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Exogenous and endogenous ghrelin counteracts GLP-1 action to stimulate cAMP signaling and insulin secretion in islet β-cells

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ABSTRACT

We studied interactive effects of insulinotropic GLP-1 and insulinostatic ghrelin on rat pancreatic islets. GLP-1 potentiated glucose-induced insulin release and cAMP production in isolated islets and $[Ca^{2+}]_i$ increases in single β-cells, and these potentiations were attenuated by ghrelin. Ghrelin suppressed $[Ca^{2+}]_i$ responses to an adenylate cyclase activator forskolin. Moreover, GLP-1-induced insulin release and cAMP production were markedly enhanced by [D-lys^3]-GHRP-6, a ghrelin receptor antagonist, in isolated islets. These results indicate that both exogenous and endogenous islet-derived ghrelin counteracts glucose-dependent GLP-1 action to increase cAMP production, $[Ca^{2+}]_i$ and insulin release in islet β-cells, positioning ghrelin as a modulator of insulinotropic GLP-1.

1. Introduction

Insulin secretion from pancreatic islet β-cells is regulated by a variety of inhibitory and stimulatory gastro-intestinal hormones including ghrelin and glucagon-like peptide-1 (GLP-1). Ghrelin, a stomach-derived 28-amino acid hormone discovered as the endogenous ligand for the growth hormone (GH) secretagogue-receptor (GHS-R) [1,2], potently stimulates GH release and feeding and exhibits positive cardiovascular effects, suggesting its possible clinical application [3]. Ghrelin, GHS-R [4–6] and ghrelin *O*-acyltransferase (GOAT), the enzyme that acylates the third serine residue of ghrelin [7,8], are all expressed in pancreatic islets [9]. Administration of ghrelin inhibits insulin release and increases blood glucose levels in rodents [6,10] and humans [11]. Ghrelin suppresses glucose-induced insulin release in perfused pancreas and isolated islets and glucose-induced increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in single β-cells [6,12]. Conversely, ghrelin immunoneutralization, GHS-R antagonists and ghrelin deficiency all enhance glucose-induced insulin release from perfused pancreas and isolated islets [12]. Administration of GHS-R antagonist [6,12,13] and GOAT inhibitors [14] enhances insulin responses and lowers glucose concentrations during glucose tolerance tests (GTTs). These findings indicate that ghrelin is an important regulator of insulin release and glucose homeostasis [15,16].

We previously reported that ghrelin stimulates pertussis toxin (PTX)-sensitive $G\alpha_{i2}$, an inhibitory subtype of GTP-binding proteins [17], attenuates cAMP-PKA signaling, and activates 2.1 subtype of voltage-dependent K^+ (Kv2.1) channels, thereby inhibiting glucose-induced $[Ca^{2+}]_i$ increases and insulin release in islet β-cells [18]. These results suggested that ghrelin could counteract the action of GLP-1, a physiological incretin hormone that activates cAMP and Ca^{2+} signaling in islet β-cells to promote glucose-induced insulin release [19–21]. Clarifying the interaction between ghrelin and GLP-1, the physiological insulinostatic and insulinotropic hormones, on the β-cell signaling pathway and insulin release, would promote the understanding of the physiological regulation of insulin release and glycemia and of the effectiveness of anti-diabetic medicines, GLP-1 agonists and inhibitors of dipeptidyl peptidase-4 (DPP-4). This study aimed to clarify whether exogenous ghrelin counteracts and blockade of endogenous ghrelin enhances GLP-1 effects on insulin release, cAMP and $[Ca^{2+}]_i$ signaling in rat islet β-cells.

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2. Materials and methods

2.1. Animals

Male Wistar rats (Japan SLC, Hamamatsu) were housed under a controlled temperature (26 °C) and photoperiod (12L:12D). The rats received pellet-type food (CE-2, Japan Clea, Tokyo, Japan) and tap water ad libitum. The animal protocols were in accordance with the Japanese Physiological Society's guidelines for animal care.

2.2. Preparation of pancreatic islets and single β-cells

Islets of Langerhans were isolated by collagenase digestion from male Wistar rats aged 8 weeks, as reported previously [6,22] with slight modification. Animals were anaesthetized by intraperitoneal injection of pentobarbitone at 80 mg/kg, followed by injection of collagenase at 1.05 mg/ml (Sigma–Aldrich) into the common bile duct. Collagenase was dissolved in 5 mM Ca^{2+} containing HEPES-added Krebs–Ringer bicarbonate buffer (HKRB) solution (in mM): NaCl 129, $NaHCO_3$ 5.0, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.0, $MgSO_4$ 1.2 and HEPES 10 at pH 7.4 with NaOH, supplemented with 0.1% BSA when needed. Pancreas was dissected out and incubated at 37 °C for 16 min. Islets were hand collected under a microscope and were immediately used for the measurement of insulin secretion and cAMP production. For β-cell experiments, islets were dispersed into single cells in Ca^{2+} free HKRB, and the single cells were plated sparsely on coverslips and maintained for 1 day at 37 °C in an atmosphere of 5% CO_2 and 95% air in Eagle's minimal essential medium containing 5.6 mM glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin.

2.3. Measurements of insulin release and cAMP productions in isolated islets of rats

For measurements of insulin release, each tube containing 12–15 islets was incubated for 1 h at 37 °C in HKRB with 2.8 mM glucose for stabilization, followed by test incubation for 1 h in HKRB with 2.8 or 8.3 mM glucose. For the experiments with GOAT inhibitor, islets were preincubated for 24 h with 5 μM GO-CoA-Tat, a peptide-based bisubstrate analog that lowers acyl ghrelin level [14], or vehicle in the culture medium. In cAMP measurements, each tube containing 10 islets was incubated for 1 h in HKRB with 2.8, 5.6 or 8.3 mM glucose containing 500 μM 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase (PDE) (Sigma–Aldrich), to avoid degradation of cAMP in the samples. Insulin release and total cAMP content in isolated islets were determined by ELISA kit (Morinaga, Yokohama, Japan) and EIA kit (GE Healthcare, Buckinghamshire, UK), respectively. The isolated islets were prepared from four to five Wistar rats in each experiment.

2.4. Measurements of $[Ca^{2+}]_i$ in single β-cells

Single β-cells on coverslips were mounted. $[Ca^{2+}]_i$ in β-cells was measured according to previous reports [17,19]. Briefly, single β-cells were superfused with HKRB at 36 °C and $[Ca^{2+}]_i$ was measured by duel-wavelength fura-2 microfluorometry with excitation at 340/380 nm and emission at 510 nm using a cooled charge-coupled device camera. The ratio image was produced with an Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan). In single cell experiments, data were taken from the cells which fulfilled the reported morphological and physiological criteria for insulin-positive β-cells, including the diameter and responsiveness to glucose and tolbutamide [19]. The effect of GLP-1 on $[Ca^{2+}]_i$ was investigated exclusively in the cells that responded to glucose with increases in $[Ca^{2+}]_i$ in a β-cell specific manner and to tolbutamide at the end of recording. For measurements of $[Ca^{2+}]_i$, β-cells were prepared from three Wistar rats in each experiment.

2.5. Statistical analysis

Data represent the mean ± s.e.m. Statistical analyses were performed using the Student's *t*-test for comparison between two groups on measurements of $[Ca^{2+}]_i$ in single β-cells or one-way ANOVA followed by multiple comparison tests for experiments of islet insulin release or cAMP productions. *P* values below 0.05 were considered statistically significant.

3. Results

3.1. GLP-1 enhances glucose-induced insulin release and cAMP production in pancreatic islets and ghrelin counteracts the GLP-1 effects

In rat isolated islets under static incubation, glucose (8.3 mM)-induced insulin release was potentiated by 10 nM GLP-1, and this potentiation was suppressed by ghrelin at 10 nM (Fig. 1A). A rise in glucose concentration from 2.8 to 8.3 mM stimulated cAMP production in islets under static incubation in the presence of 500 μM IBMX, an inhibitor of PDE. The glucose (8.3 mM)-induced cAMP production was enhanced by GLP-1 (10 nM), and this enhancement was blocked by ghrelin (10 nM) (Fig. 1B).

3.2. Ghrelin attenuates GLP-1-induced $[Ca^{2+}]_i$ increases in single β-cells

A rise in the glucose concentration from 2.8 to 8.3 mM increased $[Ca^{2+}]_i$ in rat single β-cells. After the glucose-induced first-phase $[Ca^{2+}]_i$ increases were terminated, administration of GLP-1 (10 nM) for 3 min evoked $[Ca^{2+}]_i$ increases (Fig. 2A), confirming previous report [19]. Ghrelin at 0.1 and 10 nM added 10 min prior to GLP-1, attenuated the $[Ca^{2+}]_i$ responses to GLP-1 (Fig. 2B and C). The peak amplitude of GLP-1-induced $[Ca^{2+}]_i$ increases was decreased by ghrelin in a concentration-dependent manner (Fig. 2D). In the presence of 16.7 mM glucose, GLP-1-induced $[Ca^{2+}]_i$ increases were attenuated by ghrelin at 10 nM (Fig. 2E–G).

3.3. Ghrelin inhibits forskolin-induced $[Ca^{2+}]_i$ increases in islet single β-cells

In the presence of 8.3 mM glucose, an adenylate cyclase activator, forskolin at 5 μM, and a membrane-permeable specific protein kinase-A (PKA) activator, 6-Phe-cAMP at 10 μM, increased $[Ca^{2+}]_i$ in single β-cells (Fig. 3A and B). The peak amplitude of forskolin-induced $[Ca^{2+}]_i$ increases was significantly ($P < 0.01$) suppressed by pretreatment with 10 nM ghrelin (Fig. 3C and E), while that of 6-Phe-cAMP-induced $[Ca^{2+}]_i$ increases was not affected (Fig. 3D and F).

3.4. Blockade of endogenous ghrelin potentiates GLP-1-induced insulin release and cAMP production in isolated islets

Glucose (8.3 mM)-induced insulin release in islets was significantly enhanced by incubation with GLP-1 (10 nM) and with a GHS-R antagonist, [D-lys^3]-GHRP-6 (1 μM). Notably, the GLP-1-enhanced level of insulin release was further elevated by [D-lys^3]-GHRP-6 (1 μM) to a significantly ($P < 0.05$) higher level (Fig. 4A). Glucose at 5.6 mM stimulated cAMP production in islets in the presence of 500 μM IBMX, and the glucose-induced cAMP produc-

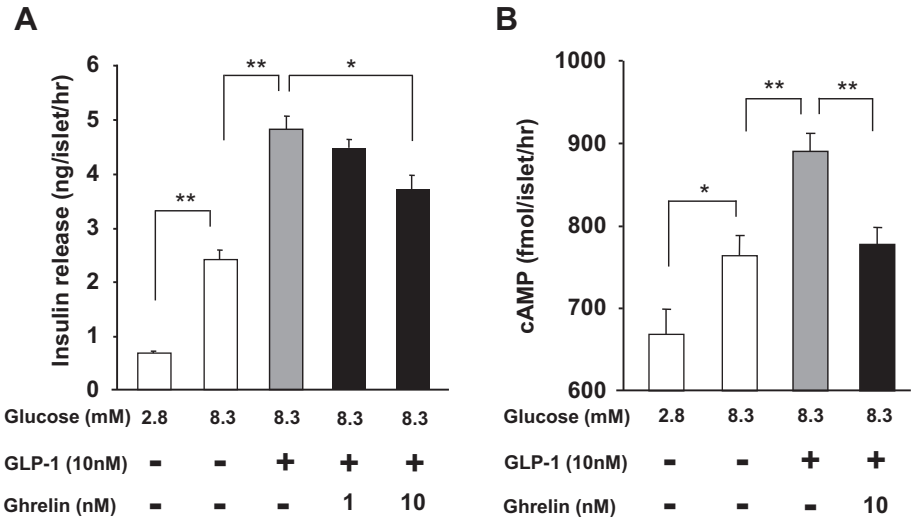

Fig. 1. Ghrelin inhibits GLP-1-induced insulin release and cAMP production in isolated islets. (A) Glucose (8.3 mM)-induced insulin release was potentiated by GLP-1 (10 nM), and this potentiation was attenuated by exogenous ghrelin at 10 nM in isolated pancreatic islets. (B) Glucose (8.3 mM)-induced cAMP productions in islets in the presence of phosphodiesterase inhibitor IBMX (500 μM) was augmented by GLP-1 (10 nM), and this augmentation was inhibited by exogenous ghrelin (10 nM). $n = 8–11$, number of tubes in each bar. One tube included 12–15 islets for insulin measurement and 10 islets for cAMP measurement $^{*}P < 0.05$; $^{**}P < 0.01$.

tion was enhanced either by GLP-1 (10 nM) or by [D-lys^3]-GHRP-6 (1 μM). The combination of these two agents further increased the cAMP production (Fig. 4B). Glucose (8.3 mM)-induced insulin release in isolated islets was significantly elevated by pretreatment with an inhibitor of GOAT, GO-CoA-Tat at 5 μM, to a level comparable to that obtained with [D-lys^3]-GHRP-6 (Fig. 4C vs. A).

4. Discussion

In this study, we clarified the interaction between insulinostatic peptide ghrelin and insulinotropic peptide GLP-1, and underlying signal transduction mechanism in rat pancreatic islets and single β-cells. GLP-1 enhanced insulin release and cAMP production in the presence of 8.3 mM glucose in islets and increased $[Ca^{2+}]_i$ in the presence of 8.3 and 16.7 mM glucose in single β-cells, and all these effects were markedly attenuated by ghrelin. Moreover, blockade of endogenous ghrelin by [D-lys^3]-GHRP-6, a GHS-R antagonist, markedly enhanced the ability of GLP-1 to increase insulin release and cAMP production in islets.

It is known that GLP-1 increases insulin release by increasing $[Ca^{2+}]_i$ in β-cells via multiple mechanisms including inhibition of ATP-sensitive K^+ (K_{ATP}) channels [21], inhibition of Kv channels [23], and facilitation of Ca^{2+} influx through voltage-dependent Ca^{2+} channels [19,24]. These effects could be mediated by cAMP production and cAMP-stimulated PKA-dependent and/or Epac-dependent mechanisms [21,25]. In the present study, GLP-1 in the presence of IBMX augmented cAMP production in islets, and this effect was suppressed by ghrelin. GLP-1 and an adenylate cyclase activator forskolin evoked increases in $[Ca^{2+}]_i$ in β-cells, and they were attenuated by ghrelin. In contrast, $[Ca^{2+}]_i$ responses to 6-phe-cAMP, a membrane-permeable selective activator of PKA, were not affected by ghrelin in islet β-cells, suggesting that ghrelin acts on the signaling process upstream but not downstream of PKA. In our previous reports [17,18], the effects of ghrelin on the glucose-induced insulin release and $[Ca^{2+}]_i$ increases and on Kv channel currents were all blunted by treatment with an adenylate cyclase inhibitor MDL-12330A, a cAMP analog dibutyryl-cAMP and a PKA activator 6-phe-cAMP in perfused pancreas, isolated islets and single β-cells, suggesting that ghrelin acts on the adenylate cyclase-PKA signaling route. Taken together, our results support that ghrelin interacts with adenylate cyclase to inhibit its activity and downstream PKA pathway, and that this inhibitory signaling attenuates $[Ca^{2+}]_i$ increases and insulin release in response to GLP-1 (Fig. 5), although an additional involvement of Epac in the ghrelin signaling remains to be established.

It was reported that exogenous ghrelin enhanced glucagon release from pancreatic islets and from pancreatic α-cell lines [26]. Hence, a possibility can not be excluded that a part of the effect of ghrelin on glucose- and GLP-1-induced insulin release results from the effect of ghrelin on glucagon release. However, it is not highly likely since glucagon enhances insulin release and it has been reported that GHS-R antagonist has no effect on glucagon release from isolated islets in the presence of 5.6 mM glucose [6] and that ghrelin has no effect on glucagon release in human subjects and perfused pancreas [27,28].

GLP-1 release into the circulation is increased after food ingestion and proglucagon expression is decreased by fasting [29]. Conversely, circulating ghrelin concentrations rise before meals and fall after eating [30]. The present study clearly indicated opposite actions of GLP-1 and ghrelin on insulin release, cAMP production and $[Ca^{2+}]_i$ in islets and β-cells. These findings by us and others suggest that the inverse relationship between circulating concentrations of ghrelin and GLP-1 at both preprandial and postprandial periods together with their reciprocal effects on insulin secretion from islets cooperate to optimize insulin levels to achieve fine glucose homeostasis: it produces the ghrelin dominant state in preprandial periods to effectively restrict insulin secretion and the GLP-1 dominant state in postprandial periods to effectively promote insulin secretion for effective disposal of blood glucose. Moreover, our present finding that ghrelin counteracts the effect of GLP-1 suggests that it might also counteract the effect of other hormones that also stimulate cAMP signaling in islet β-cells, which include pituitary adenylate cyclase activating polypeptide (PACAP) and gastric inhibitory polypeptide (GIP).

Regarding the expression of ghrelin in pancreatic islets, it has been reported that the ghrelin immunoreactivity is localized in α-cells of adult Wistar rat islets at 8–12 weeks old and of adult human islets [5,6], and in β-cells of adult human islets [31], and in novel ε-cells of adult Sprague–Dawley rat islets at 8–20 weeks [32], and of adult human islets at 35–75 years [33,34]. Developmental

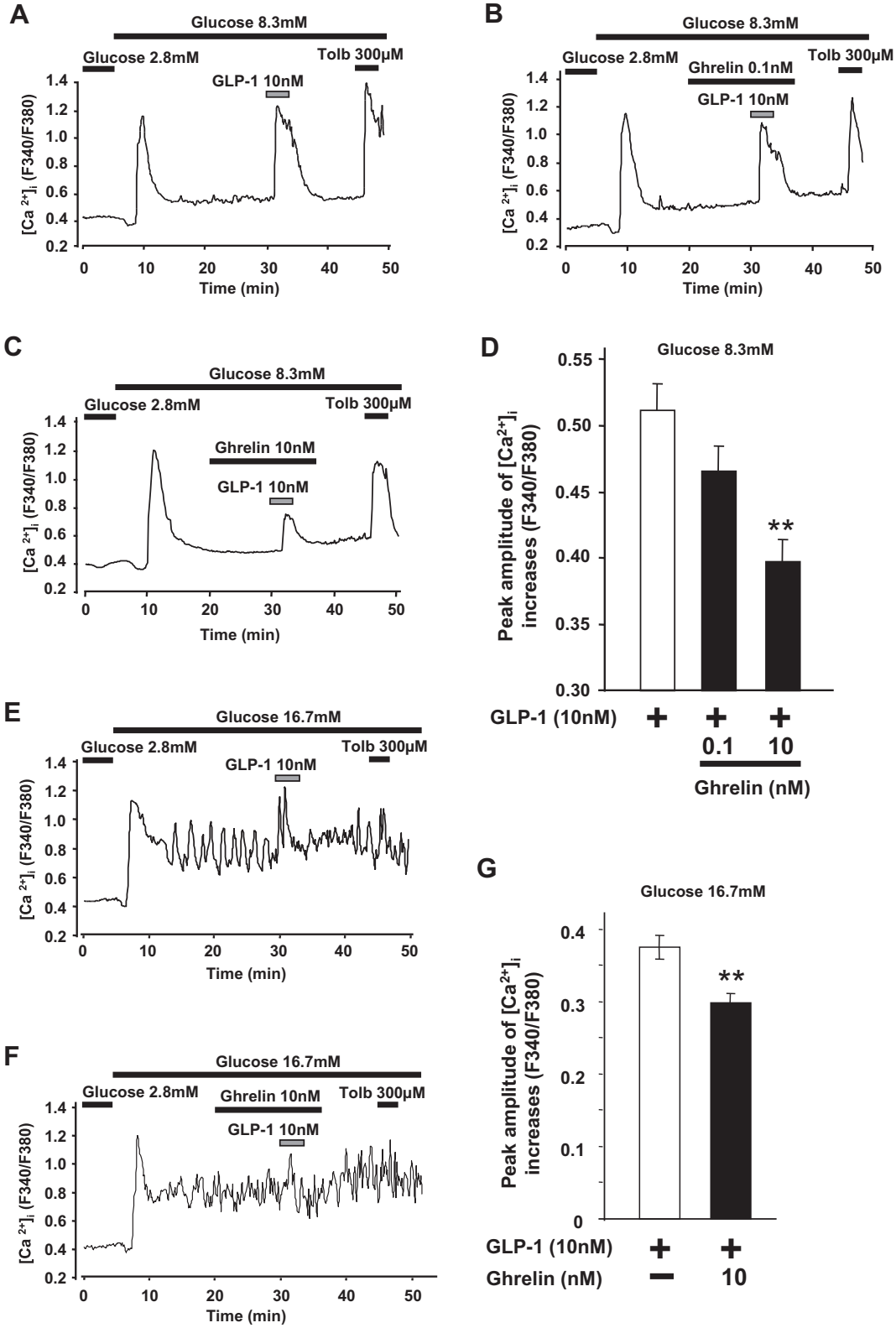

Fig. 2. Ghrelin inhibits GLP-1-induced $[Ca^{2+}]_i$ increases in islets β-cells. (A–C) Representative traces of $[Ca^{2+}]_i$ in single β-cell are expressed by dual-wavelength fura-2 fluorescence ratio (F340/F380). (A and E) In the presence of 8.3 or 16.7 mM glucose, GLP-1 (10 nM) induced increases in $[Ca^{2+}]_i$. (B, C and F) Ghrelin at 0.1–10 nM administrated 10 min prior to the GLP-1 administration attenuated the $[Ca^{2+}]_i$ increases to GLP-1. (D and G) The peak amplitude of GLP-1-induced $[Ca^{2+}]_i$ increases was significantly suppressed by ghrelin in the presence of 8.3 and 16.7 mM glucose. (n = 101–115, number of single β-cells examined each group). $^{**}P < 0.01$ vs. GLP-1 alone.

change has also been reported: abundant expression of ghrelin and large number of ghrelin expressing cells are observed in fetal pancreatic islets in mice [35], rats [32] and humans [33,34], and they significantly decrease in the adult pancreas or pancreatic islets in rats [32] and humans [33,34]. Taken together, it is consistent among the literatures that ghrelin is expressed in islet cells of adult

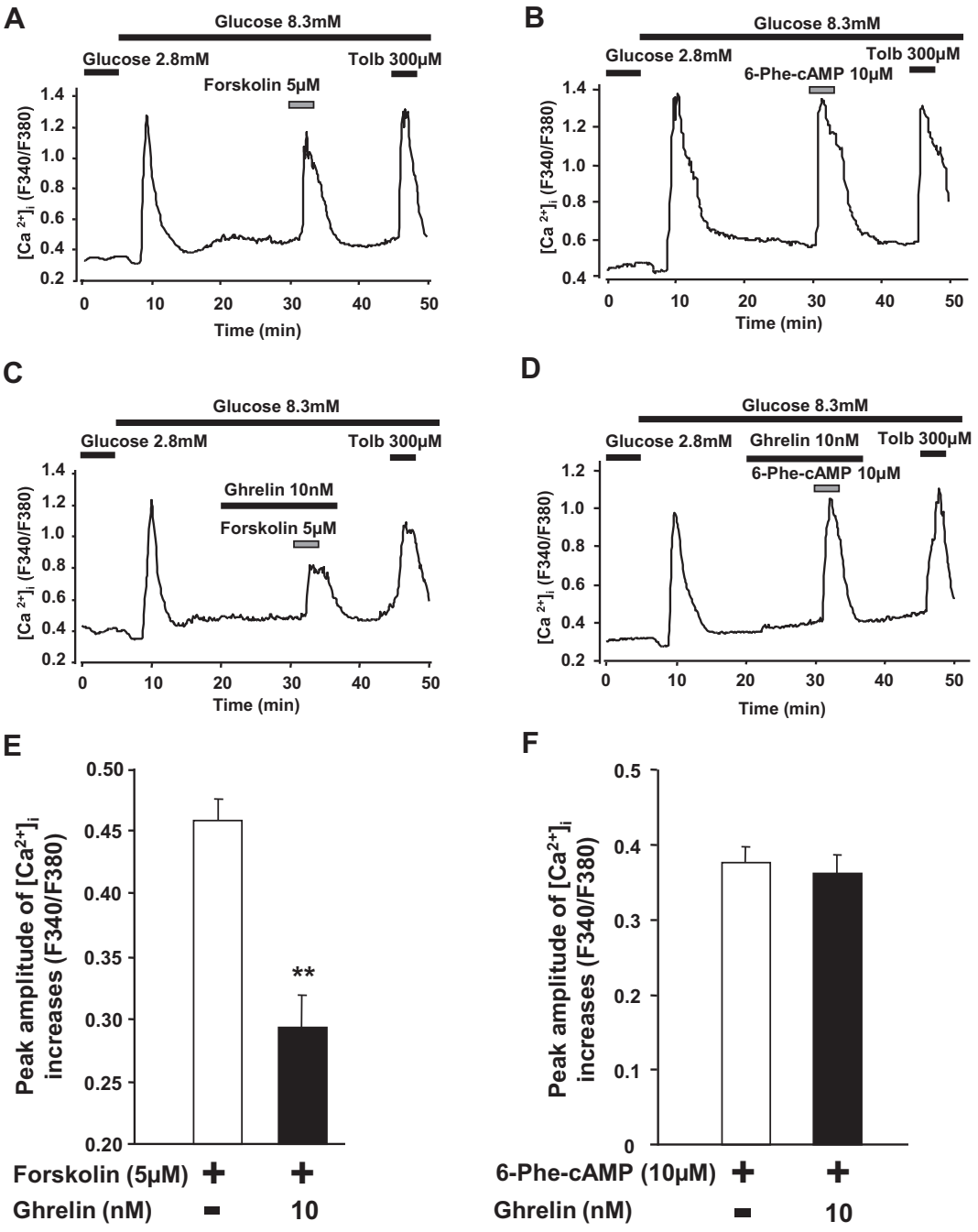

Fig. 3. Ghrelin inhibits forskolin- but not 6-Phe-cAMP-induced $[Ca^{2+}]_i$ increases in islet β-cells. (A and B) An adenylate cyclise activator forskolin at 5 μM (A) and a PKA activator 6-Phe-cAMP at 10 μM (B) evoked $[Ca^{2+}]_i$ increases in the presence 8.3 mM glucose. (C and E) Ghrelin at 10 nM attenuated forskolin-induced $[Ca^{2+}]_i$ increases in single β-cells. (D and F) The 6-Phe-cAMP-induced $[Ca^{2+}]_i$ increases were not affected by ghrelin at 10 nM. (n = 112–117, number of single β-cells examined in each group), $^{**}P < 0.01$ vs. forskolin.

rats at the age of 8–20 weeks and in adult human islets though to a lesser extent than that expressed in fetal stages. The cell species that expresses ghrelin is reportedly α-cell, β-cell and/or ε-cell, showing a discrepancy. To support the action of ghrelin on islets cells, GHS-R is expressed in α-cells of adult mice pancreatic islets at 3 months old [26] and of adult rat pancreatic islets [36], and in β-cells of adult rat pancreatic islets [36]. Both ghrelin and GHS-R mRNAs are expressed in the adult rat pancreas at 8–12 weeks [5] and adult human pancreas [4,31]. mRNAs for GOAT are expressed in adult mice and rat pancreas at 8 weeks [9] and adult human pancreas [37]. Moreover, plasma ghrelin level is higher in the pancreatic vein than pancreatic artery in anesthetized adult rats [12], suggesting that ghrelin is released from the pancreas. These documents taken together indicate that ghrelin is expressed at least in islet cells of rats at 8 weeks used in our study and suggest that the cell types that express ghrelin and the amount of ghrelin produced depend on the species, age and condition of animals/humans.

In this study, [D-lys^3]-GHRP-6, a GHS-R antagonist, was used for blockade of endogenous islet-derived ghrelin. [D-lys^3]-GHRP-6 enhanced glucose-induced insulin release and cAMP production in rat islets as previously reported [18]. It should be mentioned that

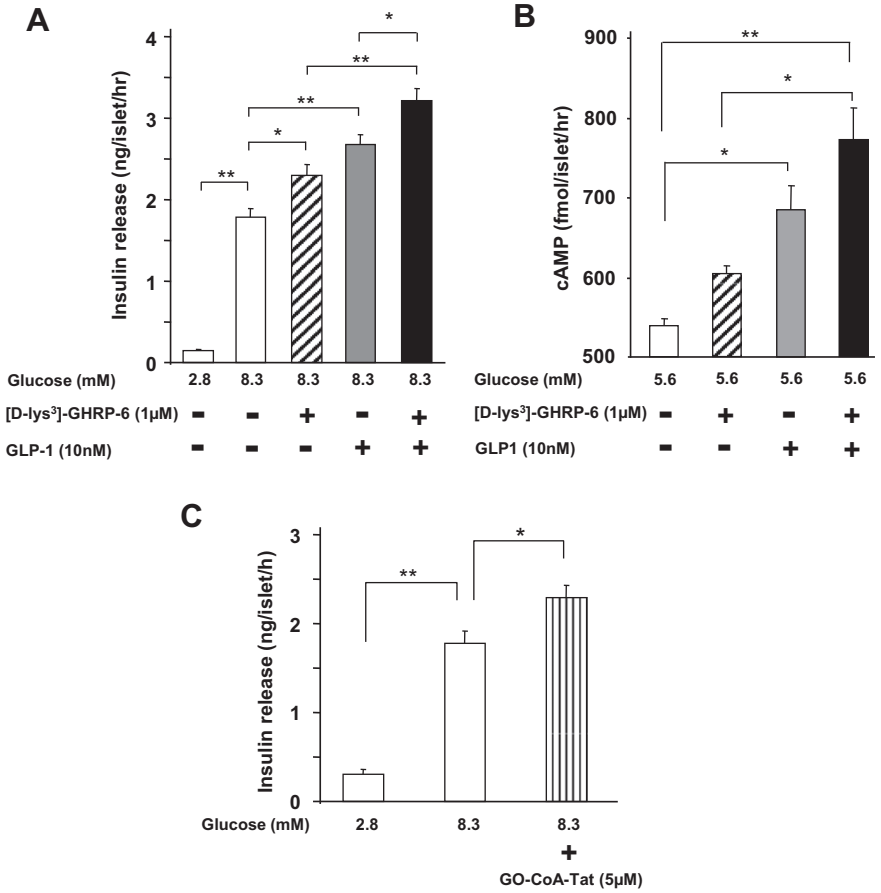

Fig. 4. GHS-R antagonist potentiates GLP-1-induced insulin release by elevating cAMP production in isolated islets. (A) Endogenous ghrelin blockade by a GHS-R antagonist [D-lys^3]-GHRP-6 (1 μM), as well as GLP-1 (10 nM), enhanced glucose (8.3 mM)-induced insulin release in isolated islets. Insulin release in response to GLP-1 was significantly potentiated by [D-lys^3]-GHRP-6. (B) Glucose (5.6 mM)-induced cAMP production was potentiated by both [D-lys^3]-GHRP-6 (1 μM) and GLP-1 (10 nM) in isolated islets. The combination of GLP-1 plus [D-lys^3]-GHRP-6 further elevated cAMP production. (C) Glucose (8.3 mM)-induced insulin release was significantly increased by pretreatment with GO-CoA-Tat (5 μM), a GOAT inhibitor, in isolated islets. n = 5–11, number of tubes in each bar. One tube included 12–15 islets for insulin measurement and 10 islets for cAMP measurement. $^*P < 0.05$; $^{**}P < 0.01$.

the effect of [D-lys^3]-GHRP-6 does not necessarily indicate the involvement of endogenous ghrelin but could reflect a possible inverse agonist action on GHS-R that decreases the constitutive activity of GHS-R [38] and non-specific effects of this antagonist on other receptors [39,40]. It has been shown by many laboratories including us that different approaches to diminish the (effect of) possible endogenous ghrelin consistently result in increases in insulin secretion and associated β-cell signaling. In isolated islets, [D-lys^3]-GHRP-6, a GHS-R antagonist, and antiserum against ghrelin markedly increased glucose-stimulated insulin release [6] and cAMP production [18]. The glucose-induced insulin release from isolated islets of ghrelin knockout mice was significantly greater than that of wild-type mice, while basal insulin release was not altered [12]. This increase in glucose-induced insulin release reflects the enhanced secretory activity, because no difference was observed between ghrelin knockout and wild-type mice in insulin content per islet and expressions of insulin 1 and insulin 2 mRNAs [12]. Moreover, pretreatment with GO-CoA-Tat, an inhibitor of GOAT, significantly increases insulin response to 8.3 mM glucose in rat islets in the present study, and increases insulin response to a glucose challenge in human islets [14]. These findings suggest that the endogenous ghrelin suppresses glucose-induced insulin secretion within islets of rodents and humans at least in vitro.

We found that ghrelin inhibits not only cAMP production in islets but also cAMP action as evidenced by inhibition of forskolin-induced $[Ca^{2+}]_i$ increases, confirming previous report [18]. Therefore, it is likely that the blockade of insulinostatic activity of islet-derived ghrelin effectively cooperates with GLP-1 in potentiating cAMP production, thereby promoting insulin release in islet β-cells. It has been reported that the plasma level of GLP-1 and incretin action of GLP-1 are impaired in type 2 diabetic patients [41–43], which may contribute to reduction of insulin release. Circulating GLP-1 is rapidly degraded by DPP-4 with a half life around 1–2 min. Currently, chemically modified DPP-4-resistant GLP-1 agonists or long-acting GLP-1 analogues and DPP-4 inhibitors are clinically used in treating type 2 diabetic patients. However, during the treatment with these incretin mimetics or enhancers, side effects including nausea and hypoglycemia have been reported in patients with type 2 diabetes [44]. An important finding of the present study is that the blockade of islet-derived ghrelin by GHS-R antagonist markedly elevates the GLP-1-induced insulin release and cAMP production in isolated islets (Fig. 4). These results suggest that blockade of endogenous ghrelin provides a novel tool to enhance therapeutic ability of GLP-1-based drugs, thereby minimizing the doses of the drugs to reduce their side effects.

In conclusion, ghrelin potently attenuates GLP-1-induced cAMP generation, $[Ca^{2+}]_i$ increases and insulin release in islet β-cells, while ghrelin receptor antagonist potentiates the GLP-1-induced cAMP generation and insulin release. These findings indicate that interaction between ghrelin and GLP-1 plays an important role in physiological regulation of glucose-induced insulin release in islet

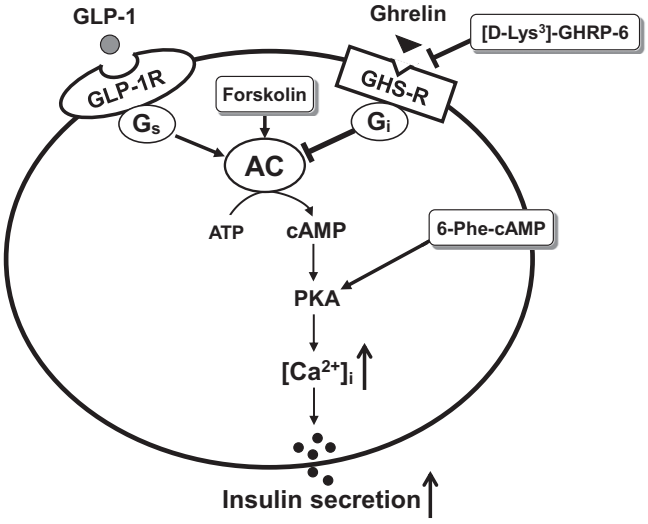

Fig. 5. The opposing effects of ghrelin and GLP-1 on islet β-cell signaling and insulin secretion. Binding of GLP-1 to its receptor in islet β-cells stimulates the cAMP cascade: activation of Gs protein and adenylate cyclase (AC) cause cAMP production and PKA activation, resulting in increases in $[Ca^{2+}]_i$ and insulin secretion. Conversely, the ghrelin binding to GHS-R in β-cells activates Gi protein to attenuate cAMP production, thereby counteracting the GLP-1-induced increases in $[Ca^{2+}]_i$ and insulin secretion. Ghrelin inhibits $[Ca^{2+}]_i$ increases induced by an adenylate cyclase activator forskolin but not by 6-Phe-cAMP, a PKA activator, indicating that ghrelin suppresses AC activity but not downstream signaling of PKA in β-cells. Thus, exogenous and endogenous ghrelin inhibit and its blockade by [D-lys^3]-GHRP-6 promotes the effects of GLP-1 on cAMP, $[Ca^{2+}]_i$ and insulin release in islet β-cells.

β-cells, and suggest that ghrelin antagonism provides a novel strategy to treat type 2 diabetes with dysregulated insulin release.

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