Liraglutide Activates AMPK Signaling and Partially Restores Normal Circadian Rhythm and Insulin Secretion in Pancreatic Islets in Diabetic Mice

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Received January 9, 2015; accepted May 1, 2015; advance publication released online June 2, 2015

β-Cell insufficiency plays an important role in the development of diabetes. Environmental factors, including lifestyle, play a critical role in β-cell dysfunction. Modern lifestyles affect the inherent circadian clock in central and peripheral organs. Recent studies have demonstrated that the normal intrinsic circadian clock in islets was essential for the viability of β cells and their insulin secretory function. Overall, however, the data are inconclusive. Our study demonstrated that the disrupted circadian rhythm of islets in streptozotocin induced type1 diabetic mice may be associated with impaired β-cell function and glucose intolerance. Liraglutide, a glucagon-like peptide-1 (GLP-1) analogue, could partially restore the normal circadian rhythm and activate the 5' AMP-activated protein kinase (AMPK) signaling pathway. Our study provided evidence demonstrating that Liraglutide might restore β-cell function and protect against the development of diabetes in a mouse model by attenuating the disruption of the intrinsic circadian rhythm in islets and by activating AMPK signaling.

Key words β-cell function; glucagon-like peptide-1 (GLP-1); circadian clock; 5' AMP-activated protein kinase (AMPK)

Diabetes mellitus (DM) is a metabolic disease with a high global prevalence; it is characterized by the progressive destruction of pancreatic islet cells. In the literature, it has been well established that pancreatic β-cell function plays an important role in maintaining glucose homeostasis and in preventing the onset of diabetes. Therefore, studies investigating how to maintain or restore β-cell function have recently attracted more attention. The increased prevalence of diabetes has topped the list of global epidemic health concerns worldwide. Although the detailed mechanisms underlying the increased prevalence of diabetes remain unknown, environmental factors, particularly contemporary lifestyles, are considered to be the chief culprits. The modern lifestyle disrupts the normal circadian rhythm. Epidemiological studies and animal models have provided strong evidence implicating the disturbed clock rhythms of the genes expressed in the pathogenesis of obesity and type 2 diabetes. Therefore, exploring the possible mechanism by which islet clock genes are regulated in the pathogenesis of diabetes may open a new avenue for treating DM.

The circadian clock, or circadian oscillator, makes it possible for organisms to coordinate their biologies and behaviors with daily and seasonal changes in the day-night cycle. It drives the cycles of physiology regulation, behavior, and morphological shifts in the human body, thereby reflecting the inner rhythm of life activities. As the environment changes, the circadian clock is reset through an organism’s ability to sense external time cues, the primary cue being light. Circadian clocks are ubiquitous in body tissues, in which they are synchronized by both endogenous and external signals to regulate transcriptional activity throughout the day in a tissue-specific manner and are intertwined with most cellular metabolic processes. The majority of identified clock components are transcriptional activators or repressors that modulate protein stability and nuclear translocation and create interlocking feedback loops. Currently, the best-understood clock genes include BMAL1 (aryl hydrocarbon receptor nuclear translocator-like, also referred to as ARNTL), circadian locomotor output cycles kaput (CLOCK), period circadian clock (PER), and cryptochrome (CRY). The normal rhythm of clock gene expression is regulated in a negative feedback pattern (i.e., a transcription-translation-transcription suppression loop composed of related genes and gene products). CLOCK/ BMAL1 heterodimers enhance the binding of the enhancer box with the PER and CRY promoter regions, thereby, leading to the subsequent gene expression, whose products form the PER/CRY heterodimer. PER/CRY heterodimers, however, inhibit CLOCK and BMAL1 expression, thus forming a loop or cycle of CLOCK/BMAL1-PER/CRY expression levels. The circadian system includes the central clock and peripheral clock. The central clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus; the SCN is considered to be the master circadian pacemaker that controls biological rhythms. Moreover, most tissues and organs have their own intrinsic circadian clocks that are known as peripheral clocks. The islet autonomous clock was reported for the first time in 2010, and it was subsequently proven to regulate the rhythm expression of genes associated with insulin secretion. Disruption of islet β-cell circadian clock genes led to decreased glucose-stimulated insulin secretion function, and β-cell specific
AMPK activation leads to circadian rhythm changes by destabi-
lyzing the negative modulators of the circadian clock, PERs
and CRYs.11) Here, we investigated the expression of circa-
dian clock genes in the islets of streptozotocin (STZ)-induced
diabetic mice with or without Liraglutide treatment. We found
that, while demonstrating significant effects on the gene ex-
pression of rhythm-related islet proteins (CLOCK, BMAL1, PER1, PER2, and CRY1), Liraglutide also notably regulated
the AMPK pathway in an STZ-induced diabetic mouse model.
Based on previous studies, as well as our phenotypic observ-
tions, we postulated that GLP-1 analogue could improve β-cell
function in diabetic animals by modifying the circadian clock
rhythm and AMPK pathway.

MATERIALS AND METHODS

Animals and Experiment Design Six-week old male
C57BL/6J mice were purchased from Shanghai Slac Labora-
tory Animal Co., Ltd., China and raised in a temperature-
controlled environment (22 ± 1°C) with 50–60% humidity. The
mice were subjected to an artificial light 12:12 light:dark
regimen (lights on at 6:00 a.m.).

An STZ-induced type 1 diabetic (T1DM) mouse model was
established, according to the protocols of the JAX laboratory.
The mice were individually marked, weighed, and intraperi-
toneally (i.p.) injected with STZ (50 mg/kg body weight) daily
(Cat. No. 50130, Sigma-Aldrich) dissolved in sodium citrate or
vehicle sodium citrate for 5 consecutive days. Blood glu-
cose levels were monitored with the OneTouch Ultra Blood
Glucose Monitoring System (LifeScan, Milpitas, CA, U.S.A.).
The mice were considered to be diabetic if their random blood
glucose levels were >16.7 mmol/L.

The mice were randomly assigned to 3 groups (i.e., the con-
trol, diabetes and diabetes groups), and they received GLP-1
analogue treatment on the 7th day after the last STZ injection.
GLP-1 analogue (Liraglutide, 0.3 mg/kg/d, Novo Nordisk A/S,
Bagsaerd, Denmark) was administered i.p. After 4 weeks of
administration, intraperitoneal glucose tolerance tests (IPGTTs)
were conducted, blood samples for the insulin assay were col-
lected by left ventricular puncture, and the pancreases were
prepared for immunoassaying of the islets. The other mice
were sacrificed for islet isolation every 4 h during the circadian
cycle. Five independent tests were conducted at each time
point (we defined 6:00 a.m. as the beginning of the circadian
cycle, indicating the beginning of the circadian time, CT0).
The mice were anesthetized with an i.p. injection of pento-
barbital sodium (100 mg/kg) under a dim red light in total
darkness. The freshly isolated mice islets were collected and
stored in liquid nitrogen for Western blot and real-time reverse
transcription polymerase chain reaction (RT-PCR) analysis.

The testing and handling of the animals were approved by
the Ethics Committee of Qilu Hospital, Shandong University.

Isolation, Purification and Identification of Mice Islets
Mouse islet isolation was performed using the pancreas in situ
perfusion method. Generally, the mice were anesthetized with
an i.p. injection of sodium pentobarbital. The common bile
duct was punctured using a syringe with a 28 G needle and
injected with 3 mL cold 1 mg/mL collagen type V (Sigma, Cat.
No: C9263). The inflated pancreas was removed and incubated
in Hanks solution for 13 min at 37°C and then vortexed into a
sand-like mixture. The sand-like mixture was infiltrated with
a 50-mesh sieve and washed with cold Hanks solution.

The isolated islets were identified with dithizone (D108724, Alad-
din, China), a chelatometric indicator conjugated with zinc
(specifically in islet β-cells), resulting in a scarlet color after
10 min at room temperature. The stock solution was prepared
by dissolving 10 mg dithizone in 3 mL alcoholic, with 50 μL
NH4OH, at 4°C in the dark.

Intraperitoneal Glucose Tolerance Test The IPGTT
was performed using the protocol described by Lamia et al.11) After
8 h of fasting, mice from all 3 groups were i.p. adminis-
tered with a glucose load of 3 g/kg. Blood glucose and plasma
insulin were tested at fasting and at 5, 10, 30, 60, and 120 min
after the glucose load was administered.

Analysis of Peripheral Blood Glucose and Plasma
Insulin After snipping the tail tip, peripheral blood glu-
cose was analyzed with the OneTouch Ultra Blood Glucose
Monitoring System. Plasma was separated from whole blood
by centrifugation at 1000×g for 10 min at 4°C and stored at
−80°C. Plasma insulin levels were measured with an enzyme-
linked immunosorbent assay (ELISA) kit (Mercodia, Cat. No.
10-1249-01), according to the manufacturer’s instructions.
Standard concentrations ranged from 41.14 to 2807.41 pmol/L.
All presented measurements were performed in duplicate.

Protein Quantification The isolated mice islets were
lysed with radio immunoprecipitation assay (RIPA) lysis
buffer (strong) for 45 min, and the protein concentrations of the cell
lysates were determined using the Bradford assay. Lysate
aliquots (20 mg/well) were separated by 10% sodium dodecyl
sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and
then transferred onto polyvinylidene fluoride membranes.
The membranes were blocked in 5% non-fat dry milk in
Tris-buffered saline Tween-20 (TBS-T; 10 mM Tris, pH 7.5,
150 mM NaCl, 0.05% Tween-20) for 1 h and then incubated
with AMPK/pAMPK (Cat. No. sc-130394, Santa Cruz), ACC/
ACC/ 

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TRIzol® Reagent Invitrogen, CA, U.S.A.), and genomic DNA was digested with DNase. Total RNA was extracted and purified from isolated islets for each sample (purity > 1.75) and synthesized at once into cDNA using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer’s instructions. The cDNA products were stored at −80°C to quantify the expression of the related genes.

Real-time PCR was performed using the LightCycler® 480 System (Roche Applied Science) using SYBR green (Toyobo) as a dye reagent, with the following protocol: denaturation at 95°C for 15 s, annealing at 62°C for 15 s, and extension at 72°C for 45 s. The primers were as follows.

- **CLOCK** forward: 5’-CAA ATC CTT CCA AGC GGC ATA-3’; reverse: 5’-CGC TGA GCC TAA GAA CTG AAA G-3’;
- **BMAL1** forward: 5’-CCA CCT CAG AGC CAT TGA TAC A-3’; reverse: 5’-GAG CAG GTT TAG TTC CAC TTT GTC T-3’;
- **PER1** forward: 5’-TGA AGC AAG ACC GGG AGA G-3’; reverse: 5’-CTG AAG CAA AAA TCG CCA CCT-3’;
- **PER2** forward: 5’-TGT GCG ATG ATG ATT CGT GA-3’; reverse: 5’-GGT GAA GGT ACG TTT GGT TTG C-3’;
- **CRY1** forward: 5’-CAC TGG TGC ATA GAG GGA GCT-3’; reverse: 5’-CTG AAG CAA AAA TCG CCA CCT-3’;
- **glyceraldehyde-3-phosphate dehydrogenase (GAPDH)** forward: 5’-AAG GTG AAG GTC GGA TGC AAC-3’; and reverse: 5’-GGG TCA TGA GGC ACA ATA-3’.

The gene expression levels were displayed as the ratio of the GAPDH transcripts used as the internal reference.

**Immunohistochemical Staining** Pancreatic tissues were fixed in 10% formalin, embedded in paraffin, and sectioned into 5µm thick tissue sections. After heat-induced antigen retrieval at 95°C for 30 min, the sections were dipped in 0.3% H₂O₂ for 10 min to quench the endogenous peroxidase and then incubated in 1% BSA/PBS for 10 min, followed by overnight incubation with primary antibodies at 4°C. The primary antibodies included anti-insulin and proinsulin (ab8304, Abcam, 1:500). Thereafter, the sections were incubated with HRP-conjugated secondary antibodies at room temperature for 15 min. For immunofluorescent staining, the sections incubated with the primary antibodies were further reacted with secondary antibodies, including green flu antibody (Cat. No. 828814, Invitrogen) and red fluoro antibody (Cat. No. 835723, Invitrogen) for 1 h in dark, and labeled with DAPI for 15 min. Then, images were acquired under a confocal microscope with the Velocity software (Perkin-Elmer, Waltham, MA, U.S.A.).

**Statistical Analysis** Quantitative data are presented as the means±standard deviation (S.D.) of three independent experiments. A t-test and two-way ANOVA were used to check the differences between 2 or more groups using SPSS 18.0, and p<0.05 was considered to be statistically significant.
RESULTS

Liraglutide Improved Mice Glucose Tolerance and Insulin Secretion after Glucose Loading We used STZ-induced diabetic mice as a T1DM model. To explore the effect of Liraglutide on islet function, we performed IPGTT after 4 weeks of Liraglutide treatment. As shown in Fig. 1A, Liraglutide significantly improved blood glucose tolerance at most time points (p < 0.5, p < 0.05, p < 0.01, and p < 0.05 for 5, 10, 30, 60, and 120 min, respectively), while the T1DM mice displayed higher blood glucose profiles. As for the insulin secretion, compared to the flat and low insulin-secreting curve observed in the T1DM mice, the Liraglutide-treated group showed significantly improved islet function, with a significant insulin release peak at 30 min after the glucose load. The quantified areas under the curve (AUC) for blood glucose and insulin secretion further demonstrated better outcomes after the Liraglutide treatment, indicating vigorous restoration of STZ-induced islets function (Fig. 1B).

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GLP-1 Analogue Attenuated STZ-Induced Pancreatic Islet β-Cell Damage Immunofluorescence staining was used for the sectioned pancreases to determine the composition and distribution of the islet insulin-producing cells in the 3 groups after 4 weeks of treatment. Our study demonstrated significant differences among the 3 groups. The control group showed intact morphology and the most intense insulin staining, while the T1DM group had nearly no intact islets. In situ, it was clear that the Liraglutide-treated group had greater islet mass, more islets and stronger insulin staining compared with the T1DM mice (see Fig. 2A). The restoration of the morphologically observed β-cell area aligned with the improved glucose tolerance and insulin secretion, as shown in Fig. 1. In addition, Fig. 2B shows a quantitative comparison of the ratio of insulin-staining areas in the pancreases among the three groups. In our study, Liraglutide was administered after successful induction of diabetes by STZ (on the 7th day of STZ administration). At this time point, most β cells were destroyed by STZ-induced insulitis (as shown in the STZ only group). Thus, the improved glucose tolerance and insulin secretion was believed to be related to the restoration of beta cells after 4 weeks of Liraglutide treatment instead of direct protection against STZ, which is consistent with a previous study.12)

GLP-1 Analogue Restored Circadian Rhythms and Average mRNA Expression of Clock Genes in the Mice Islets To uncover the change in circadian clock rhythms, we performed real-time-PCR analysis of all clock genes described above. We observed physical oscillations in the expression of intrinsic clock genes, including CLOCK, BMAL1, PER1, PER2 and CRY1, in the islets of healthy mice. The expression levels of the core circadian clock components (i.e., CLOCK and BMAL1) were blunted in the T1DM mice, which were
characterized by lower levels of these components and major delays or losses of peaks in the daily curve, and these levels were accompanied by elevated expression levels of inhibitory regulators, such as PER1, PER2 and CRY1 (Fig. 3A). Furthermore, Liraglutide treatment could attenuate the overexpression of these negative regulators, including PER1, PER2 and CRY1, and could also partially restore their rhythms, with a significant peak and shortened delay of the CLOCK and BMAL1 cycles compared with the T1DM group. These data indicated that the disrupted oscillations of the intrinsic clock gene expression levels in the T1DM model were characterized by increased expression levels of negative regulators (PER1, PER2 and CRY1) and thus blunted CLOCK/BMAL1 cycles.

To further determine the effect of Liraglutide treatment, we also quantified the daily average mRNA expression levels of the circadian clock genes. In the mice, the daily CLOCK mRNA level in the islets was clearly increased after the Liraglutide treatment, while BMAL1 showed no between-group differences ($p>0.05$). The daily mRNA levels of PER1, PER2 and CRY1 were significantly inhibited, which may at least partially explain the restoration of the expression oscillation of the normal clock genes (Fig. 3B). Note that the degree of disruption in the rhythms of the circadian genes expression levels closely correlated with the glucose tolerance impairment.9)

Effect of GLP-1 Analogue on Circadian Rhythms and Average Daily Protein Levels of the AMPK Pathway in the Mice Islets

To explore the mechanism underlying how Liraglutide acted on circadian clock oscillation, we used Western blot analysis to examine the expression and activation of major components in AMPK signaling and its correlation with circadian clock restoration. As shown in Fig. 4, the islets from the T1DM mice had lower average daily protein levels and lower cycle amplitudes for most of the related proteins compared to those from the control group, which is consis-
tent with the rhythms of circadian clock genes. However, the AMPK phosphorylation level in the Liraglutide-treated diabetic mice islets fluctuated and significantly increased in amplitude at CT4 (10 a.m. in the natural daily cycle) compared to the T1DM group. The relative average level of AMPK phosphorylation was also clearly elevated, thus implying the increased activation of AMPK. Similarly, we verified that LKB1, an upstream regulator of AMPK signaling that promoted the activation of AMPK, demonstrated consistent trends. When we further studied the rhythm of pLKB1, it was proven to oscillate with an 8 h phase advance to CT8 and with greater amplitude after the Liraglutide treatment (Fig. 4). The delay of pLKB1 expression, however, may indicate an independent regulation of AMPK signaling by Liraglutide. Moreover, phosphorylation of ACC, a downstream substrate of AMPK, also exhibited the same tendency as the AMPK and LKB1 protein levels. The pACC daily protein level induced by Liraglutide was increased and oscillated with a 4 h peak advance at CT16 (Fig. 4). The changes in the pAMPK and pACC protein levels indicated enhanced AMPK signaling. The latter would lead to phosphorylation and, thus, destabilization of the negative modulators of CLOCK/BMAL1 complex, PER2 and CRY1 proteins, as previously reported. Together, this evidence demonstrates that GLP-1 could potentiate AMPK signaling, thereby destabilizing the negative modulators and restoring the blunted rhythm of the circadian gene expression.

**DISCUSSION**

Diabetes mellitus occurs when the progressive loss of β-cell function finally fails to compensate for the increased demand for insulin. Although the mechanisms underlying the explosive spread of diabetes remain largely unknown, environmental factors and lifestyle changes associated with modern society correlate well with the increased risk of diabetes. The modern lifestyle adversely affects traditional routines and, therefore, the oscillation of the body’s circadian clock. Recent studies have demonstrated that peripheral tissues or organs contain intrinsic circadian oscillators that are essential for maintaining their normal functions. Marcheva et al. first reported the autonomous expression of clock genes. Further *in vitro* studies of mammalian cells have also revealed that CLOCK or BMAL1 deficiencies result in impaired insulin secretion from islet β cells. The abovementioned findings suggest that glucose metabolism is tightly bound with circadian oscillators. However, it remains inconclusive whether diabetes is related to the disrupted circadian oscillators, and there are no data concerning the benefit of restoring circadian rhythm in diabetes patients. For the first time, our study provided novel evidence suggesting that restoring normal circadian rhythm using GLP-1 analogue might be associated with therapeutic effects in a diabetic animal model.

Although GLP-1 has been found to play an important role in islet function restoration, the exact mechanisms associated with this role remain unknown. Recent studies have suggested that GLP-1 exerts effects on the peripheral circadian clocks.
in mouse liver and adipose tissue. These effects could be blocked by GLP-1R antagonist. Consistent with previous studies, our results confirmed that GLP-1 ameliorated islet damage, improved blood glucose profiles and facilitated insulin secretion in TIDM mice. Moreover, our data revealed a physical oscillation of the clock gene expression in the islets of healthy mice. However, the oscillation of clock gene expression in diabetic mice was disrupted. In addition, Liraglutide administration could partially restore the rhythm, thereby leading to favorable glucose control. Previous studies have reported that isolated islets from pancreas-specific Bmal1 knockout mice showed diminished insulin responsiveness to glucose load, suggesting that the autonomous circadian clock is essential for β-cell function. AMPK has long been considered to be a crucial sensor and regulator of energy metabolism. Numerous studies have shown that the activation of AMPK signaling improved blood glucose homeostasis and lipid profiles in vivo. AMPK signaling is a plausible candidate for conveying a cell’s metabolic state to the core clock circuitry. Lamia et al. first reported that AMPK is an important regulator of the circadian clock through cryptochrome phosphorylation and degradation. In addition, recent studies have demonstrated the important role of AMPK in regulating circadian clock genes in diabetes and obesity. As the essential energy sensor, AMPK plays a role in the phosphorylation and degradation of CRYs and PERs, which are the inhibitors of CLOCK/BMAL1. Therapeutically, metformin has been found to result in a phase shift of the circadian clock genes in the liver and muscle. Considering the well-established role of AMPK in diverse aspects of metabolic physiology, the reciprocal regulation of AMPK and circadian clocks might represent a fundamental link within the two-process model. It is plausible that the benefits of GLP-1 and its analogue might be related with AMPK activation and, thus, also related to restoration of normal circadian clock rhythms in islets. In the present study, we first reported that Liraglutide effectively influenced the circadian clock rhythm gene expression. Liraglutide can significantly regulate the expression and/or phosphorylation of AMPK, ACC and LKB1 in diabetic mice. Considering previous studies of the role of AMPK signaling in circadian genes oscillation, these results strongly suggest that Liraglutide might prevent β-cell dysfunction and attenuate the disruption of the intrinsic circadian rhythm in islets by regulating AMPK signaling. However, our work primarily focused on phenotypic observations and revealed the correlation between AMPK signaling, circadian rhythm and islet function. It remains largely uncertain whether the restoration of circadian rhythm was dependent upon AMPK, and the exact role that the circadian clock plays in islet function restoration requires further study.

In conclusion, our study demonstrated that GLP-1 analogues could attenuate the disruption of the intrinsic circadian rhythm in islets and activate AMPK signaling in a diabetic mouse model. Our study provides evidence that disrupted circadian rhythm might play a role in the development of diabetes. Intervention targeting the disrupted islet clock may provide a promising diabetes treatment strategy.

Acknowledgments This study is supported by the National Natural Science Foundation of China (Grant Nos. 81370943, 81400770, 81471020 and 81200608), Shandong Natural Science Foundation (Grant No. ZR2013HQ054), Jinan Young Science and Technology Stars Project (Grant No. 20120139), Shandong Medical and Health Technology Development Project (Grant No. 2014WS0148), Shandong University Basic Scientific Research Funding (Qilu Hospital Clinical Research Project) (Grant No. 2014QLKY21), Innovation Funding of Shandong University (Grant No. 2012TS141).

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES


13. Vieira E, Marroqui L, Batista TM, Caballero-Garrido E, Carneiro


