Glucagon-like peptide-1 attenuates liver injury in the rat diabetes model

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Abstract: The aim of present study is to examine the effect of glucagon-like peptide-1(GLP-1) on diabetes-induced liver injury and explore detailed mechanisms of GLP-1 hepatoprotective effect. 150 male Sprague-Dawley rats were randomly assigned into three groups with equal number, including Sham group, diabetes group and GLP-1 intervention group. Diabetes rat model was performed with intraperitoneal injection of streptozotocin (STZ, 65mg/kg). Fasting blood-glucose of rat model was assessed at 72h after STZ injection to verify diabetes rat model. Rats in Sham group were normally fed. Rats in GLP-1 intervention group received 2 ng/kg GLP-1 intervention, at 2, 4, 6 and 8 weeks after intervention, TUNEL staining were performed to examine apoptosis of liver tissue. PCR and Western blot were performed to examine insulin, GLP-1R, autophagy-associated gene and HDAC-1. Compared with diabetes group, insulin expression of GLP-1 intervention group increased significantly ($P<0.05$). TUNEL staining at different time showed apoptosis levels of liver tissues were reduced gradually after GLP-1 intervention ($P<0.05$). Compared with diabetes groups, the expressions of BCL2 and GLP-1R were increased, while the levels of caspase3 and LC3 were reduced in GLP-1 intervention group ($P<0.05$). GLP-1 treatment decreased levels of phosphorylated AKT, phosphorylated ERK1/2, and HDAC6 in liver tissues ($P<0.05$). GLP-1 treatment alleviated diabetes-induced liver injury via regulating autophagy. The mechanism of GLP-1 hepatoprotective effect could be via GLP-1R-ERK1/2-HDAC6 signaling pathway.

Keywords: Diabetes, liver injury, glucagon-like peptide, autophagy.

INTRODUCTION

Diabetes has become a great threat on human health with development of social and economic development (Dabelea 2009) and clinical studies showed that diabetes could cause multiple organ injury. For example, aberrant angiogenesis of diabetes patients not only exacerbates diabetic foot injury, but also induces potential myocardial damage in clinical scenario (Fu *et al*., 2013; He *et al*., 2014). Although classic anti-diabetes treatments have improved living qualities of patients, there is some limitation for such treatments (Shigihara *et al*., 2006; Thorand *et al*., 2005). Moreover, latest studies demonstrated that diabetes patients also suffered from liver injury, which was consequently proved to be caused by diabetes (Schulthess *et al*., 2009), while classic anti-diabetes treatments cannot alleviate diabetes-induced liver injury and even some anti-diabetic drug had potential hepatotoxicity (Hultcrantz *et al*., 2009; Frigerio *et al*., 2002). Thus, endogenous medicinal substances were warranted for treatment of diabetes-induced liver injury.

Glucagon-like peptide-1(GLP-1) is an on endogenous substance which was proved with potential protective effect on energy metabolism (Roep *et al*., 2010). Some animal studies showed that GLP-1 could improve glycometabolism so as to mitigate diabetes symptoms (Tanaka *et al*., 2009). There were still reports that GLP-1 was correlated with HDAC related pathways, but the details remain unknown (Chang 2010). In addition, HDAC pathways played a pivotal role in hepatoprotective effect. However, the effect of GLP-1 on diabetes-induced liver injury and detailed mechanisms of GLP-1 hepatoprotective effect was unclear.

Our study was aimed to explore the effect of GLP-1 on diabetes-induced liver injury in rat diabetes model and detailed HDAC related mechanisms for GLP-1.

MAERIALS AND METHODS

General materials

150 male SD rats with 5 weeks old were offered by experimental animal center, after adaptive feed for 1 week, all experimental animals weight of 220±18.65g were randomly divided into normal control group and model group, the TUNNEL kit for cell apoptosis was produced by Beyotime, total RNA extraction kit(Trizol) for tissue and cells was from TAKARA, RNA reverse transcription kit (Tian Gen), all the qRT-PCR Reagents and consumables were purchased from Promega, real time PCR was IQ5 from BioRad, data was analyzed by version 2.1 software, the fluorescent dye was produced by Roche, all the rest of reagents were the lab self-saved.

Streptozotocin (STZ) was purchased from Sigma (St. Louis, MO, USA). Formaldehyde and xylene were purchased from Guangzhou Chemical Reagent Factory

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(Guangzhou, China). Caspase3 antibody, LC3B antibody, p-Akt antibody, p-ERK1/2 and HDAC6 antibody were purchased from Abcam. Inc. (USA).

Experimental animal model: Type 1 diabetes rats

Remove all the substandard rats (weight>200g) after weighing, then made up to ensure the same number of each group, all animals were feed after randomized caging in the unit SPF laboratory animal research center, rearing condition: room temperature (25±1°C), humidity range(50±2%), sterilized drinking water and di*et al*l adopted freely, after 1w adaptive feeding and animal body weight increased to more than 220 g, injected STZ with the dose of 65mg/kg intraperitoneally in the case of animal empty stomach for 12 h, collecting tail vein blood after 72h injection, the glycemic index were monitored to confirm the further study, the empty stomach glycemic index which exceed 16.67mmol/L, maintained for above 4 weeks and displayed no difference, means the successful animal model. Animal experiment was registered and approved by ethics committee of local hospital and the approval number was 2017MH037671.

TUNEL assay for liver apoptosis

Injecting GLP-1 with dose of 2 ng/g into the successful model, the model and control group liver tissue samples were collected at 2-week, 4-week, 6-week, 8-week after anesthesia animal with ethyl carbamate, took 5mg of sample into liquid nitrogen and the rest of tissue fixed in 4% paraformaldehyde quickly. Paraffin section was performed by routine method, and the liver cell apoptosis was assessed by the TUNEL assay.

qRT-PCR assay for insulin express

After cutting the 5mg sample mentioned above into pieces in mortar, the specimen were added liquid nitrogen and ground into powder, then transfered into nucleic acid-free and 1ml Trizol were added in to EP tubes. The complex were mixed sufficiently, after placed at room temperature for 5 minutes, adding 200μl chloroform, covering EP tube tightly and rocking violently for 15s, centrifuged 20 minutes at 12000rpm, 4°C taking the upper water phase into a new EP tube, adding 500μl pre-cooling chloroform, mixed up and down, after storing in -20°C for 2h, centrifuged 10 minutes at 12000rpm, discarded the supernatant, added 1ml 75% ethanol, vortex mixing, centrifuged 5 minutes at 12000rpm, 4°C, discarded supernatant, drying 10 minutes at room temperature, precipitation were dissolved with 35μl RNA enzyme-free water, RNA stored in -80°C freezer after detecting concentration.

After detecting the RNA concentration, took 1μg RNA for cDNA reserve transcription, specific steps were described in RNA reverse transcription kit, complemented with nucleic acid-free water for the different concentration in different specimen. After the reverse transcription, took 1μl sample for qRT-PCR to detect the expression of insulin. Procedure of qRT-PCR is shown as follow: 95°C 3min; 95°C 10 s, 55°C 30s, 40 cycles, primer used in this experiment was designed and synthesized by Genomics company, sequences are shown in table 1.

Western blot

Proteins were extracted from transfected cells for Western blot with routine protocols. The brief protocols were as follows:

Protein suspension (15μg protein) was prepared for electrophoresis. Block proteins for 1.5 hours after transferring. Incubate with primary antibody (1: 800) at 4 for 12 hours. React with second antibody (1:1500) at 37 for 2 hours. Gel imaging system was used for analysis of specific protein bands.

STATISTICAL ANALYSIS

All test results were summarized and analyzed by SPSS 16.0 software, the results were shown as mean value ± standard deviation. The intra-group comparison of before and after treatment was analyzed by t test. Comparison between groups was performed with One-way ANOVA analysis. P value <0.05 was considered to be statistically significant.

RESULTS

Establishment of Diabetes rat model

80 male Sprague-Dawley rats were randomly raised in separate cages. Intraperitoneal injection was performed with 65mg/kg STZ at 12 hours fasting. 40 rats in sham group were normally bred, and all other food and drinking condition is the same among three groups. Caudal venous sampling was obtained to assess fast blood glucose. Examination result was showed in table 2. After 72 hours STZ administration, fast blood glucose levels of all modeled rats were above 16.67mmol/L to assure model stability. During 4-week observation period (weekly observation), all modeled rats had onset of typical diabetes "three polys and one little" symptom after 24 hours STZ administration, including clinical polydipsia, polyuria, and more food and weight loss. There were no significant differences among fast blood glucose levels of all modeled rats in observation period (P>0.05). Moreover, compared with sham group, Diabetes group rats had lower level of insulin expression (fig. 1). All above confirmed STZ-Diabetes rat model was successful.

TUNEL staining to assess apoptosis

Maintenance rats-feeding condition after model was confirmed successful. 40 rats were randomly selected to receive GLP-1 intervention among Diabetes modeled rats. GLP-1 intervention rats were harvest for pancreatic specimen under isoflurane anesthesia at 2-week, 4-week, 6-week and 8-week after GLP-1 intervention, respectively. Examine apoptosis of pancreatic specimen

by TUNEL apoptosis assays kit protocol. Use apoptosis index to assess apoptosis of liver cells, especially β-cells. Result of TUNEL staining was showed in table 3. GLP-1 intervention decreased apoptosis index of liver cells, and apoptosis index of GLP-1 intervention group was significantly lower than Diabetes group (P<0.05). Moreover, there was a time-dependent relationship between apoptosis and GLP-1 intervention period and this suggested GLP-1 intervention could slow down apoptosis of liver cells.

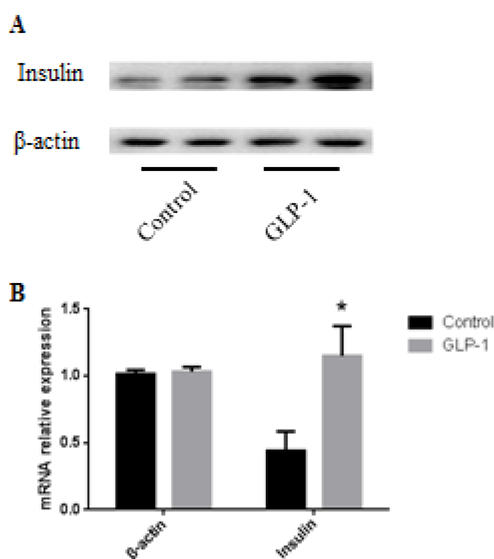

Fig. 1: (A) Western Blot of insulin in two groups; (B) Analysis of mRNA relative expression of insulin, *P<0.05 vs control group.

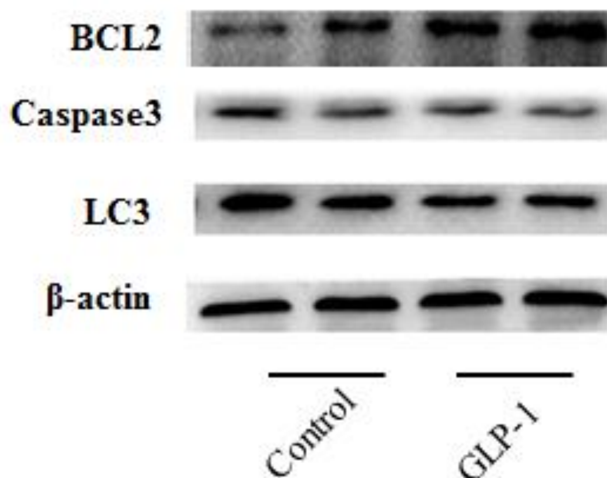

Fig. 2: Western Blot of BCL2, caspase3 and LC3 in two groups.

GLP-1 treatment increased expression of insulin

Both PCR and Western blot results showed that compared with Diabetes group, the GLP-1 intervention group had a higher level of insulin, suggesting GLP-1 treatment improved pancreas islet function (fig. 1, P<0.05).

GLP-1 maintained liver autophagy homeostasis in diabetes

Compared with diabetes group, the expression of BCL2 was increased in GLP-1 intervention group, while the levels of caspase3 and LC3 were significantly reduced in GLP-1 intervention group, verified by western blot (fig. 2, P<0.05). These results indicated that GLP-1 alleviated diabetes –induced liver autophagy dysfunction.

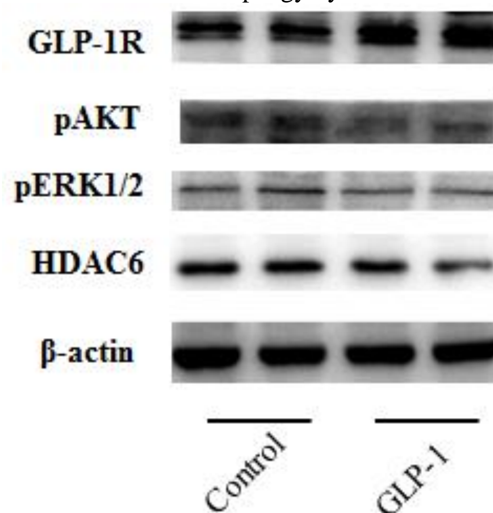

Fig. 3: Western Blot of GLP-1R, phosphorylated AKT, phosphorylated ERK1/2, and HDAC6 in two groups.

GLP-1 treatment regulated liver GLP-1R-ERK1/2-HDAC6 Signaling Pathway

Compared with Diabetes group, GLP-1 treatment significantly increased expression of GLP-1R (fig. 3, P＜0.05). Moreover, the GLP-1 intervention group had decreased levels of phosphorylated AKT, phosphorylated ERK1/2, and HDAC6 in liver tissues (fig. 3, P<0.05). These results indicated that GLP-1R-ERK1/2-HDAC6 signaling pathway was alternatively regulated under GLP-1treatment, which was responsible for GLP-1 hepatoprotective effect.

DISCUSSION

Our study demonstrated that glucagon-like peptide-1(GLP-1) alleviated diabetes-induced liver injury. GLP-1 treatment not only increased baseline insulin expression in diabetes, but also inhibited liver apoptosis and maintained autophagy homeostasis via GLP-1R-ERK1/2-HDAC6 signaling pathway.

As one kind of non-infectious diseases, diabetes significantly affects the daily lives and health of patients alongside changing lifestyles and dietary habit, and the number of diabetes patients is on rise year by year (Kieffer and Habener 1999). Diabetes patients have more severe prognosis, including renal dysfunction, heart failure and retinopathy. Consequently, studies about clinical medication and mechanism of Diabetes should be

Table 1: Primer used in this paper

name	sequence
F	ACCTTTGTGGTCCTCACCTG
R	GTGCTGCACTGATCCACAA
β-actin F	ACGAGGCCCAGAGCAAG
β-actin R	TTGGTTACAATGCCGTGTTCA

Table 2: Fast blood glucose levels of modeled rats in different period (mmol/L), *P>0.05

Group	Before administration	72h	1week	2 week	3 week	4 week
Sham	5.6±0.8	5.5±0.5	5.6±0.5	5.4±0.9	5.7±1.0	5.5±0.7
STZ-T1DM	5.5±0.6	26.3±2.8*	29.6±2.6*	30.2±2.4*	29.9±2.5*	30.3±3.1*

Table 3: Comparison of apoptosis index of three groups at different period (%) ($\bar{x}$ ±s)

Time	Sham	GLP-1	Diabetes
2-week(n=10)	2.08±0.34	38.08±3.34*	46.22±4.55
4-week (n=10)	2.42±0.23	32.08±4.2*	44.18±3.14
6-week (n=10)	2.11±0.31	20.08±3.42*	46.67±5.84
8-week (n=10)	2.25±0.21	8.08±1.23*	55.73±6.24

focused on restoration of insulin-secretion function and tissue damage repairing. Latest clinical trials indicated liver dysfunction and liver injury were also severe complication in diabetes patients, while there is not sufficient experiment evidence for the strategy to alleviate diabetes-induce liver injury (Stoffers *et al*., 2000; Farilla *et al*., 2003).

GLP-1 is a member of incretin hormone family with abilities to enhance insulin secretion (Kawasaki *et al*., 2010). GLP-1 is produced through degradation of proglucagon expression product in Langerhans cell of intestinal mucosa, and has similar protein structure and nucleotide sequence with glucagon (Ferdaoussi *et al*., 2008). In addition, GLP-1 is generally found in various organs with highly conserved nucleotide sequence (Sherry *et al*., 2007). Our study showed that GLP-1 indeed improved diabetes prognosis by increasing insulin, consistent previous report. In addition, GLP-1 decreased liver tissue apoptosis in diabetes rat model, suggesting GLP-1 alleviated liver injury.

Autophagy dysfunction is one of features for diabetes-induced liver injury, and ERK1/2-HDAC6 signaling pathway played a pivotal role in this pathological process, which is characterized by decreased BCL2 and enhanced LC3 (Hadjiyanni *et al*, 2008; Perez-Arana *et al*., 2010). Our study showed that GLP-1 treatment increased BCL2, but decreased LC3, suggesting GLP-1 maintained liver autophagy homeostasis in diabetes. As reported previously, GLP-1 regulated cellular events mainly via binding its receptor, GLP-1R. On the other hand, it has been proved that ERK1/2-HDAC6 was the downstream of GLP-1R pathways, which is named as GLP-1R-ERK1/2-HDAC6 signaling pathway [20]. Our study showed that GLP-1 treatment not only maintained liver autophagy homeostasis in diabetes, but also regulated liver GLP-1R-ERK1/2-HDAC6 Signaling Pathway.

CONCLUSION

GLP-1 treatment alleviated diabetes-induced liver injury via regulating autophagy. The mechanism of GLP-1 hepatoprotective effect could be via GLP-1R-ERK1/2-HDAC6 signaling pathway.

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