

Implications of ghrelin and hexarelin in diabetes and diabetes-associated heart diseases

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Received: 3 September 2014 / Accepted: 12 January 2015 / Published online: 4 February 2015
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Abstract Ghrelin and its synthetic analog hexarelin are specific ligands of growth hormone secretagogue (GHS) receptor. GHS have strong growth hormone-releasing effect and other neuroendocrine activities such as stimulatory effects on prolactin and adrenocorticotrophic hormone secretion. Recently, several studies have reported other beneficial functions of GHS that are independent of GH. Ghrelin and hexarelin, for examples, have been shown to exert GH-independent cardiovascular activity. Hexarelin has been reported to regulate peroxisome proliferator-activated receptor gamma (PPAR- γ) in macrophages and adipocytes. PPAR- γ is an important regulator of adipogenesis, lipid metabolism, and insulin sensitization. Ghrelin also shows protective effects on beta cells against lipotoxicity through activation of phosphatidylinositol-3 kinase/protein kinase B, c-Jun N-terminal kinase (JNK) inhibition, and nuclear exclusion of forkhead box protein O1. Acylated ghrelin (AG) and unacylated ghrelin (UAG) administration reduces glucose levels and increases insulin-producing beta cell number, and insulin secretion in pancreatectomized rats and in newborn rats treated with streptozotocin, suggesting a possible role of GHS in pancreatic regeneration. Therefore, the discovery of GHS has opened many new perspectives in endocrine, metabolic,

and cardiovascular research areas, suggesting the possible therapeutic application in diabetes and diabetic complications especially diabetic cardiomyopathy. Here, we review the physiological roles of ghrelin and hexarelin in the protection and regeneration of beta cells and their roles in the regulation of insulin release, glucose, and fat metabolism and present their potential therapeutic effects in the treatment of diabetes and diabetic-associated heart diseases.

Keywords Growth hormone secretagogues · Beta cell apoptosis · PPAR- γ and diabetic cardiomyopathy

Introduction

Diabetes mellitus is one of the leading causes of morbidity and mortality worldwide. Currently, about 382 million people suffer from this disease and by 2035, this will rise to 592 million [1]. Diabetes is classified as type 1 diabetes (T1D), characterized by absolute loss of insulin production, and type 2 diabetes (T2D), which is a result of both chronic insulin resistance and loss of pancreatic cell mass and function [2]. T2D is the most common form, accounting for about 95 % of diabetes [3]. The pathogenesis of T2D involves both genetic and environmental factors. Obesity is the most common cause of developing T2D as it represents about 75 % of the causes [4]. Obesity can lead to insulin resistance and impairment in energy metabolism of most tissues such as skeletal muscles, liver, adipose tissue, and pancreatic islets. However, insulin resistance does not lead to T2D unless it is accompanied by pancreatic beta cell dysfunction [5–7].

What are still unknown are the time points when beta cell dysfunction starts and the relative contribution of beta cell dysfunction in the development of T2D. Current evidence suggested that islet function was about 50 % of

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normal at the time of diagnosis [8]. The reduction of beta cell function most probably starts early about 10–12 years before diagnosis of diabetes [9]. The major factors for causing progressive decrease in beta cell structure and function during the course of the disease are glucotoxicity, lipotoxicity, proinflammatory cytokines, and islet cell amyloidosis [10]. The combined increased flux of free fatty acids and glucose into the beta cell has detrimental consequences on beta cells [11]. The excess free fatty acid entering beta cells inhibits proper glucose utilization in the mitochondria. Additionally, it is reported that these lipids are metabolized in a different way forming lipid intermediates that cause abnormal signaling and beta cell dysfunction. The glucotoxicity and lipotoxicity also may cause an increase in reactive oxygen species (ROS) which can damage the cell and then lead to beta cell death [12]. The role of the proinflammatory cytokines in causing beta cell apoptosis had been demonstrated [13, 14]. Macrophages may infiltrate beta cells when they start becoming damaged. This causes cytokine release from the macrophages, which may alter the ability of the beta cell to proliferate. Impaired beta cell function and mass could be reversible at early stages of the disease [15].

Insulin and its downstream signaling pathway play important roles in the homeostasis of blood levels of glucose, and the disruption of the signaling pathway plays an important role in the pathophysiology of T2D. Insulin reduces blood glucose levels through increasing glucose uptake in muscle and fat and decreasing hepatic glucose production. Skeletal muscle consumes most glucose, accounting for about 75 % of insulin-dependent glucose uptake while adipose tissue accounts for only a small fraction [16]. The adipose tissue, however, is crucial in the normal regulation of the insulin action all over the body. Adipocytes can store excess lipids in obesity but when they become saturated, lipids begin to accumulate inside other organs, and tissues making them insulin resistant. Adipocytes also can produce adipokines such as leptin and adiponectin which have been proved as insulin sensitizers due to their ability to decrease triglycerides (TG) synthesis, to stimulate beta oxidation of fatty acids, and thus to enhance insulin action in both skeletal muscle and liver [17–19].

Genetically modified animals deficient in white adipose tissue usually have severe insulin resistance in liver and muscle [20]. Transplantation of normal fat tissue into white adipose tissue deficient mice restores the insulin sensitivity [21]. Mice with a knockout of the insulin receptor in muscle have normal glucose tolerance [22], whereas those with a knockout of the insulin-sensitive GLUT4 glucose transporter in adipose tissue have impaired glucose tolerance, apparently due to insulin resistance being induced in muscle and liver [23]. Other recent Studies reported that obesity promotes inflammatory signals especially in the

adipose tissues that disrupt insulin action and mediate insulin resistance [24]. Accordingly, the therapeutic targets of T2D will be preservation of beta cell structure and function, enhancing metabolic activity especially of the adipose tissues and decreasing lipid content in different tissues. In this review, we will focus on the possible roles of ghrelin, a natural ligand of the growth hormone secretagogue receptor type 1a (GHS-R1a), and hexarelin, a synthetic peptidyl GHS in the treatment of diabetes and diabetic-associated heart diseases.

Growth Hormone Secretagogues (GHS)

GHS are substances which stimulate the release of GH from the pituitary. They include the natural endogenous one, ghrelin, which is predominantly produced by A-like cells of the stomach from its precursor proghrelin [25] and synthetic peptidyl and non-peptidyl molecules. The ghrelin peptide is acylated to acylated ghrelin (AG) by the enzyme ghrelin-*O*-acyltransferase (GOAT) [26]. AG is present in serum at a 2.5-fold lower concentration than unacylated ghrelin (UAG) [27]. UAG has been thought for a long time to be an inactive metabolite of AG [25, 27]. However, it is now recognized that UAG may exert physiologically relevant effects through an unidentified receptor. UAG can antagonize the effect of AG [28, 29]. However, in some cases UAG acts synergistically with AG [30] or have AG-independent effects [31]. UAG seems to oppose the effect of AG at the metabolic levels such as insulin secretion and food intake but cannot counteract it at the neuroendocrine levels like GH release, prolactin or adrenocorticotrophic response [27]. Most of the effects of AG appear to be mediated through activation of GHS-R1a, as the specific receptor antagonist, [D-Lys3]-GHRP-6 completely blocks the protective effects of ghrelin against oxygen–glucose deprivation insult [32]. In contrast, Baldanzi et al. reported that in cardiomyocytes ghrelin exhibits an antiapoptotic effect through binding to a novel, unidentified receptor that is distinct from GHS-R1a [33].

The GHS-R is a G protein-coupled seven-transmembrane domain receptor and was initially identified as a receptor for small synthetic molecules GHS, such as hexarelin, L-692,429, GHRP-6, and MK-0677, all of which stimulate GH secretion from the pituitary [34, 35]. The GHS-R has two forms GHS-R1a and GHS-R1b. The GHS-R1a is the functionally active and signal transducing form of the GHS-R, whereas the GHS-R1b is devoid of high-affinity ligand-binding and signal transduction activities [36]. Expression of the GHS-R1a receptor was shown in the hypothalamus and anterior pituitary gland which is consistent with its role in regulating GH release [35, 37]. GHS-R1a receptor is also expressed in many peripheral organs. GHS-R1a receptor mRNA was shown in the

stomach and intestine [38], pancreas [34], kidney [39], heart, and aorta [40], as well as in different human pituitary adenoma and various endocrine neoplasms of different organs [41–43]. These findings indicate that ghrelin and synthetic GHS have many functions other than the control of GH release. Ghrelin and synthetic GHS are potent stimulators of GH release [44–46]. However, the activity of both ghrelin and synthetic GHS is not fully specific for GH and includes stimulatory effect on both lactotroph and corticotroph secretion [46, 47]. Ghrelin enhances Appetite and increases food intake in humans [48] and animals [49] either injected centrally [50, 51] or peripherally [48, 52]. Ghrelin has been shown to have many protective effects on the cardiovascular system [53–56]. Ghrelin also has been proved to be a potent anti-inflammatory mediator both in vitro and in vivo in lymphocytes, monocytes, and dendritic cells and promoting IL-10 expression and cell migration [57].

The synthetic, peptide GHS are also known as growth hormone-releasing peptides (GHRPs). GHRP-6 was the first hexapeptide shown to release GH in vivo, especially in humans after oral administration with low bioavailability and short-lasting effect [58, 59]. Recently, heptapeptide, GHRP-1, and two other hexapeptides, hexarelin and GHRP-2, have been now synthesized [60, 61]. Hexarelin (His-D-2MeTrp-Ala-Trp-D-Phe-Lys-NH₂) differs from growth hormone-releasing peptides GHRP-6 by having a methyl group in position 2 of the DTrp [62]. This minor modification was beneficial, making hexarelin more stable and longer acting in vivo [63]. Hexarelin is more potent than GHRP-6 as a GH releaser [64–66]. Hexarelin has many physiological actions independent of growth hormone such as the protection against cardiac ischemia and impairment of vascular endothelium function in the hearts of GH-deficient rats [67, 68]. GHS-R1a seems to mediate the action of hexarelin. However, some studies have reported that the CD36 receptor mediates the action of hexarelin in the heart and mouse 3T3-L1 preadipocytes [69, 70]. Our review will concern on the functions of ghrelin and hexarelin in the protection, regeneration of beta cells, regulation of insulin release, glucose metabolism, and their possible uses in treatment of diabetes and heart diseases.

Effects of ghrelin on pancreatic beta cells

Ghrelin has been shown to promote survival and inhibit apoptosis in many cell types. Ghrelin protected human endothelial cells against apoptosis caused by high glucose [71]. Treatment of 3T3-L1 preadipocytes with ghrelin inhibited adipocyte apoptosis induced by serum deprivation, induced cellular proliferation and differentiation to

mature adipocytes. Ghrelin also increased the basal and insulin-stimulated glucose transport in these adipocytes [72]. Ghrelin has been confirmed to have protective effects against different stressful factors including TNF-alpha [73] and doxorubicin [33]. Hexarelin is able to both increase cell proliferation and protect adult rat hippocampal progenitor (AHP) cells from apoptosis and necrosis induced by growth factor deprivation [74]. Interestingly, ghrelin is recently shown to protect beta cells from apoptotic effect of saturated fatty acids [75]. It is obvious in many studies that prolonged exposure of isolated islets or beta cell lines to saturated fatty acids is associated with beta cell apoptosis [76–78]. The signaling pathways that regulate pancreatic beta cell apoptosis during lipotoxicity or the oxidative stress could be inactivation of phosphatidylinositol-3 kinase/protein kinase B (PI3K/AKT), sustained c-Jun N-terminal kinase (JNK) activation, phosphorylation of forkhead box protein O1 (Foxo1) at serine and threonine sites distinct from those phosphorylated by AKT, enhancing the nuclear retention of Foxo1 [79, 80]. However, it is unclear whether inhibition of AKT or activation of JNK precedes inhibition of Foxo1 nuclear translocation or if there is a crosstalk between PI3K/AKT activation and JNK inhibition. Therefore, further studies are needed to clarify the mechanisms of these signaling molecules in the protection of beta cells from apoptosis. Ghrelin has been shown to exert the antiapoptotic effects on beta cells via G protein-coupled receptor/CAMP/PKA, activation of PI3K/AKT, and extracellular signal-regulated kinase (ERK) [81, 82] (See Fig. 1). Wang et al. has demonstrated that ghrelin inhibits the apoptosis and promotes beta cell proliferation through nuclear exclusion of Foxo1 and inhibition of endoplasmic reticulum (ER) stress pathway [83].

AG increased insulin expression and secretion and PDX1 mRNA in the pancreas of STZ-treated rat [84]. PDX1 is a transcriptional factor which is essential for insulin transcription and maintenance of beta cell mass [85]. Similarly, UAG and obstatin, which is encoded in the same gene as ghrelin, increased islet area, islet number, and beta cell mass and insulin and PDX1 mRNA compared to the pancreas of STZ-treated rat and also increased the expression of antiapoptotic gene, BCL-2 [86]. Both UAG and AG induced cell survival and protection against apoptosis in isolated human islets of Langerhans [82, 87]. The insulin-positive beta cells in these islets expressed GHS-R1a, explaining the mechanism of action for AG. However, the radiolabelled binding studies for UAG suggest that these islets also express UAG and AG binding sites that are probably not the classical GHS-R1a [82]. Therefore, the effects of UAG and AG may also occur via mechanisms independent of the GHS-R1a, likely mediated by specific AG and UAG binding sites. Taken together, these findings indicate that the binding sites of AG and

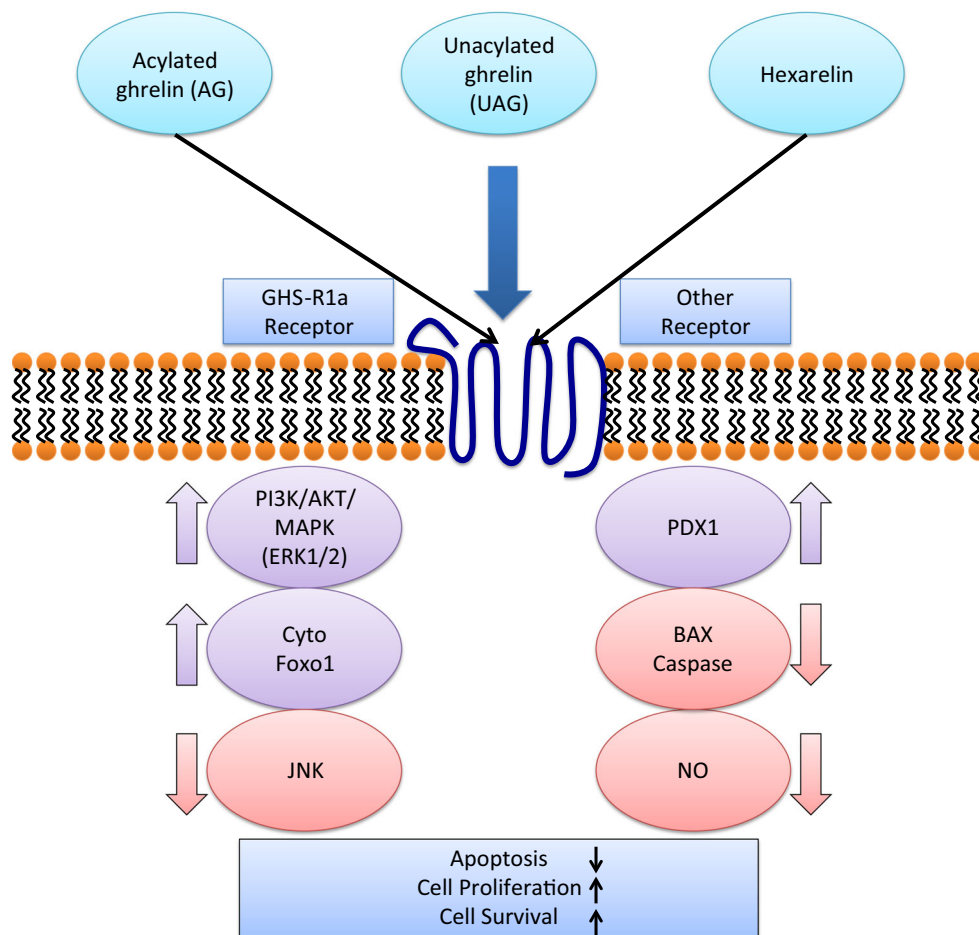


Fig. 1 The antiapoptotic, proliferative, and survival effects of AG, UAG, and hexarelin on beta cells. AG, UAG, and hexarelin bind to GHS-R1a and/or unknown other receptor, which are involved in the activation of PI3K/AKT and MAPK (ERK1/2) pathways, JNK inhibition and nuclear extrusion of Foxo1 through activation of the GHS-R1a or other receptor. Ghrelin and hexarelin increase PDX1 mRNA, which is essential for insulin transcription and maintenance of beta cell mass. Furthermore, AG and UAG decrease NO, downregulated active caspase-9 expression, and proapoptotic protein

BAX, which have been associated with beta cell dysfunction and death. All these effects contribute to reduced apoptosis, increased beta cell, and islet cell proliferation and survival (*GHS-R1a* GH secretagogue receptor type 1a, *PI3K* phosphatidylinositol 3-kinase, *MAPK* Mitogen-activated protein kinases (MAPK), *ERK1/2* extracellular signal-regulated kinase 1/2, *NO* nitric oxide, *PDX1* pancreatic and duodenal homeobox-1, *JNK* Jun-N-terminal kinase, *Foxo1* Forkhead transcription factors of the FoxO family)

UAG are different or may be some GHS-R subtypes could recognize and bind ghrelin independently of its acylation. However, the downstream signaling mechanisms in protecting beta cells may be the same for both peptides via the activation of PI3K/AKT and extracellular signal-regulated kinase (ERK) (as shown in Fig. 1).

Moreover, some studies have reported the potential roles of ghrelin in the regeneration of beta cells. Beta cell regeneration is an alternative strategy to replace islets transplantation especially with the shortage of pancreatic donors [88]. Pancreatic regeneration requires expansion of beta cells through beta cell neogenesis, dedifferentiation of acinar cell to beta cells, and proliferation of progenitor beta cells [89, 90]. Various agents have been studied to increase pancreatic regenerative capacity; the most important ones

are glucagon-like peptide 1, actin, members of the transforming growth factor beta family, and the polypeptide growth factor beta [91–93]. Interestingly, ghrelin has been reported to influence the embryologic development of the pancreas and to regulate insulin secretion [94, 95]. Rats received 90 % pancreatectomy (surgical removal of 90 % of pancreas) were shown to have severe hyperglycemia and decreased beta cell mass and insulin levels. AG administration strongly reduced glucose levels, increased insulin-producing beta cell number, and insulin secretion. On the contrary, ghrelin receptor antagonist [D-Lys3]-GHRP-6 administration in these pancreatectomized rats worsens glucose levels and decreases beta cell mass [96]. AG, UAG, and obstatin counteracted the hyperglycaemia and improved plasma and pancreatic insulin levels, which were

reduced by the STZ compound in newborn rats treated with STZ [84]. UAG and Obstatin increased islet area, islet number, and beta cell mass with respect to STZ treatment alone [86]. These findings strongly suggest that beta cells damaged by STZ administration during the neonatal stage or reduced by pancreatectomy can be regenerated or replicated following ghrelin treatment. This ability of GHS to regenerate beta cells may be supported by previous studies that have shown the regeneration effect of GH itself on the pancreas [97]. In conclusion, these findings provide evidence that GHS may function as a survival and regenerative factor for beta cells and offer a new perspective on the potential role of these peptides in diabetes.

Effects of ghrelin and hexarelin on insulin secretion and glucose homeostasis

The role of the ghrelin in the regulation of insulin secretion and insulin action remains a controversial topic. Ghrelin is reported to either inhibit or stimulate insulin secretion in animals (as shown in Table 1) and humans (as shown in Table 2). The discrepancies of ghrelin effects in different studies may be due to the following reasons: (1) the implications of ghrelin on insulin secretion and glucose metabolism could be different in different energy states like fasting, fed, obese, and diabetic, (2) Ghrelin, GHS-R, and GOAT are all expressed in pancreatic islets, suggesting a role for locally expressed ghrelin that could be different from that of the systemic ghrelin in the regulation of insulin release, (3) some of effects of ghrelin are independent of GHS-R1a and this highlight the complexity of the ghrelin-signaling pathway. This can be indicated by the paradoxical effects of ghrelin ablation and GHS-R ablation in ob/ob mice [98], (4) the controversy could be also due to differences in experimental design (different doses, different times of observation, mode of injection, in vitro versus in vivo experiments, etc.). This is obvious in one study that tested the effect of different concentration of ghrelin on insulin release. The study showed that ghrelin concentrations in the physiological range had no effect on glucose-stimulated insulin secretion (GSIS), while low ghrelin concentrations inhibited and high stimulated it [99]. The insulin response to glucose was enhanced in the presence of a high ghrelin concentration. Another study showed that the subcutaneous administration of ghrelin induced relatively specific GH release and enhanced cardiac performance in humans without significant adverse effects [99], and (5) long-term versus short-term actions of the peptide. In one study, the stimulatory action of ghrelin on insulin levels in rats was observed at 15 min but not at 5 min after ghrelin administration [100]. Therefore, the net effect on insulin sensitivity of manipulation of the ghrelin

system still remains to be determined, although the overall systemic effects suggest promoting insulin resistance.

AG levels may be an important predictor of the glucose control under physiological conditions [101]. Ghrelin clearly stimulated insulin-mediated glucose disposal, an observation that was consistent with enhanced 2-deoxy-D-[3H]glucose (2DG) uptake in muscle and adipose tissue during hyperinsulinemia in ghrelin-treated animals [102]. In contrast, ghrelin hampered inhibition of endogenous glucose production by insulin. Furthermore, simultaneous administration of UAG abolishes the inhibitory effect of ghrelin on hepatic insulin action [102]. AG and UAG have been proposed to have opposite effects on glucose metabolism, which suggests a potentially significant influence of them on glucose metabolism [103]. Transgenic mice overexpressing ghrelin under the rat insulin II promoter had a pancreatic UAG content that was 1,000 times higher than the control mice. Such mice showed reduced GSIS and lower blood glucose levels and triglyceride levels during insulin tolerance test [104]. The suppression of insulin secretion is likely due to the enhancing effect of UAG on insulin sensitivity. Insulin secretion from isolated islets was instead indistinguishable from that of non-transgenic mice. These results imply that UAG, opposite to AG, increases insulin sensitivity. Gauna et al. reported that administration of AG in humans reduces insulin sensitivity, whereas the combination of AG plus UAG strongly improves insulin sensitivity [105]. Another studies reported that the dysregulation of AG/UAG ratio could play a role in pathogenesis of insulin resistance and diabetes [106, 107].

Ghrelin influences insulin and glucose homeostasis through a range of central and peripheral GHS-R-mediated mechanisms. Glucose and insulin also influence ghrelin secretion [108, 109]. Ghrelin influences glucose homeostasis centrally through medial hypothalamus. Re-expression of GHS-R in hypothalamic neurons expressing agouti-related peptide (AgRP) of adult GHS-R-null mice restores the orexigenic response to administered ghrelin and lowered blood glucose levels during caloric restriction [110]. This normalized glucoregulatory response was associated with glucagon rises and hepatic gluconeogenesis, indicating that hypothalamic arcuate-expressing agouti-related peptide (AGRP) neurons are responsible for the orexigenic effects and the central effects of ghrelin on glucose homeostasis. The effect of hexarelin on blood insulin, glucose, and in general on type 2 diabetes mellitus has not been studied well yet. In one study, obese and lean male rats of the Zucker strain were treated with hexarelin (80 µg/kg, b.i.d., s.c.) or saline (1 ml/kg, b.i.d., s.c.) for 30 days [111]. The results showed an increase in plasma insulin concentration in the obese rats treated with hexarelin. Obese rats treated with saline had plasma glucose concentrations similar to those found in lean animals.

Table 1 The in vivo and in vitro effects of ghrelin on insulin secretion and glucose homeostasis in animals

Model	Treatment	Dose and mode of administration	Insulin secretion and blood glucose
Rat pancreatic islets	AG	10 ⁻¹² M	Increased GSIS [114]
Isolated pancreas from normal and STZ treated rats	AG	10 ⁻⁹ M	Increased insulin release from the pancreas of normal and diabetic rats [115]
Wistar rats with 90 % pancreatectomy	AG	I.P. (10 nM/Kg/day) in the first postoperative day	Increased plasma insulin from the 15th postoperative day and reduce plasma glucose [96]
STZ-treated neonatal rats.	AG	S.C. (100 µg/kg/day) for 7 days (from day 2 to 8) after birth	No changes in blood glucose or insulin levels between the STZ and STZ/Ghrelin groups. Significant increase in insulin expression and insulin mRNA in pancreas of STZ/Ghrelin groups [84]
HIT-T15 insulin-producing cells	AG or UAG	100 nM	Increased GSIS. UAG being more potent than AG at raising insulin levels [82]
STZ-treated neonatal rats	AG or UAG	S.C. (100 µg/kg, twice daily), from day 2 to 8 after birth	AG or UAG reduced glucose levels, restored plasma insulin and pancreatic insulin [86]
GH-deficient little mice and control wild mice	AG or UAG	I.P. (1 and 10 nM/kg)	AG decreased insulin and elevated blood glucose levels. UAG failed to significantly alter blood glucose levels. Ghrelin at 10 nmol/l, but not 0.1 nmol/l and 1 pmol/l, decrease GSIS in islets [116]
Perfused pancreas from male Wister rats and isolated islets from male ghrelin knockout and wild-type mice	AG or UAG	10 nM	AG suppressed both phases of GSIS UAG has no effect. Ghrelin-knockout mice display increased insulin and decreased glucose levels. Increase GSIS from isolated islets of ghrelin-knockout mice [117]
Min6 cells with overexpression of UCP-2	AG	10 nM	Inhibited GSIS [118]
Isolated rat pancreas	AG	10 nM	No change of basal insulin release but markedly inhibited GSIS and insulin release elicited by 1 mM carbachol [119]
Isolated rat islets and INS-1E cells	AG	10 nM for isolated islets 1 nM for INS-1E cells	Inhibited GSIS in both the isolates islets and cell line [120]
INS-1E cells	AG (rat, human or Dap octanoylated human) or UAG (rat or human)	10 nM	AG or UAG increased GSIS [121]
Male sprague Dawley rats	AG	I.V. (25 nmol) into the jugular vein	Increased insulin significantly at 15 and 60 min after iv ghrelin, compared with control [100]
Female C57BL/6J mice and isolated islets from mice	AG	I.V. (50, or 150 nM/kg) with glucose at the dose of 1 g/kg in a tail vein and 0.01–1 nM for the isolated islets	Inhibited plasma insulin. No effect on insulin sensitivity. Inhibited GSIS from isolated islets [122]
Adult male WT, ghrelin <i>-/-</i> , and GHS-R <i>-/-</i> mice			Lowered insulin concentrations in both ghrelin <i>-/-</i> and GHS-R <i>-/-</i> mice compared with WT mice during the postabsorptive state. No change in blood glucose between the three groups [123]

IP intraperitoneal injection, *SC* subcutaneous injection, *IV* intravenous injection, *DapAG* Dap–octanoylated human ghrelin, a ghrelin molecule where the octanoyl group on the third serine residue is stabilized by a α - and β -diaminopropanoic acid, *WT* (wild type), ghrelin $-/-$ (ghrelin knockout) and GHS-R $-/-$ (growth hormone secretagogue receptor knockout) mice

Table 2 The effects of ghrelin on insulin secretion and glucose homeostasis in humans

Model	Treatment	Dose and mode of administration	Insulin secretion and blood glucose
Morbidly obese non-diabetic subjects	UAG or UAG+AG	I.V.: UAG 200 µg or UAG 100 µg + AG 100 µg for 4 days	UAG+AG decreased insulin and no effect on blood glucose. UAG had no effect on blood glucose or plasma insulin [124]
Healthy subjects	AG	AG (0.3, 0.9 and 1.5 nmol/kg/h) or saline was infused for more than 65 min	AG decreases GSIS and worsens intravenous glucose tolerance in healthy humans [125]
Obese and normal women	AG	I.V. 1 µg/kg	Increased glucose and reduced insulin in obese women and increase glucose but not insulin in normal women [126]
Normal young subjects	AG	I.V. 1 µg/kg	Inhibited insulin and increased glucose levels. Ghrelin did not modify both glucose and insulin responses to OGTT [127]
Normal young volunteers	AG or UAG	I.V. 1 µg/kg	AG decreased insulin levels and increased blood glucose. UAG has no effect [128]
Healthy or hypopituitary humans	AG or AG+UAG	AG: 1 µg/kg UAG: 1 µg/kg	AG and to a lesser extent UAG induced significant hyperglycemia. No increase in blood glucose when GH was administered 15 min before the administration of AG. AG+UAG seemed to lower serum glucose levels significantly. AG+UAG induced a significant reduction in serum insulin [105]
Healthy, gastrectomized or hypopituitary humans	EHC: AG	AG: I.V. 5 pmol/kg per minute 5 h pancreatic clamp	Decrease insulin sensitivity [129–131]
Healthy humans	EHC: AG (intramuscular)	AG: I.M. 6.25 mg/l 5 h	Increase insulin sensitivity [132]

OGTT oral glucose tolerance test, EHC euglycemic/hyperinsulinemic clamp, IM intramuscular

Treatment with hexarelin increased plasma glucose concentrations in obese rats but not in lean rats. One possibility of these findings may be related to the ability of hexarelin to stimulate the hypothalamic–pituitary–adrenal (HPA) axis [112, 113]. However, the mechanism by which GHS stimulate the HPA axis is unknown.

Effects of ghrelin and hexarelin on adipose tissue

The effect of ghrelin on adipose tissue has also been reported. Ghrelin increased the insulin-stimulated deoxyglucose uptake in isolated white adipose tissue from epididymal fat at concentration of 1,000 nM compared to 0.1 nM insulin alone. Ghrelin increased slightly the glucose uptake at 100 nM without statistical significance [133]. AG did not increase the deoxyglucose uptake in peri-renal adipocytes, which does not express GSH-R1a. UAG had no effect on insulin-stimulated glucose uptake. The dose of ghrelin in this study was above the physiological plasma concentration of ghrelin which could be explained by the difference in the effect and the dose of local and systemic ghrelin. The local ghrelin concentrations in adipose tissues may be considerably higher than those in the circulation and systemic ghrelin could be less important in the modulation of glucose uptake than those

found locally. Ghrelin enhanced insulin-stimulated glucose uptake in adipocyte cell line, 3T3-L1 [72]. On the contrary, Ott et al. reported that ghrelin pre-treatment had no effect on insulin-stimulated glucose uptake in an immortalized brown adipocyte cell line [134]. Consequently, ghrelin appears to directly potentiate adipocyte insulin-stimulated glucose uptake in selective adipocyte populations and may play a role in adipocyte regulation of glucose homeostasis. Also, ghrelin regulate cholesterol efflux in macrophages through a GHS-R1a/PPAR- γ -dependent pathway [135]. Interestingly, these studies showed that PI3K/AKT pathway is mediating PPAR- γ activation by ghrelin which is the same pathway that mediates the antiapoptotic effects of ghrelin on various cells.

The potential effects of hexarelin on lipid metabolism have been studied. Hexarelin through binding to CD36 receptor induced an increase in thermogenic coactivator PGC-1 α and uncoupling protein-1 (UCP-1) in 3T3-L1 adipocytes as well as in epididymal fat of treated mice, indicating increased fatty acids metabolism in the white fat in response to hexarelin [70]. Hexarelin treatment in obese rats significantly decreased plasma cholesterol but not triglyceride levels [111]. In another study, apolipoprotein E-null mice maintained on a long-term, high-fat, and high-cholesterol diet, a condition known to cause atherosclerosis, showed a significant regression in atherosclerotic lesions

when treated with hexarelin compared to saline-treated controls [136]. This study demonstrated the activation of the PPAR- γ -liver X receptor (LXR α)-ATP-binding cassette transporters (ABC) metabolic cascade could be the signaling pathway for the action of hexarelin to cause the macrophages to mobilize excess cholesterol into the HDL cholesterol reverse pathway. These beneficial effects of hexarelin were observed under conditions in which GH was not upregulated, and similar effects observed in mice treated by EP80317, a hexarelin derivative with no GH release activity, supporting a GH-independent role for hexarelin [137].

These effects of hexarelin derived from enhanced expression of PPAR target genes, resulting in a thermogenic-like profile. PPAR- γ is considered a master regulator of fatty acid metabolism in fat through its direct role in regulating the expression of a broad range of genes involved in fatty acid and glucose metabolism [138]. Among the genes upregulated by PPAR- γ , genes related to fatty acid uptake (fatty acid transport protein FATP, CD36), glucose uptake (GLUT4), β -oxidation (acyl-CoA dehydrogenase, carnitine palmitoyltransferase CPT-1, acyl-CoA oxidase), gluconeogenesis (phosphoenolpyruvate carboxykinase PEPCK), and lipid storage (adipophilin). PPAR- γ also plays a central role in coordinating the macrophage response to lipid loading through a transcriptional cascade involving LXR α and the ABC transporters A1 and G1 [139, 140]. Similarly, PPAR- γ agonists of the thiazolidinedione family were shown to exert beneficial anti-diabetic and anti-atherosclerotic actions by promoting activation of the PPAR- γ -LXR α -ABC pathway and cholesterol efflux [141, 142]. Consequently, we suppose that ghrelin or hexarelin by modulation of PPAR- γ activity could enhance insulin sensitivity, regulate cholesterol metabolism in macrophages, and prevent atherosclerotic vascular lesion progression. Recently, hexarelin also inhibited cholesterol synthesis in liver cells through the activation of the adenine monophosphate-activated protein kinase (AMPK) pathway, reduction of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), and increase recruitment of the anchor proteins insulin-induced genes (Insig-1 and Insig-2) in the liver cells [143]. HMGR is the rate-limiting enzyme in the cholesterol biosynthesis pathway. These findings support that CD36 and its ligands such as hexarelin play a role in regulating AMPK activity fatty acid metabolism and reverse cholesterol transport.

Ghrelin and hexarelin promote mitochondrial activity and biogenesis

The cause of apoptosis and failure of beta cell that occur during T2D are still unknown. However, mitochondria may play a role in regulating apoptosis and insulin secretion

[144, 145]. Many studies have reported the beta cells in T2D do not sense glucose properly and consequently do not release enough amounts of insulin. The increased glucose and fatty acids could cause beta cell mitochondrial damage, leading to decreased ATP due to increase of mitochondrial membrane potential and production of ROS [146] (as shown in Fig. 2). Beta cells are susceptible to oxidative stress due to the weak expression of antioxidative enzymatic defenses, e.g., catalase and superoxide dismutase (SOD). Increased ROS can cause leakage of protons into the mitochondrial matrix, leading to decreased ATP production, and thus reduced GSIS [147]. Uncoupling protein 2 (UCP-2) modulates coupling efficiency and may regulate GSIS [148]. A small reduction in the mitochondrial membrane potential induced by mild uncoupling has a significant effect in attenuating ROS production [149]. However, the increase mitochondrial UCP2 decreases GSIS by decreasing intracellular ATP/ADP ratio [148].

It was interesting to observe that hexarelin and ghrelin enhanced mitochondrial structure and metabolism by upregulating many genes related to the mitochondrial metabolism of glucose and fatty acids [70]. More specifically, the upregulation of the genes which encode acetyl CoA acyl transferase, CPT-1, and several subunits of the ATP synthase and cytochrome c oxidase complexes may suggest an increased fatty acid metabolism through the mitochondrial oxidative phosphorylation pathway. The mitochondria of hexarelin-treated 3T3-L1 adipocytes

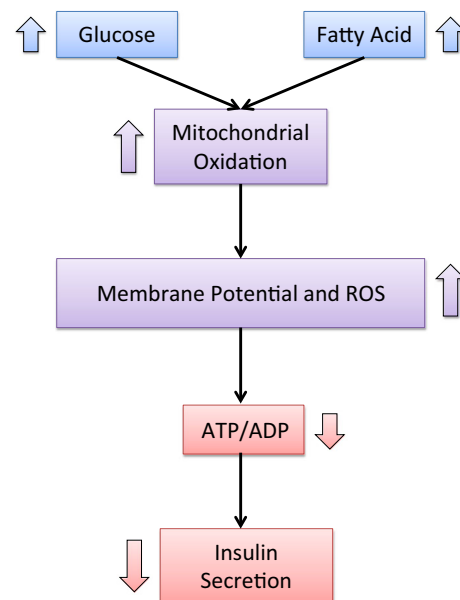


Fig. 2 The role of mitochondria in T2D. High concentration of glucose and fatty acids can lead to mitochondrial stress, which in turn results in increased production of reactive oxygen species (ROS). Increased ROS can decrease ATP production and reduced glucose-stimulated insulin secretion

showed an intense and highly organized cristae formation that spanned the entire width of mitochondria compared to that in untreated cells as shown by the electron microscopy [70]. Ghrelin also exerts a protective effect against H₂O₂-induced apoptosis in H9c2 rat cardiomyocytes by reducing the proapoptotic protein Bax and increasing Bcl-2 expression, accompanied by decreasing activation of caspase-9 and caspase-3 [150]. These results demonstrate that the antiapoptotic effect of ghrelin is probably caused by decrease H₂O₂-induced mitochondrial stress and by blocking activation of NF- κ B. The transcription factor NF- κ B is a critical signaling molecule in oxidant stress responses. UCP-2 mRNA expression was downregulated in INS-1 beta cell by ghrelin in the presence of 26.4 mM glucose [151]. Other studies showed that ghrelin decreased oxidative injury in the stomach [152], brain [153], blood vessels [154], and liver [155].

Cardioprotective effects of ghrelin and hexarelin

Individuals with diabetes are at a significantly greater risk of developing cardiovascular diseases including cardiomyopathy (CM) and heart failure [156]. Insulin resistance has been recognized as an important risk factor in the development of cardiovascular diseases [157, 158]. The diabetes-associated cardiovascular disease is responsible for 70 % of diabetes-related deaths. Diabetes can cause direct injury to myocardium, resulting in a distinct disease called “diabetic CM.” Data from experimental, epidemiologic, and clinical studies have shown that diabetes results in structural and functional cardiac changes. The structural changes of diabetic CM include higher left ventricle (LV) mass, wall thickness, and arterial stiffness [159]. These changes could be caused by deposition of advanced glycation end-products (AGEs) and collagen [160, 161]. Functionally, there are diastolic abnormalities which have been suggested as early functional abnormalities of diabetic CM [162, 163]. The cardioprotective effects of hexarelin and ghrelin have been well investigated. Many studies have shown their beneficial effects on the cardiovascular system such as protection against cardiac ischemia, cardiac fibrosis, improving cardiac function, and decreasing peripheral resistance after myocardial infarction (MI) in animals [164–166] and humans [167]. Our group showed that treatment of spontaneously hypertensive rats (SHRs) with hexarelin for 5 weeks from an age of 16 weeks significantly reduced cardiac fibrosis in SHRs by decreasing interstitial and perivascular myocardial collagen deposition and myocardial hydroxyproline content and reducing mRNA and protein expression of collagen I and III in SHR hearts [168]. In addition, hexarelin treatment significantly attenuated left ventricular (LV) hypertrophy,

LV diastolic dysfunction, and high blood pressure in SHRs. So, our data demonstrate that hexarelin reduces cardiac fibrosis in SHRs, perhaps by decreasing collagen synthesis and accelerating collagen degradation [168].

Ghrelin and hexarelin showed protective effects on cardiac ischaemia. Administration of ghrelin in vitro to ischaemia/reperfusion (I/R) rat hearts was shown to reduce the infarct size through the activation of PKC, and these effects were likely initiated by the binding of ghrelin to its receptor, GHS-R1a [169]. Pre-administration of ghrelin in vivo greatly ameliorated the damaged heart function, attenuated myocardial injury, and apoptosis through inhibition of ER stress [170]. The role of hexarelin on the protection against cardiac ischemia has been reported earlier [67, 68, 171]. Rats pre-treated with ghrelin or hexarelin for 7 days in vivo prior to in vitro I/R injury showed an improvement in cardiac function [172]. A single dose of oral hexarelin treatment at the very acute phase after MI has protective effects on chronic cardiac function [173]. Aragno et al. also showed that obestatin, which is encoded by the same gene for ghrelin, protected STZ-caused myocardial dysfunction [174]. Our group has confirmed the protective effect of hexarelin and ghrelin on cardiac ischaemia [164]. Ghrelin (10 nM) or hexarelin (1 nM) was administered in the perfusion solution of isolated hearts before or after ischemia for 10 min. It was found that relative sarcomere shortening was significantly reduced after I/R and exhibited a reduction in both the amplitude and rising rate of intracellular calcium ([Ca²⁺]_i) transients but increases in cytoplasmic [Ca²⁺]. The alteration in [Ca²⁺]_i transients and cytoplasmic [Ca²⁺] which occurs during the cardiac ischemia and reperfusion is due to reduction of sarcoplasmic reticulum (SR) Ca²⁺ ATPase and Na/K ATPase activities or due to increased oxidative stress which occurs during the reperfusion which leads to subsequent damage to the plasma and SR membranes and further increases in cytoplasmic Ca²⁺. Pre- and post-treatments with ghrelin and hexarelin protected freshly isolated mouse ventricular cardiomyocytes against these negative effects of I/R. They resulted in normal cardiomyocytes contractility, normal amplitude, and rising rate of [Ca²⁺]_i transients in the cardiomyocytes during an in vitro I/R injury, which could be attributed to normalized SR Ca²⁺ ATPase activity and SR Ca²⁺ content [164].

To compare the effect of ghrelin and hexarelin on cardiac function after infarction MI, ghrelin-knockout mice were used to show whether hexarelin could compensate for the ghrelin deficiency [175]. The results showed that hexarelin-treated mice had better cardiac function than ghrelin-treated mice, as indicated by the higher absolute values of percent of the ejection fraction (EF), peak rate of pressure rise (dP/dt max) and dP/dt min, fractional shortening (FS), preload-adjusted maximal power (PAMP), and

maximal elastance (E_{max}). Hexarelin and ghrelin treatments also significantly reduced collagen volume fraction in the non-infarcted LV walls and plasma atrial natriuretic peptide levels than vehicle administration [175]. These stronger effects of hexarelin was also confirmed by another study in which hypophysectomized rats were treated in vivo for 7 days with either ghrelin (320 g/kg) or hexarelin (80 g/kg), and then their hearts were subjected in vitro to the ischemia and reperfusion procedure [172]. Ghrelin was far less effective than hexarelin in preventing increases in LV end-diastolic pressure (15 and 60 % protection for ghrelin and hexarelin, respectively), coronary perfusion pressure (10 and 45 % reduction), and release of creatine kinase in the heart perfusate (15 and 55 % reduction). The effects of hexarelin were mediated largely by interactions with CD36 in the heart and to a lesser extent by GHS-R. However, other studies reported that ghrelin and hexarelin had similar effects [176].

In addition, AG and UAG also exert antiapoptotic effects on mouse and rat cardiomyocytes by acting on MAPK and PI3K/AKT pathways [33]. Similarly, our studies have confirmed the cardioprotective role of hexarelin in I/R models of hearts from mice [164]. GHS can preserve the electrophysiological properties of cardiomyocytes after I/R and inhibit cardiomyocyte apoptosis and promote cell survival by modification of MAPK pathways through activating GHS-R1a [176]. Hexarelin also showed a protective role on angiotensin II (ANG II)-induced apoptosis of isolated cardiomyocytes from neonatal rats [177]. Administration of hexarelin significantly decreased ANG II-induced apoptosis and DNA fragmentation and increased cardiomyocyte viability via inhibiting the increased caspase-3 activity and BAX expression and by increasing the expression of BCL-2. GHS-R mRNA was abundantly expressed in cardiomyocytes and was upregulated after administration of hexarelin.

Recently, some studies have reported that the beneficial effect of ghrelin on the cardiovascular system might be mediated by modulation of cardiac autonomic nerve activity. Ghrelin has been shown to suppress sympathetic activity and to decrease blood pressure through mechanisms involving the central nervous system [178, 179]. The beneficial effects of ghrelin were accompanied by the suppression of MI-induced increase of heart rate and plasma norepinephrine concentration [180]. These effects of ghrelin decreased with atropine pre-treatment or vagotomy. Ghrelin exerted antiarrhythmic effects in rats during acute MI via modulation of vagal activity by showing increased high-frequency (HF) component as an index of parasympathetic activity and decreased low-frequency (LF)/HF ratio as an index of sympathetic activity and restoration of phosphorylated connexin-43 protein levels [181].

As connexin-43-deficient mice have been reported to be markedly susceptible to ischemia-induced ventricular arrhythmias [182]. Other studies have been proposed to explain the reduction in blood pressure after ghrelin administration, including vasodilation via endothelium activation or a direct effect on vascular smooth muscle cells [183, 184]. Ghrelin also inhibited post-infarct myocardial remodeling, while improving cardiac function through its anti-inflammatory effects as shown by decreased mRNA and protein levels of interleukin-1beta and tumor necrosis factor-alpha [185].

Clinical studies in human with growth hormone deficiency (GHD) suggested that patients with either ischemic or non-ischemic CM might benefit from GHS therapy, mainly from reduction of LV maladaptive remodeling and cardiomyocyte loss. In healthy volunteers and in patients with GHD, an acute administration of hexarelin (2 μ g/kg) elicited a short-term increase in contractility and LV ejection fraction in a GH-independent manner [186]. Similar results were observed in patients with ischemic CM but not with dilated CM [187]. Studies with ghrelin showed an improved cardiac function following acute administration, although this effect was associated with a dose-dependent increase in GH [188]. Also, a short-term infusion of ghrelin (0.1 μ g/kg/min/60 min) decreased mean arterial pressure and increased cardiac and stroke volume index, which may be related to a reduced systemic vascular resistance in healthy volunteers and in patients with heart failure. The cardiovascular effects of GHS could be through GHS-R1a in the heart and the blood vessels or through CD36 that is a specific cardiac receptor for hexarelin [189].

Conclusions

The exact mechanisms by which GHS promote their metabolic response are not fully understood. However, it becomes clear that interacting with GHS-R1a and/or other receptors induces profound changes in metabolic activities of target tissues, especially regarding PPAR- γ -downregulated events. These metabolic effects besides the proliferative, regenerative, and antiapoptotic effects of GHS on beta cells represent a promising avenue in the treatment of diabetes and diabetic related heart diseases.

Conflict of interest The authors declare that they have no conflict of interest.

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