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## Glucose exposure pattern determines glucagon-like peptide 1 receptor expression and signaling through endoplasmic reticulum stress in rat insulinoma cells

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### ABSTRACT

Repeated fluctuation in plasma glucose levels, as well as chronic hyperglycemia, is an important phenomenon frequently observed in diabetic patients. Recently, several studies have reported that glucose fluctuation, compared to chronic hyperglycemia, mediates more adverse effects due to induced oxidative and/or endoplasmic reticulum (ER) stress. In type 2 diabetes, stimulation of insulin secretion by glucagon-like peptide-1 (GLP-1) has been found to be reduced, and the results of recent studies have shown that the expression of the GLP-1 receptor (GLP-1R) is reduced by chronic hyperglycemia. However, GLP-1R signaling in glucose fluctuation has not been elucidated clearly. In this study, we hypothesized that intermittent high glucose (IHG) conditions also reduced GLP-1-mediated cellular signaling via reduction in GLP-1R expression. To evaluate this hypothesis, rat insulinoma cells (INS-1) were exposed for 72 h to either sustained high glucose (SHG) conditions (30 mM glucose) or IHG conditions (11 and 30 mM glucose, alternating every 12 h). In comparison to both the SHG and control groups, IHG conditions induced a more significant impairment of insulin release and calcium influx in response to 1 nM GLP-1 treatment. In addition, the activity of caspase 3/7 as well as the gene expression of binding protein (Bip) and C/EBP homologous protein (CHOP), molecular markers of ER stress, was significantly higher in IHG-treated cells than in SHG-treated cells. Interestingly, the expression level of GLP-1R was significantly lower under IHG conditions than under SHG conditions. Collectively, these findings indicated that glucose fluctuation reduces GLP-1R expression through ER stress more profoundly than sustained hyperglycemia, which may contribute to the diminished response of GLP-1.

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### 1. Introduction

Hyperglycemia is closely related to  $\beta$ -cells dysfunction and known to be a critical cause of type 2 diabetes [1]. An overwhelming body of evidence suggests that glucagon-like peptide-1 (GLP-1), an incretin hormone that stimulates insulin secretion, is modestly reduced in type 2 diabetes [2]. The subsequent actions of GLP-1 are mediated by its specific receptor, GLP-1R, thereby stimulating the adenylyl cyclase pathway, which induces increases in intracellular cAMP and calcium influx [3,4]. Via this important signaling pathway, GLP-1 exerts its insulin-releasing effects in response to glucose in pancreatic  $\beta$ -cells [5].

Chronically elevated glucose concentrations result in glucose toxicity and ultimately  $\beta$ -cells death [6]. Recently, several studies

have demonstrated that repeated fluctuation from normal to high glucose concentrations, in addition to chronic and persistent hyperglycemia, is another important phenomenon inherent to type 2 diabetes [7,8]. Accumulated data appear to demonstrate that fluctuating high glucose levels influence the development of diabetic complications via the generation of reactive oxygen species, in addition to other mechanisms [9,10]. Other recent studies indicated that fluctuating high glucose levels affect  $\beta$ -cell functions and induce cell death [11,12]. Hou et al. showed that intermittent high glucose (IHG) conditions induced a higher degree of endoplasmic reticulum (ER) and oxidative stress in INS-1 cells as compared to sustained high glucose (SHG) conditions [11]. In addition, IHG conditions, compared to SHG conditions, induce a more serious impairment of insulin release in response to high glucose levels in rat islets and INS-1 cells [11]. Under SHG conditions, GLP-1R expression is downregulated, which contributes to the impaired incretin effects found in diabetes [13,14]. On the other hand, although IHG conditions seriously affect type 2 diabetic patients, the effects of IHG on the subsequent signaling of GLP-1 have not been elucidated clearly.

*Abbreviations:* ER, endoplasmic reticulum; IHG, intermittent high glucose; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; SHG, sustained high glucose.

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Therefore, in order to evaluate the effects of IHG on GLP-1-related signaling and  $\beta$ -cell functions, we measured insulin secretion and calcium influx in response to GLP-1, as well as the expression of GLP-1R in INS-1 cells under SHG or IHG conditions.

## 2. Materials and methods

### 2.1. Cell cultures and treatment conditions

INS-1 cells (rat insulinoma cell line, passages 10–20) were cultured in RPMI1640 medium supplemented with 11 mM glucose, 10% fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES pH 7.4, 50  $\mu$ M 2-mercaptoethanol, 100 U/mL penicillin G, and 100  $\mu$ g/mL streptomycin at 37 °C and 5% CO<sub>2</sub> [15]. Cells were seeded at 20,000 cells/well in 96-well multiplates in RPMI1640 medium. After 48 h, the cells were treated for 72 h with RPMI1640 medium containing 30 mM glucose to produce sustained hyperglycemic conditions. To produce IHG conditions, the cells were treated alternately (every 12 h) with media containing either 11.1 mM or 30 mM glucose. In the control and SHG cell samples, the media was also changed every 12 h.

### 2.2. Determination of insulin secretion and cellular insulin content

After 72 h of treatment, the INS-1 cells were rinsed twice in phosphate-buffered saline (PBS), then pre-incubated in Krebs–Ringer bicarbonate HEPES buffer (KRBH buffer: 11.5 mM NaCl, 24 mM NaHCO<sub>3</sub>, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 25 mM HEPES pH 7.4, and 0.5% bovine serum albumin [BSA]) without glucose at 37 °C. After 1 h, INS-1 cells were incubated for 30 min in fresh KRBH buffer supplemented with glucose and 1 nM GLP-1 at 37 °C. The insulin secreted into the medium was determined with an insulin enzyme-linked immunosorbent assay (ELISA) kit (ALPCO, Windham, NH) in accordance with the manufacturer's instructions. Finally, the value of insulin was normalized to the total protein content as measured via the bicinchoninic acid (BCA) method.

From the cell layer, insulin content was measured using the method described by Hamid et al. [16]. In brief, the cells were incubated in 200  $\mu$ L of acid-ethanol solution (1.5% (v/v) HCl, 75% (v/v) ethanol, 23.5% H<sub>2</sub>O) at 4 °C overnight. The insulin levels in the lysates were detected and normalized to the total protein content, as described above.

### 2.3. Polymerase chain reaction

After 72 h of exposure to high glucose conditions, total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's recommended protocols. First-strand DNA was synthesized using reverse transcriptase with random hexamers as primer. The amplification of the target gene was carried out using the following specific primers: binding protein (Bip) (forward, 5'-CCA CCA GGA TGC AGA CAT TG-3'; reverse, 5'-AGG GCC TCC ACT TCC ATA GA-3'), C/EBP homologous protein (CHOP) (5'-CCA GCA GAG GTC ACA AGC AC-3'; reverse, 5'-CGC ACT GAC CAC TCT GTT TC-3'), insulin (forward, 5'-TCT TCT ACA CAC CCA TGT CCC-3'; reverse, 5'-GGT GCA GCA CTG ATC CAC-3'), GLP-1R (forward, 5'-CCT GAG GAA CAG CTC CTG TC-3'; reverse, 5'-CAG TGA GGC CAG GAT AGA GC-3'), and  $\beta$ -actin (forward, 5'-GGC TGT GTT GTC CCT GTA TG-3'; reverse, 5'-AGG AAG GAA GGC TGG AAG AG-3'). Amplification cycles were optimized for each gene to be in linear range. The expression of target genes was normalized to that of  $\beta$ -actin.

### 2.4. Western blotting

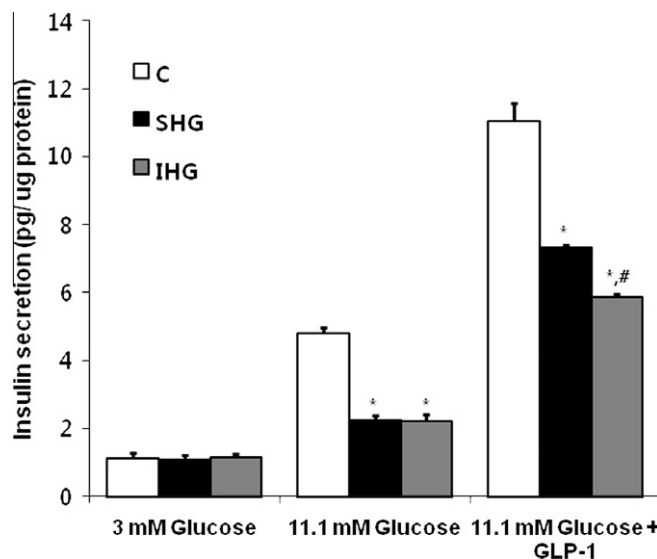
After 72 h, the INS-1 cells were washed once with PBS. Then, proteins were extracted using RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and centrifuged at 4 °C and 10,000g for 10 min. Total protein concentration in the supernatant was quantified via the BCA method. Next, 20  $\mu$ g total protein of each sample was separated on a 4–12% Bis-Tris pre-cast gel (Invitrogen, Carlsbad, CA). The separated proteins were then transferred to PVDF membranes, followed by blocking with 5% skim milk in 0.1% TBST (TBS with 0.1% Tween-20). Then, the membranes were reacted with the primary antibodies for GLP-1R (1:1000) or  $\beta$ -actin (1:3000) at 4 °C overnight, reacted sequentially with rabbit polyclonal horseradish peroxidase-conjugated secondary antibody (1:2000 for GLP-1R and 1:5000 for  $\beta$ -actin) at room temperature for 1 h, and developed via ECL chemiluminescence. The expression of GLP-1R was normalized that of  $\beta$ -actin.

### 2.5. Caspase 3/7 activity assay

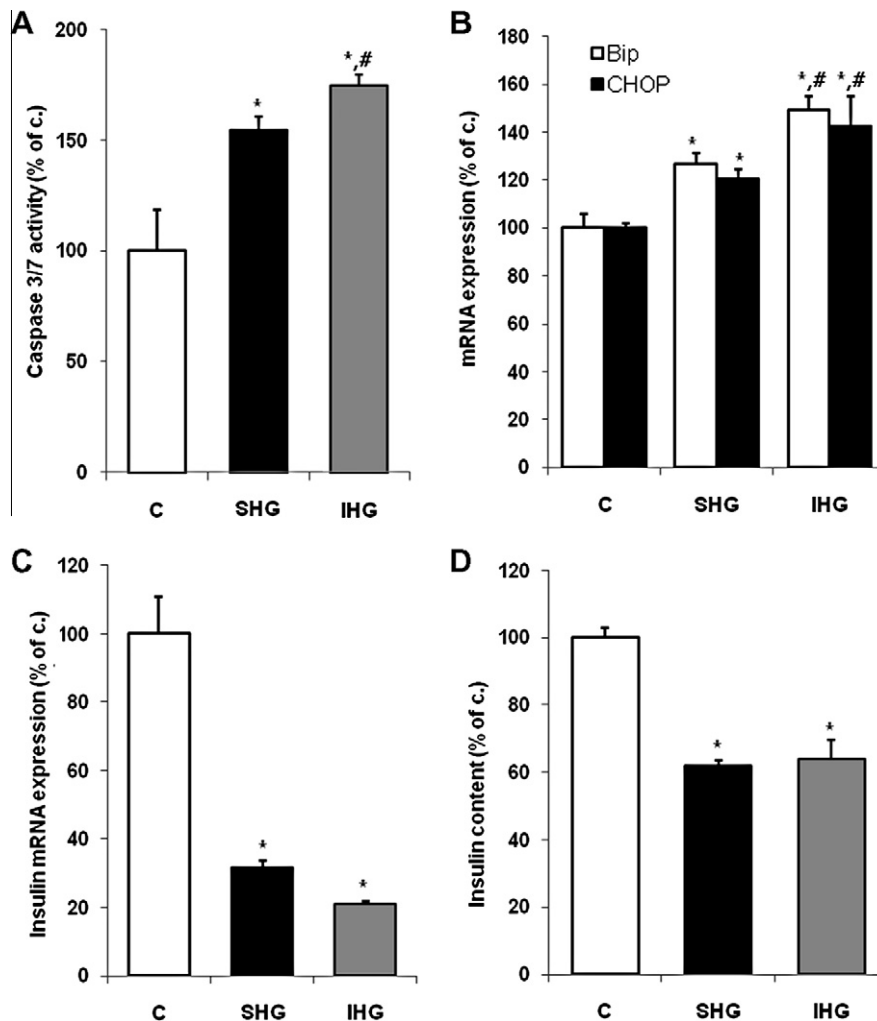
The caspase 3/7 activity assay was conducted in accordance with the manufacturer's instructions (G8091, Promega, Madison, WI). In brief, after 72 h, INS-1 cells were treated with caspase-Glo 3/7 reagent in 96-well plates. The plates were gently shaken for 30 s and incubated at room temperature for an additional 30 min. Luminescence was then measured with a luminometer (LmaxII384, Molecular Devices Inc., Sunnyvale, CA).

### 2.6. Calcium uptake assay

The calcium uptake assay was conducted using the FLIPR Calcium 5 assay in accordance with the manufacturer's instructions (R8185, Molecular Devices Inc.). In brief, after 72 h, the cells were loaded with 1 $\times$  calcium dye in Hank's Balanced Salt Solution (HBSS; containing 5.4 mM KCl, 4.2 mM NaHCO<sub>3</sub>, 1.3 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.6 mM MgSO<sub>4</sub>, 137 mM NaCl, pH 7.4) at 37 °C. After 1 h, intracellular calcium changes were recorded at an



**Fig. 1.** Insulin secretion in INS-1 cells in response to 11.1 mM glucose with or without 1 nM GLP-1. Following 3 days of exposure in the control, SHG, and IHG samples, glucose-stimulated insulin secretion in INS-1 cells was measured with an ELISA kit and then calculated. Data are expressed as means  $\pm$  SEM of 3 separate experiments. \*,  $P < 0.05$  vs. C; #,  $P < 0.05$  vs. SHG. Abbreviations: GLP-1, glucagon-like protein-1; C, control; SHG, sustained high glucose; IHG, intermittent high glucose; ELISA, enzyme-linked immunosorbent assay.



**Fig. 2.** Activity of caspase 3/7, gene expression of Bip and CHOP, and expression of insulin in INS-1 cells. (A) After the INS-1 cells were incubated for 72 h in the control, SHG, and IHG cell samples, caspase 3/7 activity was assessed. (B) After total RNA extraction and reverse transcription, Bip and CHOP expression were determined via PCR analysis. Data are expressed as the means  $\pm$  SEM of 3 separate experiments. (C) After total RNA extraction and reverse transcription, the gene expression of insulin was determined via PCR analysis. (D) Total insulin content in the INS-1 cells was extracted via the acid/ethanol method and detected with an ELISA kit. \*,  $P < 0.05$  vs. C; #,  $P < 0.05$  vs. SHG. Abbreviations: Bip, binding protein; CHOP, C/EBP homologous protein; SHG, sustained high glucose; IHG, intermittent high glucose; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

excitation wavelength of 485 nm and emission wavelength of 525 nm at 1.3 s intervals for 150 s using a multireader Flexstation-3 (Molecular Devices Inc.). GLP-1 and glucose in the HBSS solution were earlier prepared in another plate and automatically applied to the cells after 20 s of recording.

### 2.7. Data analysis

Data are expressed as means  $\pm$  SEM. One-way ANOVA using SigmaStat 2.0 (SPSS, Chicago, IL) was used for comparisons among groups. When significant differences were detected, multiple comparisons were conducted via the Student–Newman–Keuls test.

## 3. Results

### 3.1. Insulin secretion capacity by GLP-1 was reduced under IHG conditions in INS-1 cells

To examine the effect of IHG conditions on insulin secretion by GLP-1, we performed glucose-stimulated insulin secretion assays in INS-1 cells. At the end of 72 h of exposure, no significant

differences were noted among the 3 groups (control, SHG, and IHG) in the presence of 3 mM glucose. When INS-1 cells were exposed to 11.1 mM glucose, the insulin release in the 2 hyperglycemia groups was significantly reduced as compared with the controls. Under these conditions, the level of insulin secretion was almost identical between the SHG and IHG groups. However, in the presence of 1 nM GLP-1, the level of insulin secretion was more profoundly decreased in INS-1 cells exposed to IHG (by  $46.9 \pm 0.96\%$ ) compared to SHG (by  $33.8 \pm 0.56\%$ ) (Fig. 1).

### 3.2. IHG conditions increased cellular toxicity with ER stress and reduced insulin expression in INS-1 cells

To evaluate the cytotoxic effects of IHG, we subsequently assessed caspase 3/7 activity in INS-1 cells. After 72 h of exposure, the activity of caspase 3/7 increased under SHG conditions as compared with controls and this increase was even more marked under IHG conditions (Fig. 2A). To further assess the toxic effects of IHG, we used reverse transcription-polymerase chain reaction (RT-PCR) to evaluate the expression of representative ER stress markers, especially Bip and CHOP. Exposure of INS-1 cells to SHG upregulated the expression of Bip by  $1.3 \pm 0.05$ -fold and CHOP by

1.2 ± 0.08-fold over controls ( $P < 0.05$ ). IHG induced excessive activations of these 2 components of ER stress by 1.5 ± 0.06-fold and 1.4 ± 0.15-fold, respectively (Fig. 2B). With regard to the insulin expression level, although it appeared that IHG induced a more severe reduction than SHG, no statistically significant differences were noted between the hyperglycemia groups (Fig. 2C). Under SHG conditions, the insulin content in INS-1 cells was reduced by 38.1 ± 1.7% as compared with controls; however, there was no statistical significance as compared with IHG conditions (Fig. 2D).

### 3.3. Calcium influx in response to GLP-1 was decreased in INS-1 by IHG

Activated GLP-1R stimulates glucose-induced insulin secretion, which is known to be involved in the increase in intracellular calcium influx [3]. To evaluate whether glucose fluctuation affects calcium influx, we assessed calcium influx capacity in INS-1 cells in response to 1 nM GLP-1 under control, SHG, and IHG conditions. SHG reduced calcium influx levels by 30.2 ± 6.32% as compared with controls (Fig. 3A and B). IHG produced a significantly larger reduction in calcium influx levels (by 49.2 ± 5.9% vs. controls).

### 3.4. GLP-1R expression was downregulated under IHG conditions in INS-1 cells

High glucose concentrations result in the downregulation of GLP-1R expression [13,14]. To investigate the evidence of reduc-

tion in GLP-1 capacity, particularly under IHG conditions, we evaluated the expression of GLP-1R using RT-PCR and western blot. Under SHG and IHG conditions, the levels of GLP-1R mRNA in INS-1 cells were reduced by 24.3 ± 2.51% and 38.6 ± 5.52%, respectively ( $P < 0.05$ ) (Fig. 4A). In addition, the protein level of GLP-1R in INS-1 cells exposed to IHG was lower compared to that of cells exposed to SHG (Fig. 4B). These data indicate that glucose fluctuation induces a relatively more severe impairment of GLP-1R expression, which may result in reduced  $\beta$ -cell function.

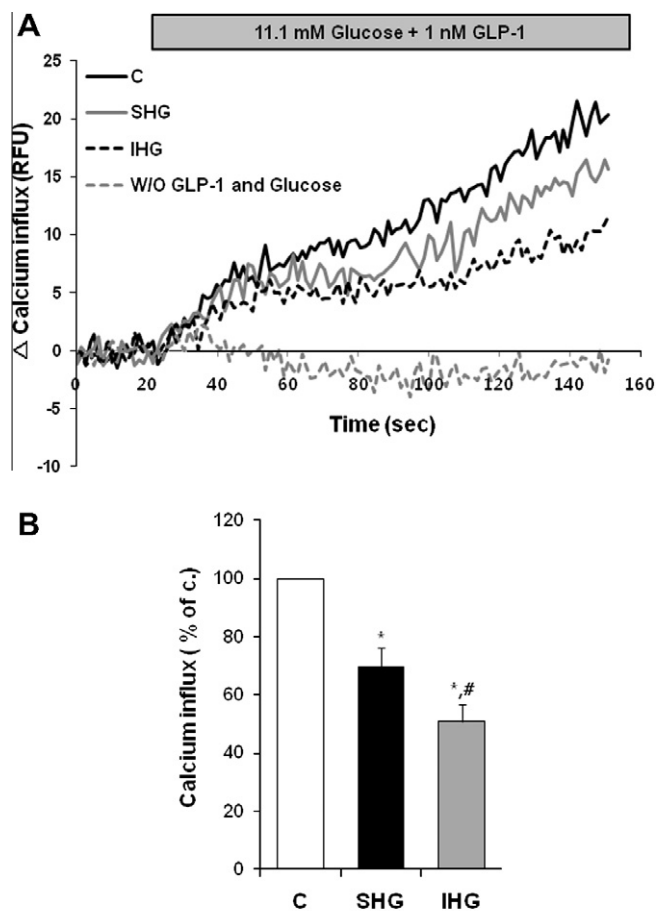
## 4. Discussion

In this study, we showed that compared to SHG, IHG exposure of rat pancreatic INS-1  $\beta$ -cells led to more marked reductions of subsequent GLP-1 signaling, as well as insulin secretion and calcium influx. Interestingly, the expression of GLP-1R at both the gene and protein levels was lower under IHG than SHG conditions.

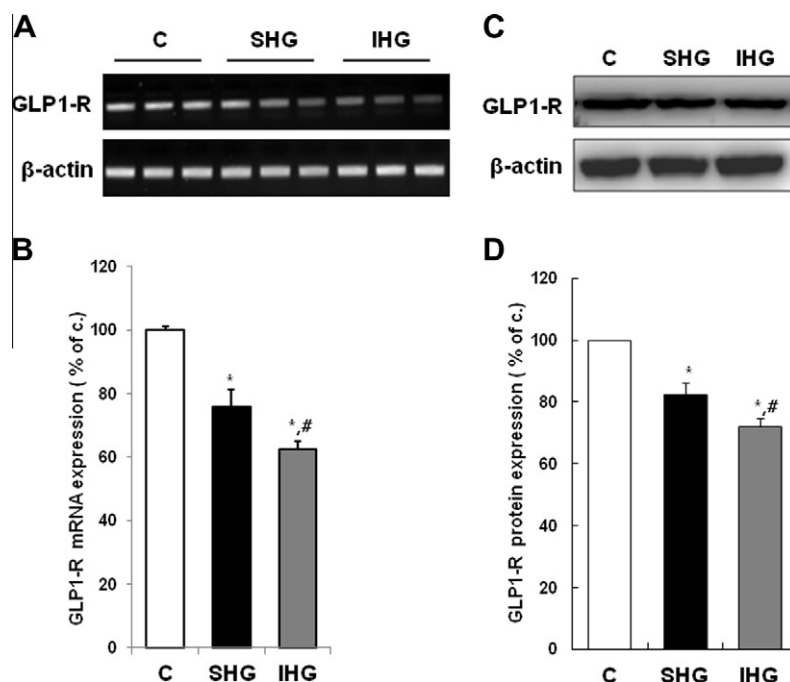
Repeated glucose fluctuation is a common phenomenon in patients with diabetes because their plasma glucose concentrations change frequently and markedly within a single day, whereas glucose concentrations are tightly regulated in normal subjects [17]. The results of several clinical studies have shown that diabetic complications, especially cardiovascular disease, increased more profoundly as the result of postprandial glucose or peak glucose levels, rather than the average blood glucose level, as indicated by hemoglobin A1c (HbA1c) levels [1,18–21]. Furthermore, Kim et al. demonstrated that glucose fluctuation also induces apoptosis and dysfunction in INS-1 cells [12]; thus, diabetic patients might experience more significant  $\beta$ -cell dysfunction under glucose fluctuation conditions than under chronic hyperglycemic conditions. However, the molecular changes associated with glucose fluctuation has not been thoroughly elucidated; therefore, studies of glucose fluctuation are clearly necessary to gain more insight into the complex phenomenon of diabetes.

An overwhelming body of evidence suggests that ER and oxidative stress inhibit gene transcription and/or translation [22,23]. In addition, recent studies have shown that IHG is a more effective activator of ER and oxidative stress [11,12]. In this study, we have confirmed that adverse ER stress increases more by glucose fluctuation than by sustained hyperglycemia, as result of higher expression levels of Bip and CHOP. Although we did not assess the changes in oxidative stress markers in this study, significant oxidative stress might also be induced under IHG conditions. Therefore, we suggest that reductions in GLP-1R expression might be affected by ER stress as well as oxidative stress. However, until now, the molecular mechanisms underlying the induction of increased adverse ER and oxidative stress in glucose fluctuation have not been clearly elucidated. One of the most likely explanations is that IHG conditions may not activate the same glucose toxicity regulators as SHG conditions [11]. Another possibility is that the more prolonged epigenetic changes attributable to glucose variability also induce greater deteriorations and dysfunction in  $\beta$ -cells [9]. These speculations need to be elucidated in further investigations.

In the islets of pancreatectomized (Px) hyperglycemic and normal rats that were exposed to high glucose levels, GLP-1R expression as well as subsequent insulin secretion were also significantly reduced [13]. In a previous study, GLP-1R knockout mice showed abnormalities in fasting glucose levels, glucose-dependent insulin secretion, and islet size [24]. Therefore, in this study, lower GLP-1R expression as the result of glucose fluctuation might more significantly affect reductions of the responses mediated by GLP-1R activation. We anticipate that type 2 diabetic patients with frequent glucose fluctuation might have reduced GLP-1R expression as well as more severe subsequent signaling in  $\beta$ -cells compared to chronic hyperglycemia patients. Furthermore, because GLP-1R



**Fig. 3.** Calcium influx in INS-1 cells in response to 11.1 mM glucose in the presence/absence of 1 nM GLP-1. (A) After 72 h of exposure, calcium influx in INS-1 cells was assessed in the control, SHG, and IHG cell samples using a FLIPR Calcium 5 assay. (B) Data are expressed as the means ± SEM of 10 separate experiments. \*,  $P < 0.05$  vs. C; #,  $P < 0.05$  vs. SHG. Abbreviations: GLP-1, glucagon-like protein-1; SHG, sustained high glucose; IHG, intermittent high glucose.



**Fig. 4.** GLP-1R expression in INS-1 cells following exposure to SHG or IHG. (A) After INS-1 cells were incubated for 72 h, the gene expression of GLP-1R was determined in the control, SHG, and IHG cell samples via RT-PCR. (C) Under the same conditions, GLP-1R expression was measured via Western blot analysis. Densitometric analysis of (B) RT-PCR or (D) western blot is reported as the means  $\pm$  SEM of 6 separate experiments. \*,  $P < 0.05$  vs. C; #,  $P < 0.05$  vs. SHG. Abbreviations: GLP-1R, glucagon-like protein-1 receptor; SHG, sustained high glucose; IHG, intermittent high glucose; RT-PCR, reverse transcription-polymerase chain reaction.

is also expressed in other tissues such as the brain, liver, and heart [25], glucose fluctuation can also affect the functions of GLP-1 in these tissues.

In addition to reductions in GLP-1R expression, chronic hyperglycemia also induces the desensitization of  $\beta$ -cells in response to glucose and, thereby, reduces glucose-stimulated insulin secretion [26]. Tsuboi et al. demonstrated that INS-1 cells cultured in the presence of 30 mM glucose for 48 h showed reduced expressions of glucokinase and glucose transporter 2 (Glut2), which are glucose sensors in  $\beta$ -cells, leading to inhibition of glucose-stimulated insulin secretion [27]. In addition, the expression of synaptotagmin V, which regulates membrane vesicle fusion by sensing calcium levels, was reduced under hyperglycemic conditions and resulted in abnormal termination of vesicle exocytosis events [27]. Although the effects of repeated glucose fluctuation on those molecules have not been elucidated yet, it might be possible that decrease of glucose-related molecules were contributed to reduced cellular events in response to GLP-1 in IHG condition. Also, we supposed that the reduction in calcium uptake by IHG in this study was attributable to the diminution of this channel or calcium channel-activating molecules such as exchange protein activated cAMP (Epac) [28] as the result of ER and/or oxidative stress. Therefore, more reduced GLP-1-related subsequent events under IHG conditions than under SHG conditions might be attributable to lower GLP-1R expression and other mechanisms; further studies are necessary to evaluate the validity of this speculation.

Taken together, the results of this study demonstrated that glucose fluctuation triggers the reduction of GLP-1R expression and subsequent GLP-1 signaling in INS-1 cells, which may contribute to reductions in insulin secretion by GLP-1 to a greater degree than is observed in chronic hyperglycemia. From a clinical standpoint, our study suggests that strict regulation of the blood glucose concentration is necessary to prevent  $\beta$ -cell dysfunction mediated by the reduction of GLP-1R expression and subsequent GLP-1 signaling.

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