Liraglutide relieves myocardial damage by promoting autophagy via AMPK-mTOR signaling pathway in zucker diabetic fatty rat

Ya Zhang [a, 1], Yuanna Ling [a, 1], Li Yang [b], Yanzhen Cheng [b], Pingzhen Yang [a], Xudong Song [a], Huixiong Tang [a], Yongkang Zhong [a], Lu Tang [a], Shangfei He [a], Shuangli Yang [b], Aihua Chen [a, **], Xianbao Wang [a, *]

[a] Department of Cardiology, Zhujiang Hospital, Southern Medical University, Guangzhou, 510282, PR China
[b] Department of Endocrinology, Zhujiang Hospital, Southern Medical University, Guangzhou, 510282, PR China

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ABSTRACT

Liraglutide, a glucose-lowering agent used to treat type 2 diabetic mellitus is reported to exert cardioprotective effects in clinical trials and animal experiments. However, the cardioprotective mechanism of liraglutide on diabetic cardiomyopathy has not been fully illustrated. The present study was performed to investigate whether liraglutide alleviates diabetic myocardium injury by promoting autophagy and its underlying mechanisms. Our results show that liraglutide significantly reduced the levels of creatine kinase (CK) and lactate dehydrogenase (LDH), improved left ventricular functional status and alleviated myocardial fibrosis in the Zucker diabetic fatty (ZDF) rat model. Liraglutide also mitigated high glucose-induced injury in NRCs. However these effects were partly reversed by the autophagic inhibitor chloroquine (CQ). Liraglutide promoted myocardial autophagy in the vivo and in the vitro models. Furthermore, liraglutide-induced enhancement of autophagy was related to increased AMPK phosphorylation and decreased mTOR phosphorylation, which was partially abolished by the AMPK inhibitor compound C (Comp C). Collectively, our data provide evidence that liraglutide mediated diabetic myocardium injury by promoting AMPK-dependent autophagy.

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1. Introduction

Diabetic cardiomyopathy (DCM), a member of cardiovascular complications of diabetes, is characterised by structural and functional abnormalities, such as left ventricular dysfunction and prominent interstitial fibrosis (Fonarow and Srikanthan, 2006). As cardiovascular complications including DCM represent the major source of morbidity and mortality in diabetic subjects, it highlights the significance of treating DCM. Nonetheless, there is no clinically available effective treatment for the DCM at present (Zhang et al., 2016a).

Liraglutide (LG), a human glucagon-like peptide-1 (GLP-1) analogue, lowers postprandial hyperglycemia and increases plasma insulin levels in the subjects with type 2 diabetic mellitus (T2DM) (Russell-Jones, 2009). GLP-1 receptor (GLP-1R) agonists have also been reported to exert cardiovascular protective effects in rodents and humans, such as improving cardiac function in patients with coronary ischemia or slowing the progression of atherosclerosis (Arturi et al., 2016; Gaspari et al., 2013). However, the actions of LG on DCM are complex and its underlying mechanisms have not yet been absolutely elucidated.

Autophagy, as an intracellular catabolic pathway, maintains cellular homeostasis under unfavorable environmental conditions, such as oxidative stress and hyperglycemia, which are both observed in diabetes. Autophagy is considered to be involved in the development of DCM (Kobayashi and Liang, 2015). Studies indicated that LG increased the viability of hepatocytes and pancreatic β-cells by promoting autophagy (Sharma et al., 2011; Wang et al., 2015), whereas the question of whether LG exerts cardioprotective effects in DCM by regulating autophagy has not been absolutely revealed. In addition, adenosine monophosphate activated protein kinase (AMPK) is a classic upstream regulator of

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autophagy and the loss of AMPK resulted in defective autophagy (Egan et al., 2011). It has been reported that LG activates AMPK in skeletal muscle cells and hepatic cells (Andreozzi et al., 2016; He et al., 2016). Similarly, AMPK is activated by GLP-1R agonists in adult cardiomyocytes (Balteau et al., 2014). Considering the close association between AMPK-regulated autophagy and LG, we wondered whether LG protects against diabetic myocardial damage by enhancing autophagy via the AMPK signaling pathway.

Therefore, the objectives of the present study were to assess the effects of LG on diabetic myocardium injury and to investigate its underlying mechanisms involving AMPK-dependent autophagy.

2. Materials and methods

2.1. Materials

Zucker diabetic fatty (ZDF) rats (ZDF-Lepr^{fa}) and their lean controls (ZDF-Crl) were obtained from the Beijing Vital River Laboratory Animal Technology Co. Ltd. at 8 weeks of age. Fifty rats were included in the study. LG was purchased from Novo Nordisk A/S (Denmark). 3-Methyladenine (3-MA), chloroquine (CQ), compound C (Comp C), 5-bromo-2-deoxyuridine (BrdU), D-(+)-Glucose powder were obtained from Sigma-Aldrich (USA). Foetal bovine serum (FBS) and Dulbecco Modified Eagle's Medium (DMEM) were purchased from Gibco Co. Ltd. (USA). MTS was purchased from Promega (Promega, Madison, WI, USA). Ad-mRFP-GFP-LC3 adenovirus was purchased from HanBio Technology (HanBio, Shanghai, China). Antibodies against LC3 (1:1000), p62 (1:1000), p-AMPK (1:1000), AMPK (1:1000), p-mTOR (1:1000) and mTOR (1:1000) were all purchased from Cell Signaling Technology (CST, Shanghai, China), and the anti-GAPDH (1:10 000) antibody (Kang Chen, China) and the goat anti-rabbit (1:8000) secondary antibody (Boster Biological Technology, China).

2.2. Type 2 diabetic mellitus animal model

All of the experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication, No. 86–23, revised 1996) and were approved by the Bioethics Committee of Southern Medical University, Guangzhou, China. The rats were adapted to their housing (21 °C ± 1 °C; 12 h day/night cycle) and had free access to food and water. The ZDF-Lepr^{fa} rats were fed with a high-fat-carbohydrate chow, whereas the ZDF-Crl rats were fed with a normal chow diet. The ZDF rats were randomly divided into five groups: (1) normal group (Control); (2) DCM group; (3) DCM + LG group (200 μg/kg of LG, subcutaneous injection); (4) DCM + CQ group (25 mg/kg of CQ, intraperitoneal injection); and (5) DCM + LG + CQ group. LG and CQ were injected once a day for 8 weeks. Rats with glucose levels ≥ 12 mmol/L were considered diabetic and at < 8 mmol/L were classified as control rats (Cai et al., 2002). At week 16, blood samples were collected for biochemical analysis before the rats were sacrificed, and the hearts were extracted for further analysis.

2.3. Hyperglucose cell model

Neonatal rat cardiomyocytes (NRCs) were isolated from newborn (1- to 3-day-old) Sprague Dawley rats. The cells were cultured in DMEM with 10% FBS and 100 units/mL penicillin and streptomycin in a 5% CO$_2$ incubator at 37 °C. To mimic a type 2 diabetic mellitus animal model, the cells were exposed to D-glucose at a final concentration of 50 mM once the cell populations reached 40%–50% confluence and were exposed to D-glucose at a concentration of 5.5 mM as a control (Tsai et al., 2015). NRCs were divided into the following groups: (1) normal group (Control): the cells were cultured in minimal essential medium with glucose (5.5 mM) for 96 h; (2) high glucose group (HG): the cells were exposed to high glucose (50 mM) for 96 h; (3) HG + LG group: the cells were primarily cultured in high glucose for 72 h and were then co-treated with LG (100 nM) for 24 h; (4) HG + CQ group: the cells were incubated in high glucose for 72 h and were then simultaneously exposed to CQ (25 μM) for 24 h; (5) HG + LG + CQ group: the cells were cultured in high glucose for 72 h and were then co-administered with LG (100 nM) and CQ (25 μM) for 24 h; (6) HG + C group: the cells were cultured in high glucose for 72 h and were then stimulated with Comp C (20 μM) for 24 h; and (7) HG + LG + C group: the cells were cultured in high glucose for 72 h and were then co-administered with LG (100 nM) and Comp C (20 μM) for 24 h. The culture medium was changed daily.

2.4. Cell viability analysis

Cell viability was evaluated by an MTS assay (Promega, Madison, WI, USA) according to the manufacturer's instructions with 3×10^4 cells/well seeded into flat-bottomed 96-well plates. To exclude a hyperosmotic effect, we added identical concentration of mannitol (Sigma, USA) in the control cultures (Osmotic control group). Following the experimental treatments, 20 μl of the MTS solution was added to each well and was incubated for 3 h at 37 °C in the dark. The viability was measured by detecting the absorbance at 490 nm using a microplate reader (Bio-Rad Laboratories), and the readings were normalised with a vehicle control.

2.5. Apoptosis assay

The evaluation of apoptosis was performed using a TUNEL (Promega) assay according to the manufacturer's instructions. Apoptotic nuclei were visualized by fluorescence microscopy. TUNEL-stained cells (%) were calculated according to the distribution of myocardial cells by microscopy, five fields were chosen in each section. Finally we calculated the average percentage of apoptotic cells as the apoptosis index.

2.6. Masson-trichrome staining

For the histological analysis, the fresh myocardial tissues were fixed in 4% formalin, buffered with PBS, dehydrated in various concentrations of ethanol and transferred to xylene; they were then embedded in paraffin, cut into 6 μm sections, and the histological stainings were performed accordingly. The images were analysed by Image-Pro plus 5.1 software. The collagen volume fraction (CVF) was measured.

2.7. Immunohistochemical (IHC) analyses

In short, the specimens were dewaxed and were incubated with 3% H$_2$O$_2$ in methanol at 37 °C for 10 min to quench endogenous peroxidase. After blocking at room temperature for 30 min, the sections were incubated with the primary antibody LC3B (Cell Signaling Technology) at 1:1000 dilution over night at 4 °C, followed by incubation with a secondary goat anti-rabbit IgG antibody (Boster) and diaminobenzidine as a chromogen before being examined under an optical microscope. The images were analysed by Image-Pro plus 6.0 and the area and integral optical density (IOD) were measured.

2.8. Echocardiography

Cardiac function was evaluated by echocardiography. An