H3 relaxin alleviated vascular injury in rats with type 1 diabetes by inhibiting endoplasmic reticulum stress and inflammation

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Abstract

Human recombinant relaxin-3 (H3 relaxin), a small molecule peptide hormone, ameliorated myocardial injury after myocardial infarction or isoprenaline injection by inhibiting apoptosis and fibrosis. However, whether H3 relaxin protects vascular function in rats with type 1 diabetes and its mechanism are unknown. In type 1 diabetes rats model induced by streptozotocin (STZ), rats were subcutaneously injected H3 relaxin (2 µg/kg/d or 0.2 µg/kg/d) for 2 weeks. At 4 or 8 weeks after STZ injection, we detected the expression of fibrosis (type I and III collagen), ERS (endoplasmic reticulum stress) and NLRP3 inflammasome activation in the aortas and inflammation markers in the plasma from rats with diabetes. Compared with the diabetic rats, H3 relaxin treatment exhibited markedly decreased plasma oxidative stress markers (TNF-α and MDA) levels. The protein expression levels of type I and III collagen in the aortas were increased in rats with diabetes, inhibited by H3 relaxin. H3 relaxin treatment inhibited ERS (GRP78 and CHOP) and NLRP3 inflammasome activation in the aortas of diabetic rats. These results suggest that H3 relaxin inhibited fibrosis, ERS and inflammation activation in the aortas of type 1 diabetic rats.

1. Introduction

Arterial stiffness is an early stage of arterial dysfunction in patients with diabetes, which pathophysiologic mechanism is that high glucose induces impaired endothelial function. Endothelial dysfunction aggravates media thickness and fibrosis, in turn worsen arterial stiffness[1]. Arterial Fibrosis is an active process that collagen, fibronectin and adhesion proteins accumulate, adhesion molecules and integrins increase, and the extracellular matrix (ECM) remodel, which are involved in the arterial complications of diabetes[2]. Another pathogenic mechanism of arterial dysfunction in diabetes is that high glucose mediated vascular inflammation[3, 4]. ROS mediated oxidative stress is considered to be responsible for the progression and development of diabetic arterial dysfunction[5, 6]. Our previous studies suggest that the ROS mediated NLRP3 inflammasome activation plays a key role in the progression of diabetic cardiomyopathy[7, 8]. The NLRP3 inflammasome, a complex, comprised of the apoptosis-associated speck-like protein (ASC), NLRP3 and pro-caspase-1[9]. Once NLRP3 inflammasome was activated, then pro-caspase-1 transformed into cleaved caspase-1, which can activate IL-1β and IL-18 involved in arterial injury in diabetes. In addition, ROS induced by high glucose promotes ERS, aggravated inflammation and insulin resistance in diabetic vascular dysfunction. ERS may be involved in the mechanism of diabetic nephropathy, for example, ERS marker CCAAT/enhancer-binding protein homologous protein (CHOP) knockout ameliorated diabetic nephropathy in streptozotocin-induced diabetes mice[10]. In the retina of rats with streptozotocin-induced diabetes, ERS resulted in increased inflammation and vascular permeability[11]. Previous investigations suggested that acute ERS inhibition in aortic rings protected mice from diabetes-induced endothelial dysfunction[12].

Relaxin-3, an ancestral peptide of the human relaxin subclass of the insulin superfamily, was predominantly expressed in the central nervous system and regulated feeding, arousal, memory, learning and central responses to physiological stressors[13–16]. Recent data indicated that H3 relaxin alleviated

ischaemic injury by reducing hypoxia-induced production of ROS[17]. H3 relaxin inhibited myocardial injury induced by isoproterenol or in cardiac-restricted transgenic overexpression of β 2-AR mice[18, 19]. Our recent study reported that H3 relaxin prevented cardiac injury and fibrosis by regulating the activation of NLRP3 inflammasome in diabetic cardiomyopathy[7, 8]. However, whether H3 relaxin protected the vascular complications of diabetes from inhibiting the activation of ERS and the NLRP3 inflammasome was unknown. In this study, we sought to define whether H3 relaxin attenuates the vascular complications of experimental diabetes. Moreover, we explored the mechanism that H3 relaxin inhibited vascular injury.

2. Materials And Methods

2.1. Animals and reagents

Male SD rats (Second Affiliated Hospital of Harbin Medical University, Harbin, China; weight, 200 ~ 250g) experiments were conducted at First Affiliated Hospital of Harbin Medical University, and the present study was approved by the Laboratory Animal Management and Ethics Review Committee of First Affiliated Hospital of Harbin Medical University. STZ was purchased from Sigma (St. Louis, MO, USA), and H3 relaxin was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA).

2.2 Experimental diabetic rats model and groups

Diabetes was prepared in SD rats by intraperitoneal injection of 65 mg/kg STZ once as described previously[7]. Rats were randomly divided into 4 groups: control group, DM group, A group and B group. A group: at 2 or 6 weeks after STZ administration, the rats were injected with subcutaneous 2 μ g/kg/d H3 relaxin for 2 weeks. B group: at 2 or 6 weeks after STZ administration, the rats were injected with subcutaneous 0.2 μ g/kg/d H3 relaxin for 2 weeks.

2.3 Hematoxylin and eosin (HE) staining and Masson staining

At 8 weeks after STZ injection, the aortas were dissected from the rats. HE-stained and Masson trichrome-stained sections were photographed using a light microscope (Olympus). The areas of fibrosis in 4 randomly selected visual fields were measured with a threshold function under 40 $\times$ magnification.

2.4 Western blotting

The aorta proteins extract (40 μ g) from four groups were separated and transferred to PVDF membrane. Membranes were incubated in antibodies, including anti- β -actin (ZSGB-BIO, BeiJing, China), anti-GRP78(Abcam, Cambridge, UK), anti-CHOP (Abcam, Cambridge, UK), anti-NLRP3(Bioss, Beijing, China), anti-IL-1 β (Novus Biologicals, CO, USA), anti-IL-18(Novus Biologicals, CO, USA), anti-I-collagen (Bioss, Beijing, China) and anti-III-collagen(Bioss, Beijing, China). Then PVDF membranes were incubated in horseradish peroxidase–linked secondary antibody for 1 hour. Bands were quantified with NIH Image Software.

2.5 Measurements of plasma MDA and TNF-a

The blood of SD rats was centrifugated into obtaining plasma at 1000g for 10 min, then MDA was detected using ELISAs (Nanjing Jiancheng) and TNF-a was measured as described previously[7].

2.6 Relaxin and receptor expressions quantified by real-time PCR

Aorta tissues in control and DM group rats were collected at 4 and 8 weeks after STZ administration. Total RNA was isolated, reverse transcribed and quantified according to the manufacturer's instructions (TaKaRa, Dalian, China) as described previously[20].

2.7 Statistical analyses

Experiments were carried out in 8 rats per group in triplicate, and data were expressed as the means $\pm$ SE by GraphPad Prism 7.0 software. Data were analysed by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. $P < 0.05$ was considered significant.

3. Results

3.1 H3 relaxin ameliorated aorta injury in diabetic rats

Histological analysis of the vascular structure was performed by HE staining of the aorta specimens at 8 weeks after STZ injection. We observed that disordered elastic fibres in the aortas of rats with diabetes compared with those of controls by HE staining; however, H3 relaxin treatment restored the vascular structure (Fig. 1a). Masson staining showed that vascular smooth muscle cells were stained red and interstitial collagen was blue. The collagen network of adjacent cells is intact in control rats. Extracellular matrix deposition in vascular wall increased significantly in diabetic group. More, the collagen network around the cell breaks and arrange disorders in diabetic rats, and the content of collagen in H3 relaxin treatment group are less than diabetes group and elastic fibres arrange more regular than diabetes group (Fig. 1b,c,d).

Compared with the controls, the contents of MDA at 8 weeks in the DM group were markedly increased, whereas were significantly decreased after H3 relaxin administration. Compared with the controls, the levels of TNF-a at 4 weeks in the DM group were markedly increased, wherea were significantly decreased after H3 relaxin adiminstration (Fig. 2).

3.2 H3 relaxin improved vascular fibrosis in the aortas of rats with diabetes

Extracellular matrix contains structural proteins, such as Type I and III collagen, adhesion molecules, integrins. The expression of types I and III collagen were increased in the aortas of STZ-treated diabetic rats, whereas were inhibited by H3 relaxin treatment in diabetic rats (Fig. 3).

3.3 H3 relaxin inhibited ERS in the aortas of rats with diabetes

To explore whether ERS participated in H3 relaxin protection against vascular injury in rats with diabetes, the levels of ERS markers, including GRP78 and CHOP, were evaluated by western blotting. We found that compared with control, the expression of GRP78 and CHOP were significantly higher in the DM group, however, were lower after H3 relaxin treatment in diabetic rats (Fig. 4).

3.4 H3 relaxin inhibited NLRP3 inflammasome activation

Previous investigations have reported that the activation of NLRP3 inflammasome was involved in the pathophysiologic mechanism in complications of diabetes. In our work, the expression levels of NLRP3, IL-1 β and IL-18 proteins were increased in DM group, decreased by H3 relaxin administration (Fig. 5).

3.5 Relaxin-1/3 and their receptor expressions in aortas

In the aortas of diabetic rats, the mRNA expression of *Relaxin-1* significantly was upregulated at 4w after STZ injection (Fig. 6a); however, the mRNA expression of *Relaxin-3* was upregulated at 4w and 8w after STZ injection (Fig. 6b). In addition, the mRNA expression of relaxin family peptide receptor 1 (*RXFP1*) was significantly upregulated at 8w after STZ injection (Fig. 6c); *RXFP3* mRNA expression was also upregulated at 4w and 8w after STZ injection (Fig. 6d).

4. Discussion

The aims of this study were to identify whether H3 relaxin protected against vascular injury induced by diabetes in vivo and potential mechanism. Our results demonstrated that vascular fibrosis, NLRP3 inflammasome activation and ERS were involved in the vascular complications of diabetes and that H3 relaxin improved vascular injury by inhibiting fibrosis, ERS and NLRP3 inflammasome activation.

In higher primates, relaxin family peptides contains seven members, which are relaxin-1, 2, 3 and insulin-like peptides (INSL) 3, 4, 5, 6. However, in rats, there are six members in relaxin family peptides, including relaxin-1 (similar to human relaxin-2), relaxin-3 and INSL-3, 4, 5, 6[13]. The relaxin family peptides receptors contains RXFP1, 2, 3, 4, natural ligand of human relaxin-2 (rats relaxin-1) is RXFP1 which plays protective effects in the cardiovascular disease. Recent studies reported that relaxin-3 can inhibit myocardial injury by binding to RXFP1 in rats with myocardial infarction[19], although RXFP3, a natural receptor of relaxin-3, is mainly located in the brain. Later, study found that in rat atrial and ventricular cells, there existed relaxin-3 mRNA which expression was up-regulated in the myocardium after isoproterenol administration[18]. Our recent study reported that H3 relaxin improved cardiac injury by regulating the activation of NLRP3 inflammasome in diabetic cardiomyopathy[7, 8]. This study focus on whether H3 relaxin inhibited diabetic vascular injury, and we choose the dose of 0.2 or 2ug/kg/day relaxin-3 treatment for diabetic rats as zhang et al. described previously[18]. Interestingly, we found both doses of H3 relaxin were effective to a similar degree, and we will choose the very low dose H3 relaxin in future experiment. This study limitations contained that we did not test plasma relaxin-1 and relaxin-3

expression, so we can not clarify the issue that why both high and low dose H3 relaxin had similar protective effects in vascular injury in type 1 diabetes rats. We will avoid this question in future.

We found that the body weight increased and the glucose level decreased after H3 relaxin injection in diabetic rats as reported in our previous study[7], and which maybe a mechanism of that H3 relaxin inhibited vascular fibrosis, need to be veriflicated. Unfortunately, we did not detected the level of insulin, we will avoid this issue in future. In addition, we found that *Relaxin-1, 3* and *RXFP1, 3* mRNA were significantly up-regulated after STZ in the aortas of diabetic rats according to real-time PCR[20]. The reason in increased expression of *RXFP1* and *RXFP3* in aortas of diabetic rats may be due to increased expression of endogenous relaxin-1/3, however, these results clarified that endogenous relaxin-1/3 and receptors participated in the mechanism of vascular injury in diabetic rats.

The mechanism of vascular complications of diabetes contains endothelial dysfunction, oxidative stress and arterial remodelling. Oxidative stress is a determining mechanism in vascular complication of diabetes. Increased plasma TNF- α and IL-6 are associated with vascular dysfunction in patients with type 2 diabetes[21]. NF- κ B activity increased, TNF- α and intercellular adhesion molecule (ICAM) upregulated in vascular tissues of type 2 diabetic rats[22]. Aljwaid H et al. found that compared with control, MDA levels were higher in patients with diabetes, which was related with higher levels of oxidized ascorbate. These studies clarified that oxidative stress was involved in the mechanism of vascular dysfunction in diabetes[23]. Recent data indicate that H3 relaxin protects against ischaemic injury by reducing the hypoxia-induced production of ROS. In our study, we found that plasma MDA and TNF- α levels were increased in rats with diabetes, and H3 relaxin inhibited vascular dysfunction by regulating MDA and TNF- α levels in rats with diabetes.

The expression of ERS markers were increased in kidney from patients with diabetes, were involved in the pathogenesis of diabetic nephropathy, however, CHOP knockout improved kidney injury in diabetic mice[10]. In addition, ER stress activated inflammatory factors and mediated increased vascular permeability in retina of diabetes[11]. In a recent study, we found that H3 relaxin inhibited the ERS mediated apoptosis of myocardial cells induced by high glucose[24]. In our study, we found that compared with the controls, the expression of CHOP and GRP78 increased in the aortas of diabetic rats, which were inhibited by H3 relaxin administration, indicated that H3 relaxin inhibited ERS-induced vascular injury.

High glucose-induced NLRP3 inflammasome activation prompted vascular complications in diabetes. In vivo, NLRP3 inflammasome was activated in the aortas of rats after a high glucose diet, and inhibited by rutin administration[25]. Aberrantly, in atherosclerotic pig aortas, the expression of NLRP3, ASC and IL-1 β were increased, along with NF- κ B activation and endothelial dysfunction. In patients with coronary heart disease, increased NLRP3 expression in aortas was positively associated the severity of coronary artery disease[27]. In addition, our findings are consistent; compared with the controls, the expression and activation of the NLRP3 inflammasome were increased in the aortas of rats with diabetes, and were inhibited by H3 relaxin.

In conclusion, H3 relaxin is a potential candidate for treating diabetes-associated vascular complications by inhibiting fibrosis, the activation of ERS and NLRP3 inflammasome.

Declarations

Author contributions: Xiaohui Zhang and Kelaier Yang: conception, design and analysis of data, performed the data analyses, and wrote the manuscript. Xiao Ma and Xinhua Yin: contributed to the conception of the study. Jinyu Chi and Wenjia Chen: contributed significantly to analysis and manuscript preparation.

Compliance with ethical standards

Ethical approval This study was conducted after obtaining First Affiliated Hospital of Harbin Medical University's ethical committee approval.

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Conflict of interest statement

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

Data statement

Study data are available.

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Figures

Figure 1

HE and Masson staining in the aortas of rats with diabetes. (a) HE staining in the aortas of rats with diabetes at 8 weeks after STZ injection($\times 200$). (b) Masson staining in the aortas of rats with diabetes at 8 weeks after STZ injection($\times 200$). (c) Masson staining in the aortas of rats with diabetes at 8 weeks after STZ injection($\times 400$). (d) Quantification of fibrosis area analysed by Masson staining.

Figure 2

H3 relaxin decreased plasma MDA and TNF- α expression in diabetic rats (a) Protein expression of MDA at 4 and 8 weeks in the plasma of diabetic rats. (b) Protein expression of TNF- α at 4 and 8 weeks in the plasma of diabetic rats. * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, # $P < 0.05$ vs. DM, ## $P < 0.01$ vs. DM.

Figure 3

Effect of H3 relaxin treatment on vascular fibrosis in the aortas of diabetic rats (a) The protein expression of fibrosis markers was analysed by western blotting. (b) The protein levels of collagen III were normalized to β -actin (collagen III/ β -actin). (c) The protein levels of collagen I were normalized to β -actin (collagen I/ β -actin). * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, # $P < 0.05$ vs. DM, ## $P < 0.01$ vs. DM.

Figure 4

Effect of H3 relaxin treatment on ERS in the aortas of diabetic rats (a) GRP78 and CHOP protein expression levels were analysed by western blotting. (b) The protein levels of GRP78 were normalized to β -actin (GRP78/ β -actin). (c) The protein levels of CHOP were normalized to β -actin (CHOP/ β -actin). * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, # $P < 0.05$ vs. DM, ## $P < 0.01$ vs. DM.

a

b

c

d

Figure 5

H3 relaxin inhibited NLRP3 inflammasome activation (a) Protein expression of NLRP3 inflammasome markers (NLRP3, IL-1 β and IL-18) at 4 and 8 weeks in the aortas of diabetic rats. (b) The protein levels of NLRP3 were normalized to β -actin (NLRP3/ β -actin). (c) The protein levels of IL-1 β were normalized to β -actin (IL-1 β / β -actin). (d) The protein levels of IL-18 were normalized to β -actin (IL-18/ β -actin). *P < 0.05 vs. control, **P < 0.01 vs. control, #P < 0.05 vs. DM, ##P < 0.01 vs. DM.

Figure 6

Relaxin-1, relaxin-3 and their receptors RXFP1 and RXFP3 levels in aortas of experimental diabetic rats. Real-time PCR detected the expression of relaxin-1 (a), relaxin-3 (b), RXFP1 (c) and RXFP3 mRNA (d) at 4 w and 8 w in the aortas of experimental diabetic rats. The results are relative to the β -actin level. * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control.