Chronic hepatitis C virus infection impairs insulin secretion by regulation of p38δ MAPK-dependent exocytosis in pancreatic β-cells

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Chronic hepatitis C virus (HCV) infection has a close association with type 2 diabetes mellitus. Although the mechanisms of insulin resistance in chronic hepatitis C (CHC) patients have been extensively studied, little attention has been given to the role of β-cell function in HCV-associated diabetes. Here, we analysed β-cell function in CHC patients and HCV-infected mouse model and found in addition to insulin resistance, impaired pancreatic β-cell function occurred in CHC patients and HCV-infected C/O10 mice, not only in diabetic individuals but also in individuals with impaired fasting glucose levels. Both first-phase and second-phase insulin secretion were impaired, at least partially due to the reduction of exocytosis of secretory insulin-containing granules following HCV infection. Up-regulated p38δ in HCV-infected β-cells resulted in inactivation of protein kinase D (PKD), which was responsible for impaired insulin secretory capacity of β-cells. Thus, impaired insulin secretion due to HCV infection in β-cells contributes to HCV-associated type 2 diabetes. These findings provided a new inspiration for the important prognostic and therapeutic implications in the management of CHC patients with impaired fasting glucose.

Introduction

Hepatitis C virus (HCV) is the leading cause of viral hepatitis, which may lead to chronic hepatitis (CHC) in up to 60–80% of infected adults and can progress to liver fibrosis, cirrhosis and eventually hepatocellular carcinoma [1,2]. Although the liver is the target organ for HCV infection, extrahepatic manifestations, including diabetes mellitus (DM), especially type 2 diabetes (T2DM), are frequently encountered in the clinical setting [3] and the prevalence rate of T2DM in CHC patients ranges from 8 to 33% [4,5].

Hepatitis C infection is associated with peripheral and hepatic insulin resistance [6,7]. The molecular mechanism by which HCV promotes insulin resistance has been extensively studied [8–11]. However, little attention has been given to the role of β-cell function and mass compared with insulin resistance as a cause of HCV-associated diabetes. It has been reported that HCV may have a direct cytopathic action, as it can infect many tissues, including pancreas [12–14]. In the present study, we investigated whether insulin secretion defects likewise manifest.

Insulin secretion by pancreatic β-cells involves sequential intracellular events. When extracellular glucose levels rise, GLUT2 mediated glucose uptake into β-cell. Then, glucose oxidation results in ATP production to closure of ATP-dependent K+ channels, leading to membrane depolarization and Ca2+ influx. Then insulin granules fuse with the plasma membrane in a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent process and release insulin. The rapid exocytosis that
In the present study, we have investigated β-cell function in both CHC patients and HCV-infected C/OTg mice. An impaired early-phase insulin response occurred even at the early stage of infection within the impaired range of fasting glucose following HCV infection. HCV infection reduced insulin secretion, at least partially by inhibiting exocytosis of insulin secretion via p38δ-protein kinase D (PKD) pathway. β-cell dysfunction induced by HCV infection could contribute to type 2 diabetes.

Materials and methods
Patients and biopsies
The study population was 46 CHC patients who had not been treated with interferon admitted to the First Hospital of Jilin University and the First Affiliated Hospital of Xinjiang Medical University. The clinical data are listed in Supplementary Table S1. Diagnosis of chronic HCV infection was based on standard serological assays and the presence of abnormal serum aminotransferase levels for at least 6 months. The following patients were excluded from the study: (1) positive for HBV surface antigen (HBsAg), (2) a family history (both parents and siblings) of diabetes, (3) taking medication known to affect glucose tolerance or insulin secretion, (4) abnormal thyroid tests (thyroid stimulating hormone, free T3 and free T4), abnormal renal function tests (serum creatine levels >1.0 mg/dl), or history of gastrectomy or chronic pancreatitis. Viral copies or genotyping was performed using HCV PCR-based assay or the Inno-Lipa HCV II assay (Qiagen). All patients underwent OGTT with 75 g of glucose according to the recommendations of the National Diabetes Data Group of the National Institutes of Health [19]. According to fasting plasma glucose (FPG), patients were subdivided into normal fasting glucose (NFG) (FPG < 5.6 mmol/l), impaired fasting glucose (IFG) (FPG 5.6–6.9 mmol/l) and DM (FPG ≥ 7.0 mmol/l). Different categories of FPG were generated as followed: FPG < 5.0 mmol/l; FPG 5.0–5.5 mmol/l; FPG 5.6–6.9 mmol/l; FPG 7.0–7.9 mmol/l; FPG ≥ 8 mmol/l. The fasting plasma glucose, insulin and C-peptide or from OGTT were shown in the Supplementary Data.

The sections of pancreatic tissue were taken from seven HCV patients with the carcinoma of head of pancreas, and the control samples were collected from four non-diabetic donor without HCV infection at the Liver Unit of the First Hospital of Jilin University and the First Affiliated Hospital of Xinjiang Medical University. With immobilization and embedded in paraffin, the pancreas was sliced to H&E or Masson stained pathological sections for investigating the pathology of islets.

Transgenic mice and metabolic studies
The transgenic C/OTg ICR mice harbouring both human CD81 and OCLN genes were maintained as described [16]. For infection in vivo, mice were tail-vein injected with 1 ml HCV (JFH-1, TCID50 = 1 × 10^8/ml), in 60–120 s to avoid liver injury as described [16]. All animal procedures in this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23, revised in 1996) and the approved regulations were set by the Laboratory Animal Care Committee at Wuhan Institute of Virology, Chinese Academy of Sciences (CAS) (permit WIVA02201602). All mice were housed in environmentally controlled conditions (25 ± 0.5°C, 12/12-h light/dark cycle with ad libitum access to food and water) in the laboratory of animal facility of Wuhan Institute of Virology, CAS. C/OTg mice (n = 70) were divided in three batches and tail-vein injected with 1 ml HCV (JFH-1, TCID50 = 1 × 10^8/ml) (Batch 1-3, Supplementary Table S3). Infection experiments were performed in a nonblind manner. Unless otherwise specified, genders of mice used in different assays were random. Mice were randomly withdrawn from each batch at the indicated time points for varies analyses, including insulin, glucose, C-peptide, viral loads, H&E and Masson’s trichrome staining and were killed using cervical dislocation after anesthetized with 0.3% pentobarbital sodium (10 μl/g) according to the experimental strategy. The virological and diabetic information of HCV-infected (n = 70) and mock-infected (n = 8) C/OTg mice raised in synchronization are listed in Supplementary Table S2.

Glucose tolerance test
Glucose tolerance tests were performed by intraperitoneal injection of mice with glucose (2.0 g/kg) according to methods described previously [17]. Glucose tolerance was evaluated as follows: normal glucose tolerance (NGT) (fasting plasma glucose (FPG) 5.6–6.9 mmol/l and 2-h (plasma glucose (PG) < 7.8 mmol/l), isolated impaired FPG (IFG) (FPG 5.6–6.9 mmol/l and 2-h PG < 7.8 mmol/l), isolated impaired glucose tolerance (IIGT) (FPG > 5.6 mmol/l and 2-h PG 5.6–7.8 mmol/l), isolated impaired glucose tolerance (IIGT) (FPG ≥ 7.0 mmol/l and 2-h PG ≥ 7.8 mmol/l), and diabetic (DM) (FPG ≥ 7.0 mmol/l).
mmol/l and 2-h PG between 7.8 and 11.0 mmol/l), IFG+IGT (FPG 5.6–6.9 mmol/l and 2-h PG 7.8–11.0 mmol/l), and DM subjects (FPG ≥ 7.0 mmol/l and/or 2-h PG ≥ 11.1 mmol/l).

**Cell culture, isolation of islets and insulin secretion**

The insulin-secreting mouse cell line MIN6 were cultured as previously described [18]. Mouse islets were isolated from adult male C57BL/6 mice (20–25 g; Shanghai Laboratory Animal Centre, CAS) as described [19]. Human islets from Institutional Review Board (WIV) approved healthy donors (with either written informed consent from the donor or the next of kin obtained) were isolated. Insulin secretion studies are performed as previously described [19].

**Virus productions and infections**

HCV (JFH1 strain) was used in the present study. Virus purification was performed as described [18]. Virus infections were performed at multiplicities of infection (MOI) of 0.1 or as indicated. A mock control (no virus input) was included in the experiment.

**Histology and immunohistochemistry (IHC)**

Serial 5-μm pancreatic sections from pancreas were fixed in 10% formalin and embedded in paraffin. Hematoxylin and Eosin (H&E) and Masson's trichrome staining of formalin-fixed paraffin-embedded pancreas tissues of CHC patients or C/O78 mice (n = 4 for each time point, 3 sections per mouse, 4 views per section) were performed according to manufacturer's instructions (Leica, Germany). IHC staining was performed as previously described with primary antibodies to CORE (1:50). All images were acquired with a Leica DS-Ri1 microscope. The representative images were shown in the indicated figures.

**Real-time PCR and Western blotting**

RNA isolation, cDNA synthesis and quantitative PCR with the indicated primers (Supplementary Table S3) as well as Western blotting were performed as described [18]. The following antibodies were used: NS5A (from Charles M. Rice); CORE, ab2740 (Abcam); GAPDH, 2118; Actin, 4970; p38δ, 2308; PKD, 90039; Phospho-PKD (Ser916), 2051 (Cell Signalling Technology); Syntaxin 1A, 66437-1-Ig, synaptosomal-associated protein 25 (SNAP25), 14903-1-AP and vesicle-associated membrane protein 2 (VAMP2), 10135-1-AP (Proteintech).

**Hyperglycaemic clamping**

First, the mice were intraperitoneally anesthetized with 0.3% pentobarbital sodium (10 μl/g). Hyperglycaemic clamping of mice was conducted as described [20].

**Perifusion assays**

Harvested islets or MIN6 cells were incubated overnight and then placed in a perifusion chamber as described [21].

**Electrophysiology and measurement of [Ca2+]i**

Whole-cell exocytosis was recorded and analysed with port-a-patch clamp amplifiers and the software Pulse+Pulsefit (Nanion Technologies, Germany). MIN6 cells were loaded with fura-2, and [Ca2+]i was measured as under a Leica S2 laser confocal scanning microscope (Leica TCS NT, Heidelberg, Germany) [22].

**Total internal reflection fluorescence (TIRF) microscopy**

MIN6 cells transfected with insulin-GFP were imaged using a custom-built TIRF microscope. The cells were infected with HCV and further cultured for 24 h before performing TIRFM. Before image acquisition, cells were pre-incubated for 30 min in KRB buffer containing 3.3 mmol/l glucose, and then were stimulated by KRB buffer containing 16.7 mmol/l glucose. All recordings were performed at 37°C.

**Stable cell line construction**

The sequences (Supplementary Table S3) encoding short hairpin RNA (shRNA) targeting the p38 gene (shp38δ) and a negative-control shRNA were cloned into the shRNA expression vector pSUPER.retro.neo (OligoEngine, Inc.) following the manufacturer’s instructions. Stable cell lines were generated as described [23].
Calculations

Insulin resistance was assessed by the homeostatic model assessment–insulin resistance (HOMA-IR) score. Insulin secretion derived from the fasting state was calculated as HOMA-β. Insulin secretion indexes derived from the OGTT were calculated as insulinoenic index (IGI1) and IGI2, and disposition index (DI). Surrogate indexes of insulin secretion and insulin resistance were calculated according to published formulae, using glucose and insulin concentrations at 0 and 30 min [24].

Statistics

Data are presented as means ± SEM. Statistical analysis was carried out using Student's t-test when comparing two groups and ANOVA with Bonferroni post hoc tests when comparing multiple groups. Statistical analyses were performed using GraphPad Prism Ver. 6.01 (San Diego, CA, U.S.A.) and SPSS 17.0 (IBM, IL, U.S.A.). Differences were considered significant at *P < 0.05, **P < 0.01; ***P < 0.001).

Results

β-cell functional defects in CHC patients

To investigate HCV-associated T2DM pathophysiology, a total of 46 participants were included to determine and evaluate the β-cell function in CHC patients (Supplementary Table S1). We subdivided the CHC patients into NFG, IFG and DM according to FPG. HOMA-IR was increased following HCV infection as compared to healthy controls. HOMA-IR in CHC patients with DM and IFG was higher than that in those with NFG as previously reported. β-cell function index, including fasting state (HOMA-β), OGTT-derived (IGI1 and IGI2) and the composite measure (DI) were significantly lower in CHC patients than in those healthy controls (Supplementary Table S4). In particular, IGIls and DI were significantly lower in CHC patients with IFG than in those with NFG, suggesting an impairment of the early-phase insulin secretion even in prediabetic CHC patients. Of note, both IGIls and DI declined along with the increase of insulin resistance following HCV infection (Supplementary Figure S1A–C).

We generated different categories of FPG in CHC patients to investigate β-cell function and observed a considerable increase in insulin resistance even within the impaired range of FPG (5.6–6.9 mmol/l), compared with the healthy control (Supplementary Figure S1D). Meanwhile, despite there was no change in HOMA-β index (Supplementary Figure S1E and Table S4), HCV infection significantly decreased the early-phase insulin response evaluated by IGI within the impaired range of FPG and further decreased in diabetes (Figure 1A,B), suggesting the loss of compensatory insulin secretion. A decrease in DI index was also substantial in the impaired FPG (Figure 1C). These data indicated that impaired β-cell function might exist following HCV infection even with impaired FPG in CHC patients and the effect of HCV infection on β-cell function was primarily on early-phase insulin response.

Moreover, we analysed the histomorphological alterations of pancreas biopsies from seven CHC patients with pancreatic head carcinoma. Compared with the regular condensed structure and vole shape of normal islets in the non-diabetic, uninfected, pancreatic head carcinoma controls (n = 4), there were a large number of cavities and disordered arrangement observed in the pancreatic islets of CHC patients (n = 7), indicated that the acinar structures were affected (Figure 1D). The Masson staining images revealed fibrosis of the pancreas from three pancreatic head carcinoma controls and six CHC patients (Figure 1E). Therefore, HCV infection induced histomorphological alterations in islet of CHC pancreases, whether HCV induced/enhanced fibrosis of pancreas need further investigation.

β-cell functional defects in HCV-infected C/O Tg mouse

We next used a total of 70 C/O Tg mice to further understand HCV-induced β-cell functional defects. Altogether, 39 subjects (56%) had NGT, 11 (16%) had IIFG, 3 (4%) had a combination of IFG and IGT (IFG+IGT), and 17 (24%) had DM, whereas an IGTT subject was not observed. Of note, the prevalence rate of diabetes among C/O Tg mice with HCV infection was similar to that in CHC patients as previously reported [5]. HOMA-IR was significantly increased in the IIFG, IFG+IGT and DM groups compared with the mock-infected control (Supplementary Figure S2A). Meanwhile, β-cell function evaluated by HOMA-β, IGI1 and IGI2 or DI were significantly decreased in the DM group compared with the mock control. In particular, IGIls and DI were significantly lower in the prediabetic (IFG and IFG+IGT) groups than in those controls (Supplementary Figure S2B and Figure 2A). Similar to CHC patients, a significant increase in insulin resistance was observed in HCV-infected mice, even within the impaired range of FPG (Supplementary Figure S2C). Meanwhile, HOMA-β, IGI1 and IGI2 or DI decreased within the impaired range of FPG and further decreased in DM (Supplementary Figure S2D and Figure 2B), suggesting an impaired β-cell function also within the impaired range of FPG in HCV-infected C/O Tg mice.

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Figure 1. β-cell functional defects in CHC patients

(A–C) IG1 (A), IG2 (B) and DI (C) across the categories of FPG. Bars display the value of insulin release relative to normal person. Cut-off values for different categories of FPG were in mmol/l. Data are represented as the means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, each group versus normal person. (D and E) H&E (D) and Masson (E) staining of pancreas sections of patients (three sections from each patient). The upper panel shows the representational photographs from the pancreatic head carcinoma patients (n = 4). The lower panel show the representational photographs from 4 CHC patients (n = 7). Islet (white/black dashes). Partial representative histomorphological alterations were indicated with white arrows (D), and the representative fibrotic areas were indicated with blue arrows (E); bar = 20 μm.

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Figure 2. β-cell functional defects in HCV-infected C/O Tg mice
C/O Tg (n = 70 total) mice were tail-vein injected with HCV. (A) IGI1, IGI2 and DI in different categories of glucose tolerance including NGT (n = 39), IIFG (n = 11), IFG+IGT (n = 3), DM (n = 17) and control (n = 8). (B) IGI1, IGI2 and DI across the categories of FPG. Bars display the value of insulin release relative to mock control. Cut-off values for different categories of FPG were in mmol/l. Bars display the value of insulin release relative to mock infection. Data are represented as the means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, each group versus mock-infected mice. (C and D) Representative H&E stains of pancreas tissues of C/O Tg mice (n = 4 for each time point) (C) and Masson’s staining of pancreas sections (n = 2 at each point) (D) of the C/O Tg mice. Islet (white/black dashes). Partial representative lymphocyte infiltrations were indicated with black arrows (C) and the representative fibrotic areas were indicated with blue arrows (D); bar = 20 μm.
To further investigate the global development of HCV-associated T2DM, HCV-infected mice were also subdivided into three stages, including early stage (months 1, 2), middle stage (months 4, 6, 10) and late stage (months 12, 16) according to the chronic manifestation progress of HCV infection from an asymptomatic state to steatosis, fibrosis and cirrhosis as previously described [16]. Each group was subdivided into NGT, IIFG, IFG+IGT and DM according to FPG and glucose tolerance. In addition to insulin resistance, there was a significant difference among three groups on β-cell function, including HOMA-β, IGIs and DI, at corresponding stages of chronic manifestation progress. Moreover, the prediabetic state occurred along with declined HOMA-β, IGIs and DI in HCV-infected C/O^Tg^ mice, even at the early infection stage (Supplementary Table S5).

Meanwhile, the obtained H&E staining results showed lymphocyte infiltration around islets at 10-month post-infection (mpi) (Figure 2C and Supplementary Figure S2E). Masson staining showed that 35.29% (12/34) of mice progressed to fibrosis after 6 mpi. This fibrosis was aggravated with the prolonged HCV infection until 10 mpi (Figure 2D and Supplementary Figure S2F). Thus, similar to CHC patients, HCV infection led to functional defects of pancreatic islets in HCV-infected C/O^Tg^ mice.

**Impaired glucose-stimulated insulin secretion in HCV-infected β-cells**

To further examine the role of HCV infection in β-cells in vivo, we assessed insulin secretion in cohorts of HCV-infected (including NGT, IIFG, and DM groups) and mock-infected C/O^Tg^ mice subjected to hyperglycaemic clamp studies (Figure 3A). Analysis of plasma insulin indicated significant impairment of insulin secretion in HCV-infected C/O^Tg^ mice during the acute (0–30 min) phases of secretion (Figure 3B). Interestingly, insulin secretion significantly increased in NGT group of HCV-infected C/O^Tg^ mice, suggesting a compensatory increase in β-cell function in response to insulin resistance. We then studied glucose-stimulated insulin secretion (GSIS) in static incubation in cultured islets from C57BL/6 mice following HCV infection for 24 h. Consistent with the observation in vivo, HCV infection reduced the insulin secretion response to stimulatory concentrations of glucose in mouse islets, human islet or MIN6. Moreover, the NS3/4 protease inhibitor telaprevir (VX-950) could rescue the inhibitory role of HCV in insulin secretion (Figure 3C and Supplementary Figure S3A–D).

To study dynamic insulin secretion in vitro, we performed perifusion assays on either isolated human and mouse islets or MIN6 cells. HCV-infected islets perfused with 16.7 mM glucose secreted significantly lower amounts of both first- and second-phase insulin than control islets, which was restored by VX950 (Figure 3D,E). In addition, the total pool of insulin released by KCl was decreased in HCV-infected MIN6 cells (Figure 3F).

**Reduced insulin exocytosis in HCV-infected β-cells**

HCV infection did not significantly change either insulin content or insulin and pdx1 mRNA levels both in infected MIN6 cells and islets (Supplementary Figure S4A–D), implying the insulin secretory defect by HCV infection occurred in the process of insulin secretion.

We then addressed at which step HCV infection interfered with the insulin secretory pathway in β-cells. We measured insulin secretion as stimulated with five secretagogues (α-ketoisocaproate, glibenclamide, tolbutamide, carbachol and K^+^) acting at various stages of the stimulus-secretion coupling in MIN6 cells. Insulin secretion was strongly reduced in response to all secretagogues (Figure 4A), suggested that HCV infection reduced insulin secretion by inhibiting at least exocytosis of insulin secretion. To address whether exocytosis is directly inhibited in HCV-infected β-cells, we performed high-resolution capacitance measurements of exocytosis on single β-cells. A train of 10 de-polarization steps from 70 to 0 mV evoked reduced responses in HCV-infected cells compared with the control, resulting in a 2-fold lower membrane capacitance relative to the control, which was restored by VX950 (Figure 4B,C). Therefore, HCV infection influenced insulin secretion by a direct effect on the exocytotic reaction.

Then, we used TIRF microscopy to monitor exocytosis of secretory granules (SGs) tagged with insulin-EGFP. At basal unstimulated state, punctate fluorescence, indicating docked SGs, did not differ between control and HCV-infected β-cells (Figure 5A). Assessment of cumulative fusion events over time (Figure 5B) showed fewer fusion events during the 20 min stimulation in HCV-infected β-cells than in the control. At 3.3 mmol/l glucose, there were few spontaneous fusion events of mainly pre-docked SGs (black in Figure 5C,D). With high glucose stimulation (16.7 mmol/l), in first-phase GSIS there was obvious inhibition of pre-docked and no-docked (white in Figure 5C,D) newcomer SGs from HCV-infected cells (Figure 5E). In the second-phase of GSIS, there was reduction in no-docked and short-docked (grey in Figure 5C,D) newcomer SGs (Figure 5F). HCV infection, therefore, affects pre-docked and newcomer SG fusion underlying first- and second-phase GSIS in β-cells, consistent with the hyperglycaemic clamp (Figure 3B).
Figure 3. HCV infection impairs glucose-stimulated insulin secretion

Plasma glucose (A) and insulin (B, left panel) levels and the area under the curve (AUC) for insulin secretion (B, right panel) during hyperglycaemic clamp studies performed on overnight fasted mock (n = 4) or HCV-infected C/O^®^ mice (including NGT (n = 10, number 9-16, 37, 38), IIFG (n = 4, number 26-29), DM group (n = 4, number 30, 50-52), VX950 (20 mg/kg/day). Islets or MIN6 cells were infected with HCV (MOI = 0.1) for 24 h. (C) Glucose (3.3 and 16.7 mM)-stimulated insulin secretion from human or mouse islets. (D and E) Islet perifusion of human (D) and mouse (E). Data are represented as the means ± SEM. AUC for insulin throughout the perifusion including first-phase and second-phase of release. (F) MIN6 cell perifusion. Data are representative of three independent experiments. Data are represented as the means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, each group versus mock-infected control.
Figure 4. Reduced insulin exocytosis in HCV-infected β-cells
MIN6 cells were infected with HCV (MOI = 0.1) at 24 hpi. (A) Insulin release in response to 10 mM α-ketoisocaproate (KIC), glibenclamide (Gli), 0.25 mM tolbutamide (Tol), 0.25 mM carbachol (Car) or KCl as measured by static incubation method. Data are represented as the means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. (B) A train of 10 successive 500 ms depolarizations from -70 to 0 mV increased capacitance (in femtofarad [fF]) in mock or HCV-infected MIN6 cells. (C) Average cumulative increase in capacitance.

Attenuated activity of PKD by MAPK p38δ and altered Golgi organization in HCV-infected β-cells
Insulin is processed in the Golgi apparatus and secreted in vesicles. Secretory vesicles are all derived from transport vesicles leaving the trans-Golgi network (TGN). As shown in (Figure 6A,B) the Golgi tracker exhibited a tightening distribution in HCV-infected β-cells by immunofluorescence staining. Indeed, the Golgi markers giantin and GM130 exhibited a characteristic crescent-shaped pattern in HCV-infected β-cells, whereas a diffuse distribution of staining was found in mock cells. Enhanced membrane fission at the TGN in the HCV-infected islet cells most probably accounts for the impaired exocytosis seen in capacitance experiments.

The MAPK family p38 (p38δ) is a negative regulator in stimulated insulin secretion through inhibition of PKD and regulation of exocytosis [25,26]. As shown in (Figure 6C), the expression level of p38δ was increased in HCV-infected MIN6 cells compared with the control along with the infection time course, as well as increased level of p38 MAPK phosphorylation. Meanwhile, levels of phosphorylated Ser916 (PKD autophosphorylation site) was decreased in HCV-infected cells (Figure 6C). Knockdown of p38δ increased insulin secretion both under basal and glucose-stimulated conditions compared with control in both mock and HCV-infected cells (Figure 6D). Furthermore, a marked increase in activatory PKD phosphorylation was also observed in MIN6 cells lacking p38δ compared with control cells in both mock and HCV-infected cells (Figure 6E), suggested p38δ-dependent PKD phosphorylation contributed to HCV-associated β-cell dysfunction.

Discussion
HCV infection has a close association with T2DM. However, the mechanism of β-cell dysfunction remains unclear. Here, we provide compelling evidence that HCV infection impaired pancreatic β-cell function. Our work supports the hypothesis that HCV infection reduces insulin secretion at least by inhibiting exocytosis of insulin secretion.

Although insulin resistance is a major problem in CHC patients with glucose intolerance, whether β-cell dysfunction plays an important role in determining the magnitude of the pathogenesis of T2DM is not well known. Our study indicates that the β-cell function is consistently decreased in CHC patients or HCV-infected mice during the pre-diabetic state or with DM. Following HCV infection, insulin release linearly decreased with increasing HOMA-IR and was lowest in the diabetic range, indicating a major defect in insulin secretion. This observation is consistent with a previous study showing that insulin secretion defect is very often the primary cause for HCV-associated T2DM [27]. Moreover, we observed that the proportion of pre-diabetic mice in the middle and later stages of infection was significantly higher than in the early stage, which to some extent reflected that the chronic infection of HCV may be associated with impaired fasting glucose state.
Figure 5. HCV infection affects pre-docked and newcomer secretory granules (SGs) fusion in β-cells

MIN6 cells were infected with HCV (MOI = 0.1) at 24 hpi. (A) TIRF images of docked insulin SGs in control (top) or HCV-infected (bottom) in MIN6; bars = 10 μm. Histogram (right) of comparison of averaged SG densities before stimulation. (B) Normalized cumulative fusion events of SGs per unit area from control and HCV-infected β-cells. (C and D) Histograms of fusion events in the first phase (first 4 min stimulation) and second phase (5–20 min) showing insulin SG exocytosis dynamics caused by 16.7 mM glucose stimulation from the control (C) and HCV-infected (D) β-cells. Black, white and grey bars indicate pre-docked, no-dock and short-dock newcomer SGs, respectively. Control: 12 cells; HCV infected: 15 cells; data expressed as the means ± SEM. (E and F) Summary of the three modes of fusion events in first (E) and second phases (F), shown as the means ± SEM; *P < 0.05; **P < 0.01. Data are representative of three independent experiments.
Figure 6. HCV infection attenuates activity of PKD by MAPK p38δ and alters Golgi organization in β-cells
MIN6 cells were infected with HCV (MOI = 0.1) at 24 hpi or indicated time. (A) Golgi-Tracker (red) and Cytoplasm-Tracker (green) followed by analysis under a confocal laser scanning microscope; bars = 10 μm. (B) Immunofluorescence using antibodies against GM130 (green) and molecular beacon probes specific to HCV positive RNA strands (red) in MIN6 cells as indicated. Nuclear DNA was stained with DAPI (blue); scale bar = 10 μm. (C) Immunoblot analysis of p38δ and phospho-p38 MAPK at indicated time. Activity of PKD was determined by Western blotting with an antibody against the activatory phosphorylation sites (Ser916). (D) Insulin secretion in response to basal (3.3 mM) and stimulatory glucose (16.7 mM) from MIN6 cells stably expressing shRNA against p38δ or a control vector following HCV infection. (E) Immunoblot analysis of p38δ, PKD and activated PKD as in (D). Data are representative of three independent experiments. Data are represented as the means ± SEM; **P < 0.01.

It is noteworthy that HOMA-β decreases in impaired range of FPG in HCV-infected C/O Tg mice, whereas there is no statistically significant difference between impaired range of IFG and NFG in human subjects. The reason might be due to the different subdivision and we have subdivided the patients only according to fasting plasma glucose, rather than subdivided according to both fasting plasma glucose and glucose tolerance. The IFG group could not exclude the normal glucose tolerance patients and β-cell function complementary might exist. However, prospective follow-up studies including CHC patients with IIFG or IIGT are needed to confirm this hypothesis. In addition, HOMA-β is an index predicting fasting steady-state β-cell function that do not reflect the dynamic state of the relationship between insulin sensitivity and secretion. IGIs and DI significantly decreased in both CHC patients and HCV-infected C/O Tg mice demonstrated HCV infection impaired insulin secretion, especially early-phase insulin release.

Virus have been implicated as one environmental factor that may initiate or trigger an autoimmune reaction that targets and destroys β-cells in genetically susceptible individuals. However, diabetes in HCV-positive patients is not specifically associated with autoantibody production. Accordingly, HCV infection may directly damage β-cells and disturb their function, resulting in T2DM. A direct cytopathogenic effect of HCV at the islet cell level has been documented [14] and HCV could replicate in human pancreatic islet cells [28]. Also, our previous study indicated the
HCV genome has been identified in pancreatic β-cells [18]. Here, we further provide evidence indicating HCV infection negatively regulates both first-phase and second-phase insulin secretion but does not affect insulin synthesis in β-cells. This effect can occur by a direct disturbance of Ca2+-induced activation of insulin granule exocytosis that correlates with increased p38δ expression and p38δ-mediated inhibitory phosphorylation, and further inhibited PKD activity. The Golgi complex is required for insulin-containing secretory granule formation by budding. Enhanced membrane fission at the TGN in the HCV-infected islet cells most probably accounts for the impaired exocytosis seen in capacitance experiments. A recent study identified the key role of the Golgi components in the HCV maturation process, during which PKD negatively regulates HCV secretion/release [29]. We observed that HCV infection appears to cause a general distortion of the Golgi compartment of β-cells and found that PKD kinase activation is impaired in HCV-infected cells, suggesting that Golgi fragmentation might impaired insulin secretion. Vesicle exocytosis requires the interaction of plasma membrane-bound proteins with proteins bound to the secretory vesicle proper. Of note, the expression level of several SNARE proteins, including Syntaxin-1, SNAP25, Synaptophysin and Munc18c, decreased in HCV-infected islets (Supplementary Figure S5A and B); however, it appeared the decrease was independent of p38δ MAPK (Supplementary Figure S5C).

In conclusion, we show here that in addition to insulin resistance, impaired pancreas β-cell function may occur at the early stage of HCV infection, even with the impaired fasting glucose. Our work suggests that HCV infection reduces insulin secretion, at least partially by directly inhibiting exocytosis of insulin secretion. These findings may have important prognostic and therapeutic implications in the management of CHC patients with impaired fasting glucose.

Clinical perspectives

- **Chronic hepatitis C virus (HCV) infection has a close association with type 2 diabetes mellitus. It has been demonstrated that HCV infection in liver results in insulin resistance. However, the role of β-cell function in HCV-associated diabetes remains unclear.**

- **We found that impaired pancreatic β-cell function occurred in CHC patients and HCV-infected C/O Tg mice, not only in diabetic individuals but also in individuals with impaired fasting glucose levels. HCV infection reduces insulin secretion, at least partially by directly inhibiting exocytosis of insulin secretion via p38δ-PKD pathway.**

- **These findings have important prognostic and prophylactic implications in the management of CHC patients even with impaired fasting glucose and provide a clue to the possible mechanisms underlying the development of HCV-induced diabetes.**

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

The study was designed by Q.W. and J.Z.C.; Experiments in HCV-infected cells were performed by J.Z.C., F.W. and Y.Z. Experiments in replicon cells were performed by Q.W., Y.Z. and F.W. Patients’ studies were performed by J.Z.C. Transgenic mice and animal study was performed by J.Z.C. and X.Z. Experimental analysis was performed by Q.W., J.Z.C., J.J., S.K., J.Z.L. and J.Q.N.; the manuscript was written by J.Z.C. and Q.W. Q.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
**Ethics Approval**

All patients and/or their relatives provided written informed consent for their clinical and pathological information to be used for research and to be stored in the hospital database; the present study, including its methods and experimental protocols, was approved by the Ethical Committee of the First Hospital of Jilin University and the First Affiliated Hospital of Xinjiang Medical University. All procedures performed in our study were done so in accordance with the ethical standards of our institutional research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Methods were carried out in accordance with the approved guidelines.

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**Abbreviations**

CHC, chronic hepatitis C; DI, disposition index; FPG, fasting plasma glucose; GSIS, glucose-stimulated insulin secretion; HCV, hepatitis C virus; H&E, Hematoxylin and Eosin; IFG, impaired fasting glucose; IIFG, isolated impaired fasting glucose; IGI, insulinogenic index; IGT, isolated impaired glucose tolerance; HIC, immunohistochemistry; MOI, multiplicities of infection; NFG, normal fasting glucose; NGT, normal glucose tolerance; PKD, protein kinase D; SNAP25, synaptosomal-associated protein 25; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; T2DM, type 2 diabetes; TIRF, total internal reflection fluorescence; VAMP, vesicle-associated membrane protein 2.

**References**


