMPs. Furthermore, IL-1β is associated with neutrophil recruitment and the proliferation of resident cells (primarily fibroblasts) [42]. IL-1β is increased during heart failure and is associated with poor exercise tolerance and with remodelling after ischaemia-reperfusion injury [43, 44]. The modulation of IL-1β attenuates myocardial enlargement and ventricular dysfunction [45]. IL-18 is constitutively expressed as a biologically inactive precursor molecule lacking a signal peptide. Increased IL-18 levels are correlated with functional class and mortality in heart failure. IL-18 is increased during acute heart failure and remains elevated after discharge [46].

Consistent with previous reports, DCM was characterised by worsened diastolic myocardial performance and enhanced myocardial apoptosis, fibrosis and NLRP3 inflammasome levels. The pharmacological inhibition of NLRP3 inflammasome activation attenuated the expression of cardiac apoptosis and collagen components associated with DCM. Similarly, recent evidence supports an emerging role of NLRP3 inflammasome activation in DCM in addition to its already established role in mediating cell apoptosis and fibrosis during myocardial injury. High glucose-induced ROS generation also activated NLRP3 inflammasome signalling pathways in diabetic hearts and activated cell apoptosis and fibrosis pathways, which in turn regulated the expression of proinflammatory cytokines, IL-1β and IL-18. Importantly, the oxidative-nitrative stress and inflammatory pathways in DCM are closely interrelated and promote the development of myocardial fibrosis. Treatment with H3 relaxin attenuated the myocardial expression of apoptosis markers, fibrosis, and NLRP3 inflammasome activation that occur in diabetic hearts. Additionally, H3 relaxin treatment attenuated/reversed (although to a lesser extent) some of the discussed DM-induced myocardial biochemical and functional changes after the establishment of DCM. These results also support the emerging role of the NLRP3 inflammasome in the development and progression of DCM.

Collectively, our results strongly suggest that H3 relaxin has therapeutic potential for the treatment of DCM by attenuating myocardial apoptosis, fibrosis and inflammation.

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Disclosure Statement

On behalf of all authors, the corresponding author states that there are no conflicts of interest.

References

1 Kannel WB, Hjortland M, Castelli WP: Role of diabetes in congestive heart failure: the Framingham study. Am J Cardiol 1974;34:29-34.
2 Poornima IG, Parikh P, Shannon RP: Diabetic cardiomyopathy: the search for a unifying hypothesis. Circ Res 2006;98:596-605.
3 Westermann D, Rutschow S, Van Linthout S, Linderer A, Bücker-Gärtner C, Sobirey M, Riad A, Pauschinger M, Schultheiss HP, Tschöpe C: Inhibition of p38 mitogen-activated protein kinase attenuates left ventricular dysfunction by mediating pro-inflammatory cardiac cytokine levels in a mouse model of diabetes mellitus. Diabetologia 2006;49:2507-2513.
4 Westermann D, Rutschow S, Jäger S, Linderer A, Anker S, Riad A, Unger T, Schultheiss HP, Pauschinger M, Tschöpe C: Contributions of inflammation and cardiac matrix metalloproteinase activity to cardiac failure in diabetic cardiomyopathy: the role of angiotensin type 1 receptor antagonism. Diabetes 2007;56:641-646.
5 Cai L: Suppression of nitrative damage by metallothionein in diabetic heart contributes to the prevention of cardiomyopathy. Free Radic Biol Med 2006;41:851-861.
6 Cai L, Wang Y, Zhou G, Chen T, Song Y, Li X, Kang YJ: Attenuation by metallothionein of early cardiac cell death via suppression of mitochondrial oxidative stress results in a prevention of diabetic cardiomyopathy. J Am Coll Cardiol 2006;48:1688-1697.
7 Raza H, John A, Howarth FC: Increased oxidative stress and mitochondrial dysfunction in zucker diabetic rat liver and brain. Cell Physiol Biochem 2015;35:1241-1251.
8 Wang H, Bei Y, Lu Y, Sun W, Liu Q, Wang Y, Cao Y, Chen P, Xiao J, Kong X: Exercise Prevents Cardiac Injury and Improves Mitochondrial Biogenesis in Advanced Diabetic Cardiomyopathy with PGC-1α and Akt Activation. Cell Physiol Biochem 2015;35:2159-2168.
9 Li H, Liu X, Ren Z, Gu J, Lu Y, Wang X, Zhang L: Effects of Diabetic Hyperglycemia on Central Ang-(1-7)-Mas-R-nNOS Pathways in Spontaneously Hypertensive Rats. Cell Physiol Biochem 2016;40:1186-1197.
10 Huang Z, Zhuang X, Xie C, Hu X, Dong X, Guo Y, Li S, Liao X: Exogenous Hydrogen Sulfide Attenuates High Glucose-Induced Cardiotoxicity by Inhibiting NLRP3 Inflammasome Activation by Suppressing TLR4/NF-κB Pathway in H9c2 Cells. Cell Physiol Biochem 2016;40:1578-1590.
11 Tsai KH, Wang WJ, Lin CW, Pai P, Lai TY, Tsai CY, Kuo WW: NADPH oxidase-derived superoxide anion-induced apoptosis is mediated via the JNK-dependent activation of NF-κB in cardiomyocytes exposed to high glucose. J Cell Physiol 2012;227:1347-1357.
12 Devi TS, Lee I, Hüttemann M, Kumar A, Nantwi KD, Singh LP: TXNIP links innate host defense mechanisms to oxidative stress and inflammation in retinal Muller gliaunder chronic hyperglycemia: implications for diabetic retinopathy. Exp Diabetes Res 2012:438238.
13 Bryant C, Fitzgerald KA: Molecular mechanisms involved in inflammasome activation. Trends Cell Biol 2009;19:455-464.
14 Lee HM, Kim JJ, Kim HJ, Shong M, Ku BJ, Jo EK: Upregulated NLRP3 inflammasome activation in patients with type 2 diabetes. Diabetes 2013;62:194-204.
15 Wang C, Pan Y, Zhang QY, Wang FM, Kong LD: Quercetin and allopurinol ameliorate kidney injury in STZ-treated rats with regulation of renal NLRP3 inflammasome activation and lipid accumulation. PLoS One 2012;7:e38285.
16 Luo B, Li B, Wang W, Liu X, Xia Y, Zhang C, Zhang M, Zhang Y, An F: NLRP3 gene silencing ameliorates diabetic cardiomyopathy in a type 2 diabetes rat model. PLoS One 2014;9:e104771.
17 Lamkanfi M, Kanneganti TD: Nlrp3: an immune sensor of cellular stress and infection. Int J Biochem Cell Biol 2010;42:792-795.
18 Franchi L, Munoz-Planillo R, Nunez G: Sensing and reacting to microbes through the inflammasomes. Nat Immunol 2012;13:325-332.
19 McGowan BM, Stanley SA, Smith KL, White NE, Connolly MM, Thompson EL, Gardiner JV, Murphy KG, Ghatei MA, Bloom SR: Central relaxin-3 administration causes hyperphagia in male Wistar rats.Endocrinology 2005;146: 3295-3300.
20 McGowan BM, Stanley SA, Smith KL, Minnion JS, Donovan J, Thompson EL, Patterson M, Connolly MM, Abbott CR, Small CJ, Gardiner JV, Ghatei MA, Bloom SR: Effects of acute and chronic relaxin-3 on food intake and energy expenditure in rats. Regul Pept 2006;136:72-77.

21 Ma S, Olucha-Bordonau FE, Hossain MA, Lin F, Kuei C, Liu C, Wade JD, Sutton SW, Nuñez A, Gundlach AL: Modulation of hippocampal theta oscillations and spatial memory by relaxin-3 neurons of the nucleus incertus. Learn Mem 2009;16:730-742.
22 Zhang J, Qi YF, Geng B, Pan CS, Zhao J, Chen L, Yang J, Chang JK, Tang CS: Effect of relaxin on myocardial ischemia injury induced by isoproterenol. Peptides 2005;26:1632-1639.
23 Hossain MA, Man BC, Zhao C, Xu Q, Du XJ, Wade JD, Samuel CS: H3 relaxin demonstrates antifibrotic properties via the RXFP1 receptor. Biochemistry 2011;50:1368-1375.
24 Zhang X, Ma X, Zhao M, Zhang B, Chi J, Liu W, Chen W, Fu Y, Liu Y, Yin X: H2 and H3 relaxin inhibit high glucose-induced apoptosis in neonatal rat ventricular myocytes. Biochimie 2015;108:59-67.
25 Li X, Du N, Zhang Q, Li J, Chen X, Liu X, Hu Y, Qin W, Shen N, Xu C, Fang Z, Wei Y, Wang R, Du Z, Zhang Y, Lu Y: MicroRNA-30d regulates cardiomyocyte pyroptosis by directly targeting foxo3a in diabetic cardiomyopathy. Cell Death Dis 2014;5:e1479.
26 Lamkanfi M, Kanneganti TD: Nlrp3: an immune sensor of cellular stress and infection. Int J Biochem Cell Biol 2010;42:792-795.
27 Drobic V, Cunnington RH, Bedosky KM, Raizman JE, Elimban VV, Rattan SG, Dixon IM: Differential and combined effects of cardiotrophin-1 and TGF-beta1 on cardiac myofibroblast proliferation and contraction. Am J Physiol Heart Circ Physiol 2007;293:H1053-1064.
28 Peterson DJ, Ju H, Hao J, Panagia M, Chapman DC, Dixon IM: Expression of Gi-2 alpha and Gs alpha in myofibroblasts localized to the infarct scar in heart failure due tomyocardial infarction. Cardiovasc Res 1999;41:575-585.
29 Petrov VV, Fagard RH, Lijnen PJ: Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 2002;39:258-263.
30 Santiago JJ, Dangerfield AL, Rattan SG, Bathe KL, Cunnington RH, Raizman JE, Bedosky KM, Freed DH, Kardami E, Dixon IM: Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. Dev Dyn 2010;239:1573-1584.
31 Hinz B: Formation and function of the myofibroblast during tissue repair. J Invest Dermatol 2007;127:526-537.
32 Willcox JM, Summerlee AJ: Relaxin protects astrocytes from hypoxia in vitro. PLoS One 2014;9:e90864.
33 Nunes S, Soares E, Fernandes J, Viana S, Carvalho E, Pereira FC, Reis F: Early cardiac changes in a rat model of prediabetes: brain natriuretic peptide overexpression seems to be the best marker. Cardiovasc Diabetol 2013;12:44.
34 Nunes S, Rolo AP, Palmeira CM, Reis F: Cardiomyopathies-Types and Treatments. Kaan Kirali Ed. InTech 2017;ISBN 978-953-51-3040-6.
35 Cao X, Sun Z, Zhang B, Li X, Xia H: The Effects of Ivabradine on Cardiac Function after Myocardial Infarction are Weaker in Diabetic Rats. Cell Physiol Biochem 2016;39:2055-2064.
36 Zhang X, Zhu M, Zhao M, Chen W, Fu Y, Liu Y, Liu W, Zhang B, Yin X, Bai B: The plasma levels of relaxin-2 and relaxin-3 in patients with diabetes. Clin Biochem. 2013;46:1713-1716.
37 Hida T, Takahashi E, Shikata K, Hirohashi T, Sawai T, Seiki T, Tanaka H, Kawai T, Ito O, Arai T, Yokoi A, Hirakawa T, Ogura H, Nagasu T, Miyamoto N, Kuromitsu J:Chronic intracerebroventricular administration of relaxin-3 increases body weight in rats. J Recept Signal Transduct Res 2006;26:147-158.
38 Sutton SW, Shelton J, Smith C, Williams J, Yun S, Motley T, Kuei C, Bonaventure P, Gundlach A, Liu C, Lovenberg T:Metabolic and neuroendocrine responses to RXFP3 modulation in the central nervous system. Ann N Y Acad Sci 2009;1160:242-249.
39 Harasiuk D, Baranowski M, Zabielski P, Chabowski A, Górski J: Liver X Receptor Agonist TO901317 Prevents Diacylglycerols Accumulation in the Heart of Streptozotocin-Diabetic Rats. Cell Physiol Biochem 2016;39:350-359.
40 Lamkanfi M, Dixit VM: Inflammasomes and their roles in health and disease. Annu Rev Cell Dev Biol 2012;28:137-61.
41 Fedak PW, Verma S, Weisel RD, Li RK: Cardiac remodeling and failure: from molecules to man (Part I). Cardiovasc Pathol 2005; 14(1):1-11.
42 Robert S, Gicquel T, Victoni T, Valença S, Barreto E, Bailly-Maître B, Boichot E, Lagente V: Involvement of matrix metalloproteinases(MMPs) and inflammasome pathway in molecular mechanisms of fibrosis. Biosci Rep 2016;36:e00360.

43 Kawaguchi M, Takahashi M, Hata T, Kashima Y, Usui F, Morimoto H, Izawa A, Takahashi Y, Masumoto J, Koyama J, Hongo M, Noda T, Nakayama J, Sagara J, Taniguchi S, Ikeda U: Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. Circulation 2011;123:594-604.
44 Van Tassell BW, Arena RA, Toldo S, Mezzaroma E, Azam T, Seropian IM, Shah K, Canada J, Voelkel NF, Dinarello CA, Abbate A: Enhanced interleukin-1 activity contributes to exercise intolerance in patients with systolic heart failure. PLoS One 2012;7:e33438.
45 Abbate A, Van Tassell BW, Seropian IM, Toldo S, Robati R, Varma A, Salloum FN, Smithson L, Dinarello CA: Interleukin-1beta modulation using a genetically engineered antibody prevents adverse cardiac remodelling following acute myocardial infarction in the mouse. Eur J Heart Fail 2010;12:319-322.
46 Mallat Z, Heymes C, Corbaz A, Logeart D, Alouani S, Cohen-Solal A, Seidler T, Hasenfuss G, Chvatchko Y, Shah AM, Tedgui A: Evidence for altered interleukin 18 (IL)-18 pathway in human heart failure. FASEB J 2004;18:1752-1754.