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Ghrelin is a physiological regulator of insulin release in pancreatic islets and glucose homeostasis

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Abbreviations:
GH, growth hormone
GHS-R, growth hormone secretagogue-receptor
mRNA, messenger ribonucleic acid
PTX, pertussis toxin
$[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration
ACh, acetylcholine
K_{ATP} channel, ATP-sensitive K^+ channel
Kv channel, delayed rectifier K^+ channel
TEA, tetraethylammonium
PLC, phospholipase C
AS, antisense oligonucleotide
i.p., intraperitoneal
ITT, insulin tolerance test
GTT, glucose tolerance test
GX, gastrectomized
Ghr-KO, ghrelin-knockout
IAP, islet-activating protein
HFD, high-fat diet
PCR, polymerase chain reaction
UCP, uncoupling protein

ABSTRACT

Ghrelin, an acylated 28-amino acid peptide, was isolated from the stomach as the endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R). Circulating ghrelin is produced predominantly in the oxyntic mucosa of stomach. Ghrelin potently stimulates GH release and feeding, and exhibits positive cardiovascular effects, suggesting a possible clinical application. Low plasma ghrelin levels are associated with elevated fasting insulin levels and insulin resistance, suggesting both physiological and pathophysiological roles for ghrelin in glucose metabolism. Here, we review the physiological role of ghrelin in the regulation of insulin release and glucose metabolism, and a potential therapeutic avenue to treat type 2 diabetes by manipulating ghrelin and/or its signaling. Ghrelin inhibits insulin release in mice, rats and humans. The signal transduction mechanisms of ghrelin in islet β-cells are distinct from those utilized in GH-releasing and/or GHS-R-expressing cells. Ghrelin is expressed in pancreatic islets and released into pancreatic microcirculations. Pharmacological and genetic blockades of islet-derived ghrelin markedly augment glucose-induced insulin release in vitro. In high-fat diet-induced mildly obese mice, ghrelin-deficiency enhances insulin release and prevents impaired glucose tolerance. Thus, manipulation of insulinostatic function of ghrelin — GHS-R system, particularly that in islets, could optimize the amount of insulin release to meet the systemic demand, providing a potential therapeutic application to prevent type 2 diabetes.

Contents

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1. Introduction

Ghrelin, a novel acylated 28-amino acid peptide, was isolated from the human and rat stomach as the endogenous ligand (Kojima et al., 1999) for the growth hormone (GH) secretagogue-receptor (GHS-R) (Howard et al., 1996). This novel peptide has an *n*-octanoylated serine residue at the third N-terminal position; this acylation is essential for ghrelin bioactivity (Kojima et al., 1999). Circulating ghrelin is produced predominantly in the stomach (Ariyasu et al., 2001), while substantially lower amounts are detected in the intestine, pancreas, kidney, immune system, placenta, testis, pituitary, lung, and hypothalamus (Kojima et al., 1999; Hosoda et al., 2000; Date et al., 2000a; Mori et al., 2000; Gualillo et al., 2001; Tanaka et al., 2001; Date et al., 2002; Gnanapavan et al., 2002; Hattori et al., 2001; Muccioli et al., 2002; Tena-Sempere et al., 2002; Volante et al., 2002a, 2002b). GHS-Rs are expressed in the hypothalamus-pituitary unit, and are also distributed in other central and peripheral tissues (Howard et al., 1996; Guan et al., 1997; Smith et al., 1997; Muccioli et al., 1998; Papotti et al., 2000; Cassoni et al., 2001; Ghigo et al., 2001; Gnanapavan et al., 2002; Katugampola et al., 2002; Muccioli et al., 2002). Ghrelin is a potent stimulator of GH release (Kojima et al., 1999; Arvat et al., 2000, 2001; Date et al., 2000b; Peino et al., 2000; Takaya et al., 2000). Ghrelin also stimulates feeding; ghrelin injected either centrally (Kamegai et al., 2001; Nakazato et al., 2001; Shintani et al., 2001; Wren et al., 2001b) or peripherally (Nakazato et al., 2001; Wren et al., 2001a) potently stimulates food intake. In humans, ghrelin peaks before meals, suggesting its role as a hunger signal (Cummings et al., 2002; Cummings & Overduin, 2007). Cardiovascular actions of ghrelin are also reported (Nagaya et al., 2001a, 2001b, 2001c; Nagaya & Kangawa, 2003a, 2003b; Lin et al., 2004; Matsumura et al., 2002). Given this wide spectrum of biological activities, the discovery of ghrelin opened many new perspectives within neuroendocrine, metabolic and cardiovascular research, thus suggesting its possible clinical application (Kojima & Kangawa, 2006). Ghrelin and GHS-R are also located in pancreatic islets (Date et al., 2002; Gnanapavan et al., 2002; Volante et al., 2002a; Wierup et al., 2002; Dezaki et al., 2004; Prado et al., 2004; Wierup et al., 2004; Wierup & Sundler, 2005). Ghrelin inhibits insulin release in mice, rats and humans (Broglio et al., 2001; Egido et al., 2002; Reimer et al., 2003; Dezaki et al., 2004). Low plasma ghrelin levels are associated with elevated fasting insulin levels and insulin resistance in humans (Ikezaki et al., 2002; Poykko et al., 2003). These findings suggest both physiological and pathophysiological roles for ghrelin in the regulation of insulin release. In addition, the plasma ghrelin level correlates inversely with obesity (Tschop et al., 2001; Ariyasu et al., 2002; Shiiya et al., 2002). Hence, ghrelin could be involved in energy and glucose metabolism, in which insulin plays a crucial role.

Here we review the physiological role of ghrelin in the regulation of insulin release and glucose metabolism, and present a potential therapeutic avenue to manipulate ghrelin signaling and thereby counteract the progression of type 2 diabetes.

2. Pancreatic islet-derived ghrelin

2.1. Expressions of ghrelin and GHS-R in islets

Immunohistochemistry with antiserum against ghrelin demonstrated the immunoreactivity for ghrelin in a fraction of human and rat islet cells, which were observed mainly in the periphery of islets. Ghrelin-immunoreactive cells highly overlapped with glucagon-immunoreactive cells (Date et al., 2002; Dezaki et al., 2004; Kageyama et al., 2005), while some of glucagon-immunoreactive cells were not immunoreactive to ghrelin. Immunoreactive ghrelin was also observed in mouse islets (Iwakura et al., 2005; Jeffery et al., 2005). Multiple experimental systems have shown ghrelin-immunoreactivity in α-cells (Date et al., 2002; Dezaki et al., 2004; Heller et al., 2005; Wang et al., 2007), β-cells (Volante et al., 2002a; Granata et al., 2007; Wang et al., 2007) and novel islet cells (Wierup et al., 2002, 2004; Wierup & Sundler, 2005) including those named ε-cells (Prado et al., 2004; Heller et al., 2005). It was also reported that ghrelin is expressed together with glucagon or pancreatic polypeptide in immature islet cells in rats (Wierup et al., 2004). Regarding receptors for ghrelin in islets, double immunohistochemistry revealed that GHS-R-like immunoreactivity mainly colocalized extensively with glucagon-immunoreactivity and partly with insulin-immunoreactivity in rat pancreatic islets (Kageyama et al., 2005), indicating expression of GHS-R in α- and β-cells. Messenger RNAs (mRNAs) encoding ghrelin and GHS-R are expressed in the pancreas of rats and humans (Kojima et al., 1999; Date et al., 2002; Gnanapavan et al., 2002; Volante et al., 2002a) as well as in β-cell lines (Colombo et al., 2003; Wierup et al., 2004; Granata et al., 2007). The expression of pancreatic ghrelin changes dramatically during fetal development. Ghrelin mRNA and total ghrelin in the pancreas are markedly elevated selectively in the peri-natal stages, at which their levels are six to seven times greater than those in the fetal stomach (Chanoine & Wong, 2004). It is therefore suggested that ghrelin may be expressed and located in different islet cell types depending upon the species, ages and conditions of animals/humans.

2.2. Ghrelin release from pancreas

Ghrelin is expressed in the pancreatic islets as mention above. Furthermore, ghrelin was detected in the pancreas and islets using radioimmunoassay (Date et al., 2002; Dezaki et al., 2004). Release of ghrelin from pancreatic islets was assessed by comparing the ghrelin level in the pancreatic vein (splenic vein) with that in the pancreatic artery (celiac artery) in anaesthetized rats (Fig. 1). The concentrations of both acylated-ghrelin and desacyl-ghrelin in the pancreatic vein were significantly higher (about 8 times and 3 times, respectively) than those in the pancreatic artery in rats, indicating that ghrelin is released from pancreas (Dezaki et al., 2006). Regulation of ghrelin release from the pancreas as compared to that from the stomach is an important issue that remains to be clarified.

3. Insulinostatic function of islet-derived ghrelin in vitro

In isolated rat islets, GHS-R antagonists ([D-Lys^3]-GHRP-6 and [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P) markedly increased insulin release in the presence of 5.6 mM glucose and this response was abolished in the absence of external Ca^{2+} (Dezaki et al., 2004), indicative of Ca^{2+}-dependent insulin release by the receptor antagonists. Furthermore, antiserum against active ghrelin also increased insulin release, while control nonimmune serum had no effect (Dezaki et al., 2004). These results suggested that endogenous ghrelin could suppress Ca^{2+}-mediated insulin release. Administration of exogenous ghrelin at relatively high concentration of 10 nM, but not 0.1 nM and 1 pM, attenuated 8.3 mM glucose-induced insulin release in islets, while it had no effect at 2.8 mM glucose (Dezaki et al., 2004). The similar concentration- and glucose-related inhibitory effects of exogenous ghrelin on insulin release in vitro are reported in the rat (Colombo et al., 2003) and mouse islets (Reimer et al., 2003). Although this concentration is higher than that of circulating ghrelin ranging 100 pM–3 nM (Toshinai et al., 2001; Shiiya et al., 2002), it is generally conceived that the level of hormone working in a paracrine/autocrine manner is higher than that in an endocrine manner. Insulinostatic effects of endogenous and exogenous ghrelin were blunted in islets isolated from rats pretreated with pertussis toxin (PTX), a specific inhibitor of Gi and Go subtypes of trimeric GTP-binding proteins (Katada & Ui, 1979) that function in islet β-cells (Sharp, 1996), whereas addition of 25 mM KCl could enhance insulin release from these islets (Dezaki et al., 2007). On the other hand, 10 nM ghrelin failed to alter glucagon release at both 2.8 mM glucose and 8.3 mM glucose (Dezaki et al., 2004). Glucagon release at 5.6 mM glucose was not significantly altered by 1 μM [D-Lys^3]-GHRP-6 (Dezaki et al., 2004).

To examine physiological roles of the pancreatic islet-produced ghrelin, we employed insulin release from the perfused rat pancreas, an in vitro system that retains the intact circulation in pancreatic islets and excludes the influence of other organs (Grodsky & Fanska, 1975). A rise in the perfusate glucose concentration from 2.8 to 8.3 mM evoked insulin release in a biphasic manner. Both the first and second phases of glucose-induced insulin release were significantly enhanced by blockade of GHS-R with a GHS-R antagonist [D-Lys^3]-GHRP-6 (1 μM), and by immunoneutralization of endogenous ghrelin with anti-ghrelin antiserum (0.1%) (Dezaki et al., 2006). Conversely, administration of exogenous ghrelin (10 nM) suppressed both phases of glucose-induced insulin release (Egido et al., 2002; Dezaki et al., 2006). None of these treatments affected basal insulin release at 2.8 mM glucose. These findings indicate the insulinostatic function of endogenous ghrelin within islets. It should be noted that the effect of GHS-R antagonist could be partly due to blockade of constitutive activity of GHS-R (Holst et al., 2003, 2004).

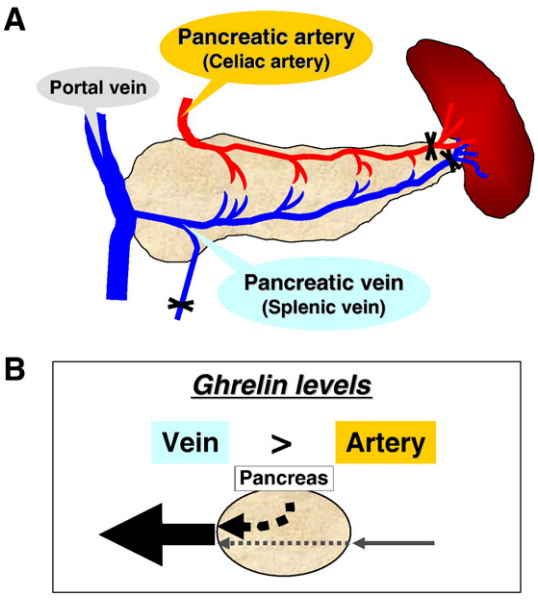

Fig. 1. Ghrelin release from pancreas. (**A**) To measure plasma ghrelin concentrations, blood samples were collected from the pancreatic arteries (celiac artery) and veins (splenic vein) of anesthetized rats. To avoid inflow of ghrelin from intestine and stomach to the splenic vein, the inferior mesenteric vein and spleen side of the splenic vein-including the short gastric and left gastroomental veins — were ligated (as denoted with ×). (**B**) The ghrelin level is higher in the pancreatic vein than in the artery, indicating that ghrelin is released from pancreas.

4. Pertussis toxin-sensitive ghrelin signaling in islet β-cells

4.1. Ghrelin attenuates $[Ca^{2+}]_i$ in β-cells

In islet β-cells, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is considered the major regulator of insulin secretion (Wollheim & Sharp, 1981; Prentki & Matschinsky, 1987). $[Ca^{2+}]_i$ measured in islets by fura-2 microfluorometry was elevated mildly by increasing glucose concentration from 2.8 to 5.6 mM. In the presence of [D-Lys^3]-GHRP-6, the peak of the first phase $[Ca^{2+}]_i$ response was enhanced and, in some islets, oscillations of $[Ca^{2+}]_i$ were induced (Dezaki et al., 2004). The peaks and integrated $[Ca^{2+}]_i$ increases of the first phase $[Ca^{2+}]_i$ responses to 5.6, 8.3, 16.7 and 22.4 mM glucose were all increased by the antagonist (Dezaki et al., 2004). Similarly, antiserum against ghrelin enhanced the $[Ca^{2+}]_i$ response to 8.3 mM glucose (Dezaki et al., 2004). These data indicate that ghrelin in islets restricts glucose-induced $[Ca^{2+}]_i$ increase and insulin secretion, presumably via a paracrine and/or autocrine route.

In rat single β-cells, ghrelin at a relatively high concentration of 10 nM, but not 0.1 nM, markedly suppressed the peak of the first phase $[Ca^{2+}]_i$ responses to 8.3 mM glucose and this effect was blocked by GHS-R antagonist (Dezaki et al., 2004, 2007). Ghrelin preincubated with antiserum against active ghrelin (immunoneutralized ghrelin) had no inhibitory effect on the $[Ca^{2+}]_i$ responses, confirming that the antiserum employed in our study neutralizes the activity of ghrelin. Ghrelin at 10 nM also attenuated $[Ca^{2+}]_i$ oscillations during the second phase responses to 8.3 mM glucose in oscillating β-cells. The attenuation of $[Ca^{2+}]_i$ oscillations by ghrelin was abolished in the presence of GHS-R antagonist (Dezaki et al., 2004). The effects of ghrelin on the first phase and second phase $[Ca^{2+}]_i$ responses were both abolished in β-cells following exposure to PTX. In contrast, 10 nM ghrelin had no significant effect on the $[Ca^{2+}]_i$ responses to 300 μM tolbutamide and 10 μM acetylcholine (ACh) at 2.8 mM glucose (Dezaki et al., 2004). Thus the counteracting action of ghrelin appeared to be specific for glucose, suggesting a possibility that ghrelin could interfere with the glucose metabolic pathway in β-cells. However, the increase in NAD(P)H in response to 8.3 mM glucose, as monitored by its autofluorescence, was not altered by 10 nM ghrelin in β-cells (Dezaki et al., 2004).

4.2. Ghrelin decreases electrical activities in β-cells

At substimulatory glucose concentrations, β-cells maintain the resting membrane potential at a hyperpolarized level of around −70 mV. Elevation of the blood glucose concentration increases glucose uptake and metabolism by β-cells, resulting in closure of the ATP-sensitive K^+ (K_{ATP}) channels. When K^+ efflux is reduced, inward currents more effectively contribute to the membrane potential and the membrane depolarizes. Under the condition of nystatin-perforated whole-cell current-clamp mode, glucose (8.3 mM) elicited firings of action potentials in rat β-cells. The firings were characterized by spike-like and repetitively occurring action potentials on top of the plateau phase of slow waves (Dezaki et al., 2007). These electrical firings were attenuated by ghrelin (10 nM) in a reversible manner. Ghrelin decreased both the frequency and amplitude of the firings, while mean membrane potentials measured at most repolarized levels between slow-wave potentials at 8.3 mM glucose were not significantly altered by ghrelin. These findings suggest that the ghrelin administration does not hyperpolarize the membrane potential but decrease the activity of action potentials in β-cells.

4.3. Ghrelin activates Kv currents in β-cells

Voltage-dependent potassium channels are involved in repolarization of excitable cells. In pancreatic β-cells, activation of delayed rectifier K^+ (Kv) channels repolarizes cells and attenuates glucose-stimulated action potentials, limiting Ca^{2+} entry through voltage-dependent Ca^{2+} channels to suppress insulin secretion (MacDonald & Wheeler, 2003). Blockade of Kv channels can promote glucose-dependent insulin secretion (MacDonald et al., 2001, 2002; Herrington et al., 2006). In patch clamp study of rat single β-cells under a perforated whole-cell clamp mode, ghrelin increased the amplitudes of K^+ currents in a reversible manner (Dezaki et al., 2004, 2007). This response occurred in the presence of tolbutamide, a blocker of K_{ATP} channel. Current–voltage relations of this current depicted that ghrelin activated the outward Kv currents at the potentials positive to −30 mV. The ghrelin enhancement of Kv currents was blunted in the entire range of potentials in β-cells treated with PTX (Dezaki et al., 2007). These results indicate that PTX-sensitive G-proteins are required for ghrelin activation of Kv currents. This enhancing effect was blunted when 10 mM tetraethylammonium (TEA), a non-selective blocker of delayed-rectifier K^+ channels, was administered during exposure to ghrelin. Furthermore, in the presence of TEA, the ability of ghrelin to inhibit glucose-induced $[Ca^{2+}]_i$ increase (Dezaki et al., 2004) and insulin release (Dezaki et al., 2007) was partially but significantly diminished, suggesting that the enhancement of Kv channel conductance is linked to insulinostatic action of ghrelin. Moreover, stromatoxin (0.1 μM), a specific blocker of the 2.1 subtype of Kv channels (Escoubas et al., 2002; Shiau et al., 2003), potentiated glucose-induced insulin release, and in the presence of stromatoxin ghrelin failed to attenuate glucose-induced insulin release (Dezaki et al., 2007). These results suggest that ghrelin inhibits $[Ca^{2+}]_i$ increases and insulin release partly via the enhancement of the TEA-sensitive Kv current, most likely passing through the stromatoxin-sensitive Kv2.1 channels. Kv2.1 is expressed at high levels in islets of various species (MacDonald et al., 2001, 2002), and immunohistochemical analysis shows expression of Kv2.1 specifically in β-cells of islets (Yan et al., 2004). Taken together, an increase in Kv2.1 channel conductance is linked to ghrelin-induced inhibition of insulin release as well as Ca^{2+} signaling.

Ghrelin did not potentiate the Kv currents in the presence of membrane-permeable cyclic AMP analogue dibutylyl-cyclic AMP, suggesting that ghrelin activation of Kv channels is mediated by cyclic AMP signaling pathway. It is known that members of the $G\alpha_i$-family are involved in transducing the information of inhibitory hormones and neurotransmitters to cyclic AMP productions (McDermott & Sharp, 1995; Seaquist et al., 1995; Urano et al., 2004), voltage-dependent Ca^{2+} channels (Keahey et al., 1989; Hsu et al., 1991) and insulin exocytosis (Sharp, 1996). Whether the ghrelin action also involves one or all of these processes is unknown and requires further studies. Possible differences in the coupling of G proteins to signaling pathways between ghrelin and other inhibitory hormones, norepinephrine and somatostatin, also remain to be clarified.

K_{ATP} channel is well known as a key molecule that determines resting membrane potentials and converts the glucose metabolism to the membrane excitation in β-cells (Aguilar-Bryan & Bryan, 1999; Tarasov et al., 2004). This channel, however, may not be the target for ghrelin signaling for the following reasons; membrane potentials which are mainly controlled by K_{ATP} channels were not significantly changed by ghrelin, and K_{ATP} channel currents at 8.3 mM glucose conditions were not altered by ghrelin application. (Dezaki et al., 2007). Therefore, the ghrelin-induced decrease in the action potential firing is caused largely, if not all, by activation of Kv channels. Ghrelin activates voltage-dependent Kv channels via PTX-sensitive mechanisms to cause a rapid repolarization and shortening of bursting action potentials, leading to attenuation of glucose-induced $[Ca^{2+}]_i$ increases and oscillations and thereby insulin secretion (Fig. 2).

4.4. Ghrelin uses G-protein $G\alpha_{i2}$ in β-cells

Expression study revealed that GHS-R is coupled to G_{11}-phospholipase C (PLC) signaling, leading to production of IP_3 and Ca^{2+} release from IP_3-sensitive stores (Howard et al., 1996). Synthetic GHSs and ghrelin evoke GH release via activation of PLC-mediated $[Ca^{2+}]_i$ increases in pituitary cells (Herrington & Hille, 1994; Bresson-Bepoldin & Dufy-Barbe, 1996; Glavaski-Joksimovic et al., 2002; Glavaski-Joksimovic et al., 2003; Malagon et al., 2003). However, ghrelin attenuation of insulin release appears not to be mediated by PLC signaling pathways, since PLC activation operated by muscarinic ACh receptor for parasympathetic nerve input (Ahrén, 2000; Gilon & Henquin, 2001) and GPR40 receptors specific for free fatty acids (Briscoe et al., 2003; Itoh et al., 2003) potentiates, but not attenuates, glucose-induced increases in $[Ca^{2+}]_i$ and insulin release in β-cells (Gilon & Henquin, 2001; Fujiwara et al., 2005; Gautam et al., 2006). Thus, ghrelin may elicit different signaling pathways in a tissue-specific manner, which may underlie the opposing effects of ghrelin on GH release and insulin release. Heterotrimeric G proteins composed of α, β and γ-subunits function as signal transducers that link the membrane receptor activation to intracellular effectors. PTX specifically ADP-ribosylates the α-subunit of G_i family of G-proteins ($G\alpha_i$) and thereby abolishes its ability to be linked to G-protein-coupled receptors. The effects of ghrelin on insulin release, Kv channels, and $[Ca^{2+}]_i$ were all blocked by PTX, suggesting that certain subtype(s) of PTX-sensitive G-proteins are crucial in the ghrelin signal transduction in β-cells. Treatment of primary cultured rat β-cells with antisense oligonucleotide (AS) specific for the $G\alpha_{i2}$ subunit of G-proteins markedly decreased $G\alpha_{i2}$ subunit expression (Dezaki et al., 2007). In the antisense-treated, but not control oligonucleotide-treated, β-cells, ghrelin failed to attenuate $[Ca^{2+}]_i$ responses to glucose (Dezaki et al., 2007). The inhibitory effect of ghrelin on $[Ca^{2+}]_i$ responses was unaltered in β-cells treated with ASs specific for $G\alpha_{i1}$ and $G\alpha_{i3}$ (Dezaki et al., 2007). Furthermore, in primary cultured β-cells treated with AS for $G\alpha_{i2}$, ghrelin failed to suppress glucose (8.3 mM)-induced insulin release, whereas significant suppression was observed in the control oligonucleotide-treated and untreated cells (Dezaki et al., 2007). These results indicate that $G\alpha_{i2}$-mediated signaling is crucial for the action of ghrelin to suppress glucose-induced $[Ca^{2+}]_i$ increase and insulin release and suggest that the ghrelin-induced attenuation of $[Ca^{2+}]_i$ increase is linked to that of insulin release (Fig. 2). The mediation by the G-protein $G\alpha_{i2}$ subtype is surprising in the light of the current concept that GHS-R signaling is mediated primarily by the G_{11} subtype

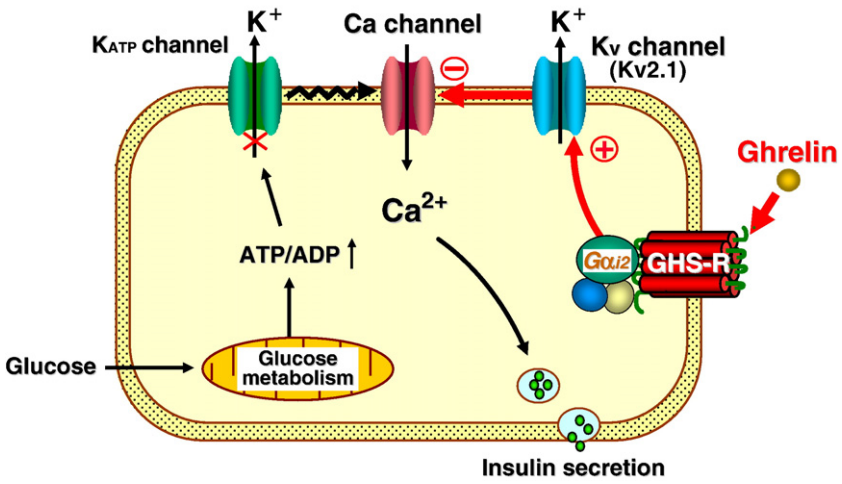

Fig. 2. Ghrelin signaling in islet β-cells. Closure of ATP-sensitive K^+ (K_{ATP}) channels by increases in ATP/ADP ratio following glucose metabolism induces membrane depolarization and increase in cytosolic Ca^{2+} concentrations via voltage-dependent Ca^{2+} channels, leading to insulin secretion in β-cells. Ghrelin activates β-cell GHS-R that is coupled with PTX-sensitive heterotrimeric G-protein $G\alpha_{i2}$, attenuates membrane excitability via activation of voltage-dependent K^+ channels (Kv2.1 subtype), and consequently suppresses Ca^{2+} influx and insulin release.

(Howard et al., 1996). However, our result is consistent with the reports that $G\alpha_{i2}$ is expressed in β-cells (Sharp, 1996; Rucha & Verspohl, 2005) while $G\alpha_{11}$ is expressed primarily in non-β-cells in islets (Astesano et al., 1999; Skoglund et al., 1999; Portela-Gomes & Abdel-Halim, 2002).

5. Systemic effects of ghrelin on the glucose metabolism

5.1. Exogenous ghrelin elevates plasma glucose and attenuates insulin levels

Systemic action of exogenous ghrelin to elevate blood glucose levels has been well documented in humans and rodents (Broglio et al., 2001, 2002, 2003a, 2003b; Dezaki et al., 2004). In mice fasted overnight, intraperitoneal (i.p.) administration of ghrelin at concentrations of 1 and 10 nmol/kg significantly elevated blood glucose levels at 30 min after administration (Dezaki et al., 2004). The hyperglycemic effect of ghrelin was completely blocked by simultaneous administration of GHS-R antagonist, [D-Lys^3]-GHRP-6. Desacyl-ghrelin failed to significantly alter blood glucose levels. These results indicate that ghrelin increases blood glucose via specific interaction with GHS-R. The following observations support that the ghrelin-induced hyperglycemia is neither caused by GH, a hyperglycemic hormone, nor by insulin resistance; ghrelin increased blood glucose in GH-deficient *little* mice and control wild mice in a similar manner, and in insulin tolerance test (ITT) i.p. injection of insulin lowered blood glucose levels in the ghrelin-administered and control mice in a similar manner. By contrast, when ghrelin at 1 and 10 nmol/kg was simultaneously injected with glucose in glucose tolerance test (GTT), the insulin responses were markedly attenuated and the glucose responses were larger in comparison to the control without ghrelin. Collectively, the hyperglycemic effect of ghrelin is neither due to the ability of ghrelin to release GH nor to induction of insulin resistance, but primarily caused by reduction of plasma insulin levels.

5.2. Endogenous ghrelin regulates plasma insulin and glucose levels

In fasted mice, i.p. administration of specific GHS-R antagonists, [D-Lys^3]-GHRP-6 and [D-Arg^1, D-Phe^5, D-$Trp^{7,9}$, Leu^{11}]-substance P (Asakawa et al., 2003), significantly reduced fasting blood glucose concentrations by 10–30 mg/ml at 30 and 60 min in a dose-dependent manner (Dezaki et al., 2004), suggesting that endogenous ghrelin is involved in the homeostatic regulation of fasting blood glucose. In ob/ob mice, a genetic model of obesity due to leptin-deficiency, chronic treatment with GHS-R antagonists reduced blood glucose and increased insulin levels (Asakawa et al., 2003). In GTT, when [D-Lys^3]-GHRP-6 was injected simultaneously with 1 g/kg glucose, increases in plasma glucose at 30 and 60 min were markedly attenuated compared to the corresponding values in saline injected control mice. Concomitantly, the insulin responses in GTT were markedly enhanced at 10 and 15 min (Dezaki et al., 2004). In ITT studies, i.p. injection of insulin lowered blood glucose levels similarly in the ghrelin receptor antagonist-injected and control mice (Dezaki et al., 2004). Esler et al. (2007) recently reported that oral administration of a novel small molecule GHS-R antagonist improved glucose tolerance in rats by stimulating insulin secretion without inducing hypoglycemia. This antagonist had no apparent effect on insulin sensitivity, confirming our data using [D-Lys^3]-GHRP-6.

5.3. Plasma insulin level is downregulated by endogenous ghrelin in gastrectomized rats

The results that glucose-induced insulin release from isolated islets and perfused pancreas was enhanced by ghrelin immunoneutralization and GHS-R antagonist suggested that ghrelin originating from pancreatic islets suppresses insulin release. As large as 70% of the circulating ghrelin originates from stomach, while the rest is derived from other tissues, including intestine and pancreas. The systemic insulinostatic function of endogenous ghrelin could be operated by the ghrelin derived from stomach and/or that derived from other tissues. Contribution of ghrelin from the stomach and other sources was assessed using gastrectomized (GX) rats lacking stomach-derived ghrelin. In GX rats, plasma concentrations of acylated-ghrelin were markedly reduced to 16% of control (5.2±0.7 fmol/ml in GX rats vs. 32.5±9.7 fmol/ml in normal rats), indicative of lack of stomach-derived ghrelin. The remaining levels of acylated-ghrelin may be derived substantially from the intestine, the second largest source of ghrelin (Hosoda et al., 2000; Gnanapavan et al., 2002). Although the circulating acylated-ghrelin was dramatically reduced in GX rats, i.p. injection of GHS-R antagonist [D-Lys^3]-GHRP-6 increased plasma insulin concentrations at 30 min in GX rats to a similar extent to that in normal rats (Dezaki et al., 2006). These results suggest that the effect of GHS-R antagonist is not due to antagonism of circulating stomach-derived ghrelin but primarily to blockade of local ghrelin including that in islets. This finding suggests that the ghrelin produced by pancreas serves as a local regulator of insulin release, although it might not contribute to the level of circulating ghrelin.

5.4. Ghrelin decreases plasma insulin concentrations in a PTX-sensitive manner

Intraperitoneal administration of ghrelin (10 nmol/kg) decreased plasma insulin concentrations. Conversely, i.p. administration of a specific GHS-R antagonist, [D-Lys^3]-GHRP-6 (10 μmol/kg), significantly increased plasma insulin levels, reflecting counteraction of endogenous ghrelin activity to inhibit insulin release (Dezaki et al., 2007). On the other hand, ghrelin (10 nmol/kg i.p.) increased plasma GH concentrations, and this effect was completely blocked by treatment with a PLC inhibitor U-73122, but not with its inactive analogue U-73343. These effects are consistent with the current concept that the effects of ghrelin are mediated by G_{11}-PLC signaling (Howard et al., 1996). In contrast, the ghrelin action to inhibit insulin release was unaffected by the PLC inhibitor (Dezaki et al., 2007). The insulinostatic effects of both endogenous and exogenous ghrelin were blunted in rats treated with PTX, while ghrelin-induced GH release was unaffected. Thus, the G-protein coupling for the ghrelin action on insulin release is distinct from that on GH release.

To further confirm the PTX sensitivity of the insulinostatic function of ghrelin, we examined GTT studies in rats fasted overnight. In control experiments without PTX, ghrelin (10 nmol/kg i.p.) injected together with glucose (2 g/kg) markedly attenuated plasma insulin responses and enhanced blood glucose responses to GTT in comparison to control values without ghrelin. In rats treated with PTX (5 μg/kg), plasma insulin responses during glucose challenge were higher and blood glucose responses were lower than those in control rats, and the actions of ghrelin to attenuate insulin responses and to enhance glucose responses were not observed (Dezaki et al., 2007).

6. Glucose metabolisms in ghrelin knockout mice

6.1. Increased plasma insulin and decreased blood glucose levels in ghrelin-KO mice

The effects of GHS-R antagonist and anti-ghrelin antiserum in the perfused pancreas, isolated islets, and the systemic insulin levels most likely result from counteraction of the action of endogenous ghrelin. To further confirm this hypothesis, ghrelin-knockout (Ghr-KO) mice were studied. In Ghr-KO mice, plasma ghrelin levels were undetectable. When fed standard chow, no significant differences between male Ghr-KO and wild-type (C57BL/6J) mice were observed at 8 weeks of age in body weights (23.4±0.7 g in Ghr-KO vs. 23.5±0.3 g in wild-type), total 24-hr food intake (3.51±0.14 g in Ghr-KO vs. 3.54±0.04 g in wild-type), and blood glucose levels in fed states (120±3.1 mg/dl in Ghr-KO vs. 127±6.0 mg/dl in wild-type), confirming previous reports on Ghr-KO mice (Sun et al., 2003; Wortley et al., 2004, 2005; De Smet et al., 2006). Behind these observations, a yet-unknown compensatory mechanism could have occurred in the knockout mice to maintain homeostatic regulation of insulin release and blood glucose levels as well as feeding behavior, since these are the life-saving functions. In GTT, however, Ghr-KO mice exhibited markedly enhanced insulin responses and attenuated glucose responses (Dezaki et al., 2006; Sun et al., 2006). The profiles of ITT exhibited little differences (Dezaki et al., 2006) or slight changes (Sun et al., 2006) between Ghr-KO and wild-type mice. Thus, the suppressed glycemic responses to GTT in Ghr-KO mice primarily result from enhanced insulin secretion, though possible additional effects of ghrelin on glucose production (Gauna et al., 2005) or insulin sensitivity (Heijboer et al., 2006; Sun et al., 2006) cannot be disregarded.

6.2. Increased insulin release in isolated islets of ghrelin-KO mice

Morphological analysis of pancreatic sections showed that the density and average size of islets were not significantly different between wild-type and Ghr-KO mice (Dezaki et al., 2006). The number and size of isolated islets obtained by collagenase digestion were not altered in Ghr-KO mice. Glucose (8.3 and 16.7 mM)-induced insulin release from isolated islets of Ghr-KO mice was significantly greater than that of wild-type mice, while basal levels of insulin release at 2.8 mM glucose were not altered. No difference was observed between Ghr-KO and wild-type mice in insulin content per islet, mRNA expression of insulin 1, and that of insulin 2 (Dezaki et al., 2006). Collectively, the larger amount of insulin release in islets of Ghr-KO mice results from greater insulin secretory response to glucose, while insulin production is unaltered.

The enhancement of glucose-induced insulin release from islets of Ghr-KO mice was undetectable after treatment with PTX (Dezaki et al., 2007), indicating that endogenous ghrelin attenuates insulin release via PTX-sensitive G proteins. PTX, an inhibitor of $G_{i/o}$ subtypes of trimeric G-proteins, was formally recognized as the islet-activating protein (IAP), because this agent increased insulin release from isolated islets (Katada & Ui, 1979). The effect of IAP has suggested the presence of intra-islet substance that activates $G_{i/o}$-protein-coupled receptors to decrease insulin release. However, the endogenous islet substance whose insulinostatic action is antagonized by PTX/IAP is as-yet unidentified. The insulinostatic effect of endogenous ghrelin in islets, as evidenced by enhanced insulin release due to ghrelin gene-knockout and GHS-R antagonist, was blunted by PTX treatment. The enhancement of insulin release by ghrelin gene-knockout or GHS-R antagonist was as large as 70–80% of the enhancement by PTX/IAP treatment (Dezaki et al., 2007). These findings indicate that a large part, but not all, of the PTX/IAP effect is exerted by blocking the action of endogenous ghrelin in islets. The apparently ghrelin-independent portion of the PTX/IAP effect could be due to inhibition of the signaling of somatostatin, an islet hormone that is released from δ-cells and inhibits insulin release via PTX-sensitive G-proteins (Sharp, 1996), although a paracrine role for somatostatin in islets has long been controversial (Samols & Stagner, 1990).

7. Effects of ghrelin gene-related peptides on the glucose homeostasis

7.1. Desacyl-ghrelin

The non-acylated form of ghrelin, desacyl-ghrelin, also exists at significant levels in pancreatic blood flow (Dezaki et al., 2006) as well as in systemic blood (Hosoda et al., 2000). In blood, desacyl-ghrelin circulates in amounts far greater than acylated ghrelin. The *n*-octanoyl group at serine 3 of the ghrelin molecule seems to be essential for the hormone's binding and bioactivity, at least in terms of endocrine actions, because the unacylated form of ghrelin does not activate the GHS-R (Kojima et al., 1999; Bednarek et al., 2000; Matsumoto et al., 2001) and is devoid of any endocrine activity including that on plasma insulin (Kojima et al., 1999, 2001; Broglio et al., 2003b). Desacyl-ghrelin did not significantly alter glucose-induced insulin release in perfused rat pancreas (Dezaki et al., 2006) and isolated rat islets (Esler et al., 2007), demonstrating that desacyl-ghrelin does not have any direct effect on the β-cell insulin release. On the other hand, desacyl-ghrelin has been reported to affect glucose homeostasis directly or indirectly. Administration of desacyl-ghrelin could totally block the hyperglycemic effects of bolus injection of ghrelin in normal subjects (Broglio et al., 2004). In isolated hepatocytes from the pig, ghrelin stimulates but desacyl-ghrelin inhibits glucose output, and desacyl-ghrelin counteracts the stimulatory effect of ghrelin on glucose output (Gauna et al., 2005). By contrast, hyperinsulinaemic-euglycaemic clamp studies in mice showed that desacyl-ghrelin, as well as ghrelin, blocked the action of insulin on endogenous glucose production (Heijboer et al., 2006). In rat adipocytes, ghrelin, but not desacyl-ghrelin, stimulates insulin-induced glucose uptake (Patel et al., 2006). Desacyl-ghrelin, as well as ghrelin inhibits isoproterenol-induced

lipolysis in rat adipocytes (Muccioli et al., 2004), and promotes adipogenesis (Thompson et al., 2004). These data suggest that desacyl-ghrelin may independently work as a hormone, or modulate ghrelin actions on glucose handling in the tissue-specific manner. Further studies are required to establish the roles of ghrelin and desacyl-ghrelin in the regulation of energy balance. Moreover, the receptor(s) mediating the metabolic signals by desacyl-ghrelin remains to be identified.

7.2. Obestatin

Obestatin is a recently identified 23 amino-acid peptide, which is derived from the preproghrelin gene (Zhang et al., 2005). It was reported that in rodents obestatin reduced food intake, body weight and jejunal contraction, the actions opposite to those of ghrelin (Zhang et al., 2005). Obestatin also reduces fluid intake by acting on the thirst center in the brain (Zhang et al., 2005). The G-protein coupled receptor GPR39 (McKee et al., 1997) was proposed to be the receptor for obestatin (Zhang et al., 2005), but this has not been confirmed by other studies (Lauwers et al., 2006; Holst et al., 2007). This receptor is present in many regions of the brain and peripheral tissues including pancreas (McKee et al., 1997; Zhang et al., 2005; Chanoine et al., 2006; Moechars et al., 2006). Furthermore, obestatin immunoreactivity positively correlated with insulin concentrations (Chanoine et al., 2006). Green et al. (2007) failed to find any effects of this peptide on the glucose homeostasis. Obestatin induced no alterations in plasma glucose or insulin responses to GTT in mice. In addition, obestatin peptides had no effect on insulin sensitivity when examined in ITT (Green et al., 2007).

8. Ghrelin as potential therapeutic target for type 2 diabetes

8.1. Ghrelin-KO counteracts glucose intolerance in high-fat diet-fed and ob/ob mice

The enhanced insulin and suppressed glycemic responses to GTT in Ghr-KO mice could be beneficial under conditions of increased demand for insulin. When wild-type and Ghr-KO mice were fed high-fat diet (HFD) for 4 weeks, both mouse lines developed moderate increases in body weight to a similar extent (Dezaki et al., 2006). In an apparent controversy, it was reported that another line of Ghr-KO mice were protected from a rapid weight gain during post-weaning exposure to HFD for 3 weeks, which was associated with decreased adiposity, increased energy expenditure and increased locomotor activity as compared to wild-type mice (Wortley et al., 2005). In our study, HFD treatment significantly increased blood glucose levels in wild-type mice but not in Ghr-KO mice (Dezaki et al., 2006). HFD treatment increased plasma insulin levels in wild-type mice, and this increment was much greater in Ghr-KO. Thus, Ghr-KO mice displayed a phenotype of enhanced insulin release and nearly normal glycemia under HFD conditions. This phenotype was even more prominent in GTT as follows. In wild-type mice, increases in blood glucose levels were exaggerated in HFD group compared to control diet group, exhibiting HFD-induced glucose intolerance. Although insulin response to GTT also tended to be enhanced in HFD group, the change was not statistically significant (Dezaki et al., 2006). In Ghr-KO mice, in contrast, increases in blood glucose levels in HFD group were not significantly different from those of control diet group, and insulin response was markedly enhanced in HFD group (Dezaki et al., 2006). Thus, ghrelin-deficiency promoted insulin release and prevented glucose intolerance in a HFD-induced obese model.

Sun et al. (2006) have recently reported that in ob/ob mice, a genetic model of obesity due to leptin-deficiency, ablation of ghrelin in ob/ob mice augmented insulin release and thereby markedly reduced hyperglycemia. Thus, the ghrelin blockade counteracts the obesity-associated glucose intolerance in two different obese models. As the underlying mechanism, we propose that lack of ghrelin and its insulinostatic activity increase the maximal capacity of glucose-induced insulin release and enable islets to secrete more insulin to meet an increased demand associated with obesity, thereby achieving normoglycemia (Fig. 3).

8.2. Chronic effects of ghrelin and its inhibition

Doi et al. (2006) have identified IA-2β as a ghrelin-induced gene by PCR-select subtraction method. IA-2β is a β-cell autoantigen for

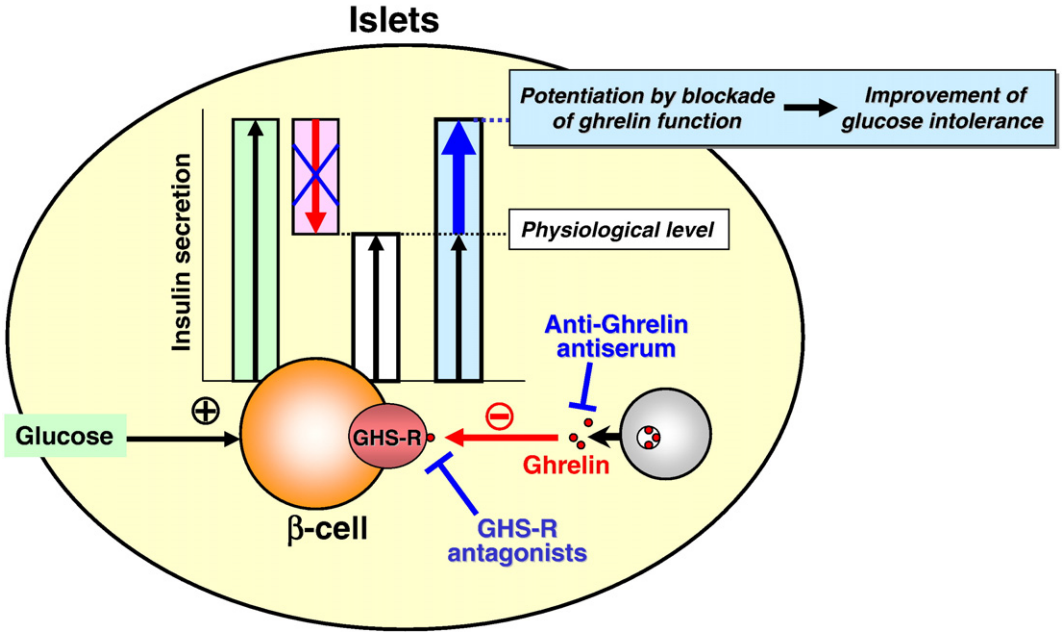

Fig. 3. Insulinostatic function of endogenous ghrelin in islets as therapeutic target for type 2 diabetes. The ghrelin of islet origin interacts with β-cell GHS-R in a paracrine/autocrine manner and attenuates glucose-induced insulin release, determining the physiological level of insulin release. Under conditions in which the systemic demand for insulin exceeds the physiological range, such as early stages of diet-induced obesity and/or insulin resistance, blockade of ghrelin function by GHS-R antagonists and anti-ghrelin antiserum can promote insulin secretion and improve glucose intolerance, providing a potential therapeutic avenue to counteract the progression of type 2 diabetes.

type 1 diabetes, also called phogrin, IAR, PTP-NP or ICAAR, which is an integral membrane glycoprotein that is widely expressed among neuroendocrine tissues, where it localizes to secretory granules (Chiang & Flanagan, 1996; Cui et al., 1996; Lu et al., 1996; Smith et al., 1996; Wasmeier & Hutton, 1996). IA-2β is a member of the receptor-type protein tyrosine phosphatase family. However, its phosphatase activity is inactive because of mutations at critical sites in the protein tyrosine phosphatase core domain, and site-directed mutagenesis can restore enzyme activity (Magistrelli et al., 1996; Drake et al., 2003). It was shown that inhibition of IA-2β expression by the RNA interference technique ameliorated ghrelin's inhibitory effects on glucose-stimulated insulin secretion in MIN6 insulinoma cells (Doi et al., 2006). Another interesting finding is that in ob/ob mice, ablation of ghrelin reduces the expression of uncoupling protein 2 (UCP2) (Sun et al., 2006). UCPs function to decrease metabolic efficiency by dissociating substrate oxidation in the mitochondrion from ATP synthesis. This is thought to be accomplished by promoting net translocation of protons from the intermembrane space, across the inner mitochondrial membrane to the matrix, thereby dissipating the potential energy available for conversion of ADP to ATP despite continued oxidation of fuels. This uncoupling effect of UCP2 then negatively regulates glucose-induced insulin release in β-cells (Chan et al., 1999, 2001; Hong et al., 2001; Zhang et al., 2001; Joseph et al., 2002). Therefore, it is likely that ghrelin inhibits insulin release via two modes of action in β-cells: it acutely activates Kv channels and suppresses Ca^{2+} signaling, while chronically it may also upregulate UCP2 and IA-2β. In ob/ob mice, the increment of insulin release due to ghrelin-KO is remarkably large (Sun et al., 2006), which could be due to that UCP2 is upregulated in ob/ob mice β-cells and therefore the magnitude of its inhibition by ghrelin-KO is greater (Zhang et al., 2001). In fact, upregulation of UCP2 in the hypothalamic feeding center of ob/ob mice has recently been documented (Parton et al., 2007). Moreover, the enhanced ghrelin action in ob/ob mice, assessed by the effect of ghrelin-deficiency, could be due to the lack of leptin (Zhang et al., 1994), since leptin and ghrelin are considered mutual antagonists. To support it, plasma levels of ghrelin and leptin are inversely correlated (Cummings & Foster, 2003). Moreover, leptin counteracts the effects of ghrelin in several systems including the regulation of feeding and the neuropeptide Y neuron activity in the hypothalamic arcuate nucleus (Kohno et al., 2003, 2007). It is worth noting that ghrelin, contrary to leptin, has several actions that could promote metabolic syndrome; it inhibits insulin release and elevates glycemia, stimulates feeding, and increases adiposity (Tschop et al., 2000). Therefore, suppression of these ghrelin actions could potentially counteract diabetes, hyperphagia and obesity simultaneously, thereby acting as anti-metabolic syndrome. However, it should be kept in mind that ghrelin also stimulates GH release (Kojima et al., 1999), a factor that decreases the fat and increases the muscle mass. The interplay between ghrelin and adpocytokines, such as leptin and adiponectin, and the impact of suppression of ghrelin — GHS-R system in the regulation of metabolism remain to be further elucidated.

9. Conclusion

The notion that the islet-derived ghrelin plays a pivotal role in the regulation of insulin release at least in rodents is supported by the following findings. (1) mRNAs and proteins for ghrelin and GHS-R are expressed in pancreatic islets. (2) The ghrelin level is higher in the pancreatic vein than in the artery, indicative of release of ghrelin from pancreas. (3) Ghrelin immunoneutralization and GHS-R antagonists augment glucose-induced insulin release from perfused pancreas and isolated islets. (4) Ghrelin directly acts on islet β-cells to inhibit glucose-induced membrane excitability, $[Ca^{2+}]_i$ increase and insulin release. (5) In gastrectomized rats with remarkably reduced plasma ghrelin level, systemic administration of GHS-R antagonist increases plasma insulin concentration and the increment was similar to that observed in normal rats. (6) Glucose-induced insulin release from isolated islets of Ghr-KO mice was greater than that of wild-type mice. Thus, pharmacological, immunological, and genetic blockade of ghrelin or ghrelin action in pancreatic islets all markedly enhanced glucose-induced insulin release. These findings support the hypothesis that ghrelin is produced and released from pancreatic islet cells and act on islet β-cells via autocrine and/or paracrine manner, thereby suppressing glucose-induced insulin release. This function of ghrelin in regulating glucose metabolism, together with inducing GH release and feeding, suggests that ghrelin underlies the integrative regulation of energy homeostasis. Under conditions in which the systemic demand for insulin exceeds the physiological range, such as early stages of diet-induced obesity and/or insulin resistance, antagonism of ghrelin function can promote insulin secretion and prevent glucose intolerance, providing a potential therapeutic avenue to counteract the progression of type 2 diabetes. As predicted, oral administration of a novel GHS-R antagonist improved glucose tolerance in GTT in HFD rats as well as lean rats (Esler et al., 2007). As a single oral administration of the antagonist in fasted animals was sufficient to improve glucose tolerance, GHS-R antagonists appear to have a direct impact on glucose homeostasis independent of the potential additional benefits that may arise from chronic dosing (Esler et al., 2007).

It is of particular importance to clarify how ghrelin and its receptor in islets are regulated under physiological and pathological conditions, including fast/fed, lean/obese and normoglycemic/diabetic states. Precise and relative roles of islet-derived and stomach-derived ghrelin in multiple steps of glucose metabolism remain to be further clarified. Although ghrelin employs unique molecules of $G\alpha_{i2}$ and Kv, its signaling mechanisms in islet β-cells remain to be further elucidated. Chronic effects of both augmentation and suppression of the ghrelin–ghrelin receptor system on islet β-cells and glucose metabolism require further studies.

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