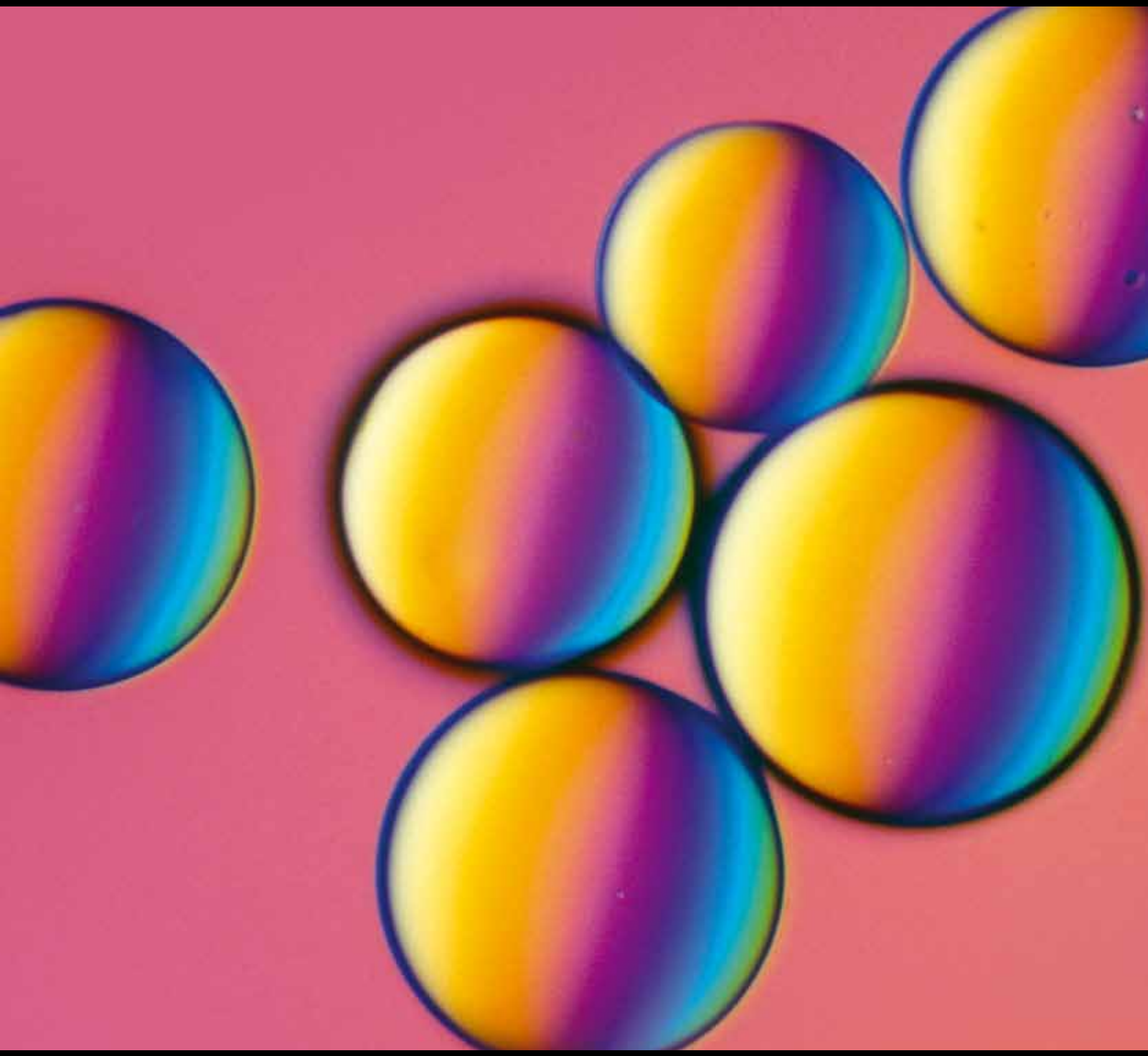
International Journal of Peptides

Update on Ghrelin

Guest Editors: Sergueï O. Fetissov, Alessandro Laviano, Satya Kalra, and Akio Inui



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Editorial

Update on Ghrelin

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Few peptide hormones have attracted as much attention of the scientific community as ghrelin, the natural secretagogue for growth hormone (GH) identified by M. Kojima et al. in 1999 [1], resulting in more than 4000 PubMed citations over the last ten years. The initial interest can be attributed to the ability of ghrelin to stimulate feeding in mammals, suggesting it as a potential target for the development of antiobesity drugs. Many studies investigating this issue have however revealed the complexity of the ghrelin system including the differential physiological effects of the three peptide products of the ghrelin gene: *ghrelin*, which is peritranslationally modified via acylation with the octanoic acid; *des-acyl ghrelin* that is not acylated or has lost its fatty acid residue; and *obestatin*, another bioactive peptide derived from the preproghrelin precursor. Most importantly, it has been realized that beside stimulation of GH secretion and increased feeding, ghrelin has multiple biological effects that would be important to preserve if aiming to antagonize ghrelin-mediated positive energy balance. Among these functions, ghrelin was found to regulate gastrointestinal motility and associated sensory functions, to modulate the reproductive and stress axes, mood and emotion, glucose metabolism, as well as affecting the cardiovascular system and renal function.

The present special issue "Update on ghrelin" includes nineteen reviews and one original study and starts with the review by J. D. Veldhuis and C. Y. Bowers who "integrate the GH axis into the ghrelin system" and discuss the biological background of the multiplicity of ghrelin's functions with their relevance to drug development [2]. Other articles, as introduced below, provide in more details some specific aspects of the ghrelin system, altogether providing a comprehensive overview of the current knowledge in the field and showing how ghrelin research has helped advance our understanding of both physiology and disease.

Ghrelin is abundantly synthesized by specialized mucosal cells in the stomach accounting for about 80% of the serum ghrelin production in rats and for 65% in humans. Some ghrelin producing cells are also found in lower parts of the gastrointestinal (GI) tract, where ghrelin expression can be increased, for example, after gastrectomy [3]. Interestingly, gastric ghrelin cells are of the closed type, whereas those in the lower GI tract are of both the closed and the open type, suggesting their regulation preferentially by hormonal or luminal factors, respectively. These cellular and also phylogenic and ontogenic aspects of ghrelin production in the GI tract are reviewed by I. Sakata and T. Sakai [4]. Moreover, Fujimiya and colleagues show that the closed type gastric cells contain ghrelin, des acyl-ghrelin, and obestatin, while the open type cells contain only des-acyl ghrelin supporting a role of luminal pH on des-acyl ghrelin secretion [5].

Although the role of ghrelin to stimulate feeding is evident in mammals, it is possible that this function of ghrelin is evolutionary late and redundant, and that ghrelin has evolved as a messenger mediating other vital functions such as activation of the somatotropic axis. In fact, in fish, as reviewed by S. E. Schwandt et al., the orexigenic effect

of ghrelin is an inconsistent finding and can be species dependent while the GH stimulating effect is preserved [6]. This phenomenon is even more evident in birds, where ghrelin stimulates GH secretion and the hypothalamo-pituitary adrenal stress axis but inhibits feeding and drinking. The avian ghrelin system is discussed in details in the review by M. P. Richards and J. P. McMurtry [7].

The integrative role of ghrelin in homeostatic regulation is evident from its function in the regulation of reproduction versus feeding. Ghrelin inhibits the activity of the hypothalamo-pituitary-gonadal axis acting both centrally and peripherally, similar to hypothalamic neuropeptide Y (NPY), a down-stream target of ghrelin. In addition, ghrelin also acts directly to inhibit reproductive function during conditions of energy deficit. The effects of ghrelin in modulation of the activity of the gonadal axis in both males and females are reviewed by J. Dupont et al. [8].

Although ghrelin stimulates feeding, it inhibits drinking and activates neurons of the hypothalamic subfornical organ usually associated with dehydration. This suggests that circulating ghrelin may signal to the brain via the circumventricular organs not necessarily associated with the median eminence. However, how peripheral ghrelin enters the brains is still an unresolved issue discussed in details by M. Fry and A. V. Ferguson who propose the relaying roles of the circumventricular organs such as the subfornical organ and area postrema [9].

In its role in food intake regulation, ghrelin interacts with gastrointestinal hormones signaling satiety such as cholecystokinin, bombesin, peptide YY, glucagon-like peptide, pancreatic polypeptide, and amylin. As discussed by A. S. Wisser et al., these peptides may inhibit ghrelin secretion or antagonize its action in the appetite regulatory neurons in the brain [10]. Since plasma levels of ghrelin normally fall after a meal, it is possible to use it as a satiety marker to evaluate the satietogenic properties of different macronutrients for the development of various nutritional antiobesity approaches. How different types of nutrients affect ghrelin secretion is reviewed by C. Koliaki et al. [11].

In addition to the appetite-related neuronal pathways activated by ghrelin, ghrelin receptors are present in many brain areas that can affect mood and emotion, and, indeed, it was found that ghrelin may interfere with the regulation of the stress response, mood, and anxiety. Again, in analogy to the orexigenic NPY, which also regulates these behavioral modalities, the situation with ghrelin is not simple, and both anxiolytic and anxiogenic effects have been attributed to ghrelin as discussed by J. C. Chuang and J. M. Zigman [12]. Further elucidation of the brain ghrelin system should help to clarify the role of ghrelin, which will be important for the designing of drugs targeting the ghrelin system.

Next, five reviews provide full coverage of the important role of ghrelin in influencing gastrointestinal contractility and motility in normal and pathological conditions, as well as showing its relevance to both new and old therapeutic approaches. H. S. Sallam and J. D. Z. Chen introduce the subject with a meticulous review of the experimental and clinical data accumulated during the last ten years related to ghrelin's prokinetic effects [13]. T. Ohno et al. discuss the possibility that changes of plasma ghrelin concentration are not relevant to the prokinetic effect of ghrelin because of the decreased plasma levels found after a meal, similar to the situation observed for motilin, a hormone structurally related to ghrelin [14]. Further, the complexity of the ghrelin system in influencing gastric motility is presented in the review by M. Fujimiya et al. who discuss the differential effects of ghrelin, des-acyl ghrelin, and obestatin, providing evidence for the implication of distinct central pathways in these effects involving hypothalamic NPY, corticotropin-releasing hormone, and urocortin [5]. As gastric motility is altered in subjects with functional dyspepsia who also experience abdominal discomfort, nausea, and decreased appetite, exploring a role of ghrelin in these patients may be worthwhile. Indeed, as discussed by T. Akamizu et al., plasma levels of ghrelin have been found to correlate with reduction of symptoms in these patients and excitedly, a preliminary study using synthetic ghrelin showed a therapeutic potential in functional dyspepsia [15]. Thus, ghrelin or its analogs may become new drugs for the treatment of functional dyspepsia.

Similarly, improvement in physiological ghrelin secretion may underlie the beneficial effects of other therapeutic approaches, for example, traditional medicine. Such an example is given in the review by T. Hattori who discusses the mechanisms behind the beneficial effects of Rikkunshito, a traditional Japanese medicine based on a mixture of eight herbs. In fact, Rikkunshito was shown to alleviate dyspepsia, for example, during cancer chemotherapy, and this improvement was associated with increased ghrelin secretion [16].

Ghrelin is also expressed in the pancreatic islets and has an intricate relation with insulin in regulation of the glucose metabolism. These effects of ghrelin with relation to the pathophysiology of type 2 diabetes are reviewed in great detail by S. Sangiao-Alvarellos and F. Cordido [17]. The authors also discuss the results obtained in ghrelin- and ghrelin receptor-knockout mice, suggesting that absence of ghrelin signaling is more relevant to glucose homeostasis than to food intake control, supporting the phylogenetic data mentioned above.

Interestingly, although ghrelin stimulates feeding, low plasma levels of ghrelin are commonly present in obesity whereas levels usually increase with weight loss. These data do not support the causative role of ghrelin in increased appetite in obesity but may suggest a link via the role of ghrelin in glucose homeostasis. L. Pulkkinen et al. in their review make an emphasis on the relation between ghrelin and insulin resistance and human genetics in the development of obesity and the metabolic syndrome [18]. Further data of the putative role of ghrelin in obesity come from studies of obese subjects who have undergone bariatric surgery and hence have reduced gastric ghrelin production. As reviewed by D. J. Pournaras and C. W. le Roux [19], ghrelin plasma levels are altered after the surgery, and both decreased and increased levels were documented. In addition, similar results have also been reported in rats [3]. As bariatric surgery is accompanied by reduced appetite and body weight, the serum ghrelin data do not support a causative role of ghrelin in these beneficial effects. However,

bariatric surgery is consistently associated with an improved insulin resistance and diabetes, taken by some as a reason to rename the surgery "metabolic" and pointing to that the surgery-associated changes of ghrelin serum levels might still have beneficial effects on the glucose metabolism.

Expression of ghrelin and the ghrelin receptor was found in vascular endothelial cells providing the background for the vascular effects of ghrelin. In fact, ghrelin was shown to decrease blood pressure, although this effect might also involve the modulation of the central sympathetic tone. The cardiovascular effects of ghrelin are discussed by M. Tesauro et al. showing that activation of the ghrelin system might be a new therapeutic approach for chronic heart failure and cardiac cachexia [20]. A. Laviano et al. review the relevance of ghrelin in another form of cachexia associated with chronic renal failure both in regard to its pathophysiology and to its putative therapeutic role [21]. The authors propose the use of exogenous ghrelin which should be able to overcome endogenous ghrelin resistance present in renal cachexia, that might improve the nutritional status in cachectic patients. In fact, synthetic ghrelin or ghrelin analogs might be considered as a new therapy for a variety of pathological conditions characterized by anorexia or cachexia. For instance, a state of ghrelin resistance is present in anorexia nervosa, and a recent pilot study showed that administration of ghrelin is accompanied by improved appetite in these patients [22]. Another possible indication of "ghrelin therapy" can be anorexia associated with gastrectomy as suggested by the experimental study in Yada's laboratory [3].

To conclude this editorial, ten years of ghrelin research indicate that this peptide initially identified as a GH secretagogue is an universal hormone. As guest editors, we thank all the authors who have contributed to this special issue for preparing excellent articles and we wish the readers a good time in exploring the ghrelin world, which we find ourselves most exciting and promising for the new discoveries and potential therapeutic spin offs.

Sergueï O. Fetissov
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Satya Kalra
Akio Inui

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Review Article

Integrating GHS into the Ghrelin System

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Oligopeptide derivatives of metenkephalin were found to stimulate growth-hormone (GH) release directly by pituitary somatotrope cells in vitro in 1977. Members of this class of peptides and nonpeptidyl mimetics are referred to as GH secretagogues (GHSs). A specific guanosine triphosphatate-binding protein-associated heptahelical transmembrane receptor for GHS was cloned in 1996. An endogenous ligand for the GHS receptor, acylghrelin, was identified in 1999. Expression of ghrelin and homonymous receptor occurs in the brain, pituitary gland, stomach, endothelium/vascular smooth muscle, pancreas, placenta, intestine, heart, bone, and other tissues. Principal actions of this peptidergic system include stimulation of GH release via combined hypothalamopituitary mechanisms, orexigenesis (appetitive enhancement), insulinostasis (inhibition of insulin secretion), cardiovascular effects (decreased mean arterial pressure and vasodilation), stimulation of gastric motility and acid secretion, adipogenesis with repression of fat oxidation, and antiapoptosis (antagonism of endothelial, neuronal, and cardiomyocyte death). The array of known and proposed interactions of ghrelin with key metabolic signals makes ghrelin and its receptor prime targets for drug development.

1. Overview

Fundamental questions in peptide biology are the extent to which any given peptide operates in isolation versus interdependently, locally or systemically, and via a single pleiotropic or multiple distinct receptors. Identification of the ghrelin/GHS family initially disclosed GH-releasing properties [1]. Investigations subsequently unveiled multiorgan expression [2–4], multivariate actions [5], and complex modulation of and by collateral effectors [1, 4]: Table 1. The burgeoning repertoire of ghrelin actions mimics that of inhibin and activin [6, 7], which were originally isolated as regulators of follicle-stimulating hormone secretion, and thereafter recognized for hematopoietic and oncologic activity. Analogously, prominent clinical applications of ghrelin/GHS may involve not only GH-stimulating effects but also appetitive, metabolic, cardiovascular, locomotive, and gastrointestinal signaling: Figure 1. Recent development of transgenic mice expressing ghrelin-eGFP (enhanced green fluorescent protein) should permit more detailed mapping of ghrelin-expressing neurons in hypothalamic arcuate and ventromedial nuclei [8–10] and ghrelin-expressing cells in gastric oxyntic glands, pancreatic islets (epsilon cells), the anterior pituitary gland, bone marrow, and other less well-studied sites [4, 11, 12].

2. Unique Facets of Ghrelin

2.1. Multiplicity of Roles. The etymology of ghrelin is "ghre" for "growth", consonant with the report by Bowers et al. of direct pituitary GH-releasing effects of metenkephalin-derived synthetic oligopeptides three decades ago [1]. The peptides were initially termed GH-releasing peptides (GHRPs), and more recently GH secretagogues (GHSs) to include congeneric molecules [2]. Multiple peptidyl and several nonpeptidyl analogs of ghrelin (GHS) have been developed and assessed functionally over the last three decades. The ghrelin receptor was cloned in 1996 by Howard

Table 1: Interactions with ghrelin.

(a) Regulation of ghrelin gene	
Stimulation	*Repression*
(1) GHRH (pituitary)	(1) Leptin (hypothalamus)
(2) Octanoate (stomach)	(2) Glucagon-like peptide (hypothalamus)
(3) Estradiol (stomach)	(3) Peptide YY (3-36) (hypothalamus)
(4) Glucagon (rat stomach)	(4) Insulin (stomach)
(5) Cholecystokinin (stomach)	(5) Somatostatin (pituitary, stomach)
(6) Hypoglycemia (stomach)	(6) Histamine (stomach)
(7) Acetylcholine (stomach)	(7) Hypoglycemia (brain)
(8) Leptin (stomach)	(8) Glucagon*
(b) Regulation of ghrelin receptor	
Activation	*Suppression*
(1) Constitutive expression	(1) Estradiol (appetitive effects)
(2) Acylghrelin	(2) GH (hypothalamus and pituitary)
(3) Estradiol (in vitro)	(3) IGF-I (pituitary)
(c) Modulation of ghrelin action	
Potentiation	*Inhibition*
(1) GHRH (GH release)	(1) Testosterone (dog and rat)
(2) Estradiol (GH release)	(2) Free Fatty acids (pituitary)
(3) L-arginine (GH release)	(3) Leptin (neurons)
	(3) Desacyl-ghrelin (hunger, insulinostasis)
(d) Mediation of ghrelin actions	
Appetitive	*Cardiovascular*
(1) NPY (↑)	(1) Nitric oxide (↑)
(2) Orexin A (↑)	(2) Extracellular-receptor activated kinases (↑)
(3) Leptin (↓)	(3) Unknown desacyl-ghrelin receptor
(4) Insulin (↓)	(4) CD36 (type B scavenger receptor)
Stomach	*Pituitary*
(1) GHS receptor-1a	(1) Phospholipase C
(2) ? CRH receptor-2	(2) Diacylglycerol
	(3) Protein-kinase C
	(4) Ca^{2+} signals
Pancreas	
(1) Inward Ca^{2+} and outward K^+ channels	
(e) Regulation of ghrelin octanoyl-acyl transferase (GOAT)	
Stimulation	*Inhibition*
(1) Long-term fasting (stomach)	(1) By acylghrelin (stomach)
(2) Acetylcholine	
(3) Leptin	

* IV glucagon suppresses serum ghrelin concentrations in humans [121].
Selected References: [9, 15, 52, 88, 122–127].

et al. based upon phospholipase C-mediated intracellular inositol triphosphatate-dependent Ca^{2+} signal generation in transfected cells [5]. In certain systems, such as pituitary somatotropes, gastric oxyntic cells, appetitive neurons, pancreatic islets, and endothelial cells, additional messengers like phosphoinositidyl-3-kinase, Akt/protein kinase B, adenosine monophosphate protein kinase (AMP kinase), and nitric oxide may modulate ghrelin's actions [13–20]: Table 1. Mitogen-activated protein kinases (MAPKs) and extracellularly regulated kinases (ERKs 1/2) may mediate certain of ghrelin's proliferative and antiapoptic effects [15, 21, 22].

2.2. GH-Releasing Mechanisms. Ghrelin and cognate receptor, GHS-R1a, are distinct entirely from GH-releasing hormone, GHRH, and its receptor isolated in 1982 by the Guillemain and Vale laboratories from human pancreatic neoplasms [23, 24]. The homotypic GHRH receptor is an adenylyl cyclase-activating seven transmembrane-spanning

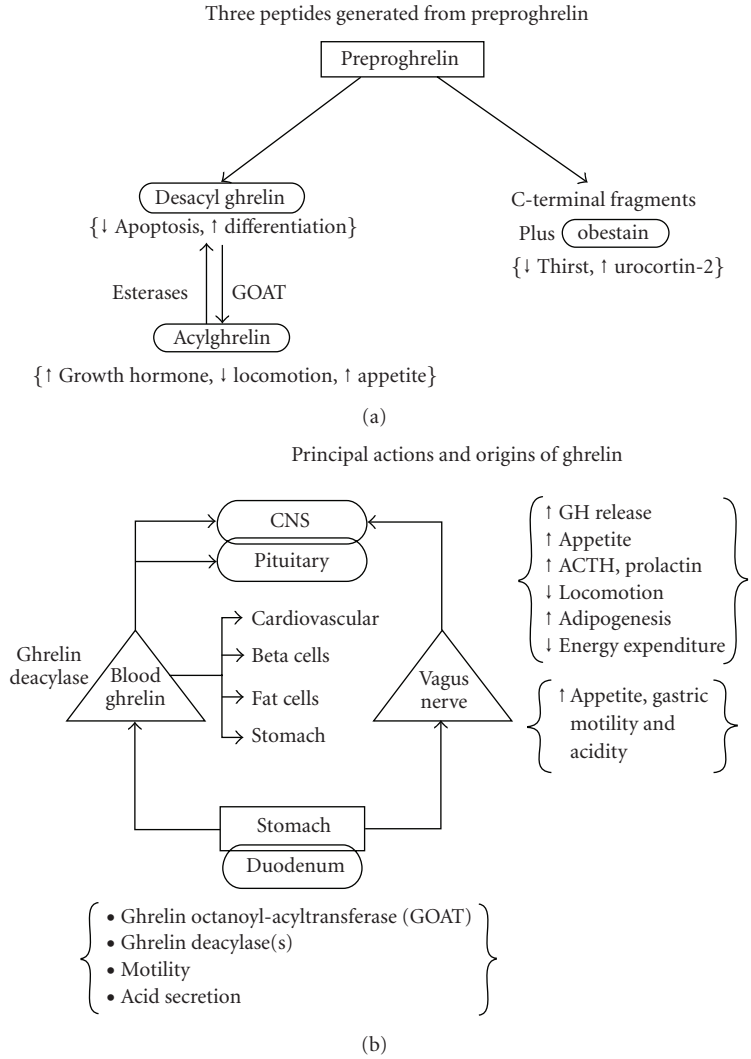

Figure 1: Principal peptide products of preproghrelin (a) and primary actions of ghrelin recognized to date (b) (unpublished line drawing).

GTP-binding protein [25]. GHRH neurons in the arcuate nucleus express GHS-R1a, but hypothalamic ghrelin neurons do not manifest GHRH receptors [4]. Both neuronal ensembles are extensive and project to the external layer of the pituitary stalk [11]. Nonetheless, to date, any direct role for hypothalamic release of ghrelin to the pituitary gland remains undocumented [26]. Ghrelin is unusual in that existence of the receptor was predicted three decades and cloned three years, before the natural ligand was identified by Kojima et al. in 1999 [3]. The ghrelin receptor exhibits high basal activity even unstimulated. Mutational disruption of constitutive GHS-R1a activity is associated with short stature in humans [27], whereas overexpression of GHS-R1a on GHRH neurons augments postweaning growth, reduces fat mass, and augments GHRH and GH gene expression in mice [28]. Thus, GHS-R1a is an amplifier of GHRH outflow as well as a direct effector of pituitary GH secretion. Exogenous GHS partially overcomes GH inhibition by glucose and infused somatostatin [29, 30]. Neurophysiological data and immediate-early gene responses have revealed two additional mechanisms of ghrelin action, namely, partial antagonism of noncompetitive hypothalamic and pituitary inhibition by somatostatin and of hypothalamic melanocortin and leptin pathways [31–40]. Figure 2 depicts a model, which incorporates several major actions of ghrelin/GHS within the GH axis [41]. This model does not include pituitary ghrelin, which is downregulated by excessive thyroxine, glucocorticoid, or brain-GH feedback and upregulated by GHRH [42]. Selective silencing of the pituitary ghrelin gene will be required to discern its physiological role.

2.3. Structural Features. Ghrelin is unique as the first acylated peptide recognized in mammals requiring a short-chain fatty-acid (octanoyl, decanoyl, or decenoyl) moiety linked to the third N-terminal amino acid (usually serine) for primary biological activity, namely. GHRH and GH release, locomotor suppression and appetite stimulation [26, 43–45]. Decanoyl and octanoyl moieties seem equipotent. The enzyme mediating Ser^3 acylation was cloned by the

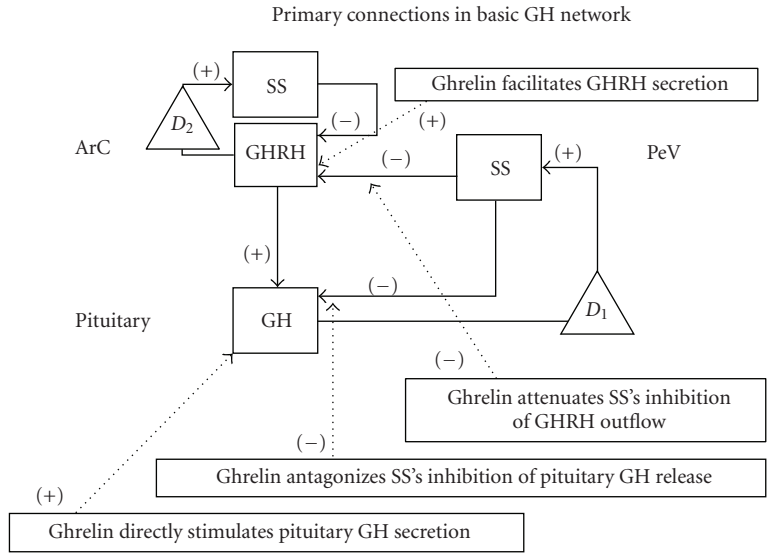

Figure 2: Model-based functional networks subserving GH secretion, showing major effects of GH-releasing hormone (GHRH) and somatostatin (SS) as modified by GHS (ghrelin). D_1 and D_2 denote time delays. ArC and PeV define arcuate and periventricular nuclei (adapted from [41]).

Brown and Goldstein laboratory in 2008 and named ghrelin octanoyl-acyltransferase (GOAT) [46–48]. GOAT is one of 16 members of membrane-bound O-acyltransferases [49]. It is expressed in pancreas, stomach, skeletal muscle, heart, intestine, bone, and other organs and is endproduct inhibited and fatty-acyl substrate and fasting induced [50–52]. GOAT protein and transcripts exist in individual ghrelin-containing gastric mucosal oxyntic cells [53]. Regulation may exist pretranslationally also, since natural antisense ghrelin RNA's can be demonstrated by analysis of human genomic DNA [54, 55]. A second acyltransferase, termed microsomal zinc-stimulated serine (Ser^2 ghrelin) octanoyltransferase, distinct from GOAT, was cloned by Ozawa et al. in 2009 [56]. This enzyme is concentrated in the endoplasmic reticulum of human erythroleukemia cells. Its in vivo role is not yet known but might include negative regulation of GOAT via the octanoylated products generated.

Unacylated ghrelin competes with acylghrelin for GHS-R1a to a negligible degree, namely, with a k_d of 13 μM, which is four orders of magnitude greater than that for bioactive peptide [57]. Deacylases (esterases) of ghrelin exist in the blood and stomach, which may be nonselective [58, 59]. GOAT can utilize both ghrelin (1–4) and ghrelin (1–5) as substrates for serine acylation, consistent with their core structure [46, 60]. Des-Gln acylghrelin (1–27), an alternative transcript lacking glutamine in N-terminal position 14, is also fully active on the GHS-R1a receptor [61]. Ghrelin's amino-acid sequence is significantly conserved in fish, reptiles, amphibia, birds, and mammals [62]. Congeneric molecules include GHRP-2, GHRP-6, hexarelin, ipamorelin, ibutamorelin, and the nonpeptide MK-0677, which like ghrelin rapidly induce inositol triphosphate, diacylglycerol, and Ca^{2+} release via the GHS-R1a [51, 63–67].

2.4. Blood-Borne Ghrelin. Ghrelin concentrations in blood comprise principally desacyl-ghrelin (85%–90%) and in lesser amounts acylghrelin (10%–15%) and C-terminal proghrelin peptides [68, 69]. In large cohorts, ghrelin is higher in women than men [70] and declines with age, body mass index (BMI), hypertension, and other markers of the metabolic syndrome [70]. The inverse relationships between ghrelin and both BMI and insulin concentrations appear to explain much of the age effect [71]. Appropriate sample collection and storage are necessary to limit ghrelin deacylation before assay [72, 73]. Although its exact role is not known, desacyl-ghrelin can exert a variety of agonistic and antagonistic actions [74–76], as discussed further in the relation to the ghrelin receptor. Octanoylated and total ghrelin levels in the stomach and blood rise between meals, during fasting, in cachexia, anorexia, or malnutrition, type I (insulinopenic) diabetes mellitus, after acute endotoxin exposure, overnight during initial deep sleep and in response to estradiol (E_2), acute hypoglycemia, glucagon infusion, vagal stimulation, and chronic octanoate ingestion [77–89]: Figure 3. In mice, plasma bioactive decanoylated ghrelin increases and octanoylated ghrelin paradoxically decreases during fasting [90], suggesting precise posttranslational control [72]. Unlike gastric ghrelin, hypothalamic ghrelin gene transcript and peptide levels fall during short-term fasting [9].

In various studies, serum total ghrelin concentrations correlate positively with E_2, IGFBP-1, and creatinine concentrations, and negatively with somatostatin, insulin, thyroxine, leptin, and testosterone (T) concentrations and arterial blood pressure [70, 82, 86, 91–98]. Thus, ghrelin levels rise not only in fasting individuals but also in estrogen/progestin-treated women [99–101], combined antiandrogen and progestin-treated men [82], and the estrogen-rich milieu of the late follicular phase of the menstrual cycle in one but

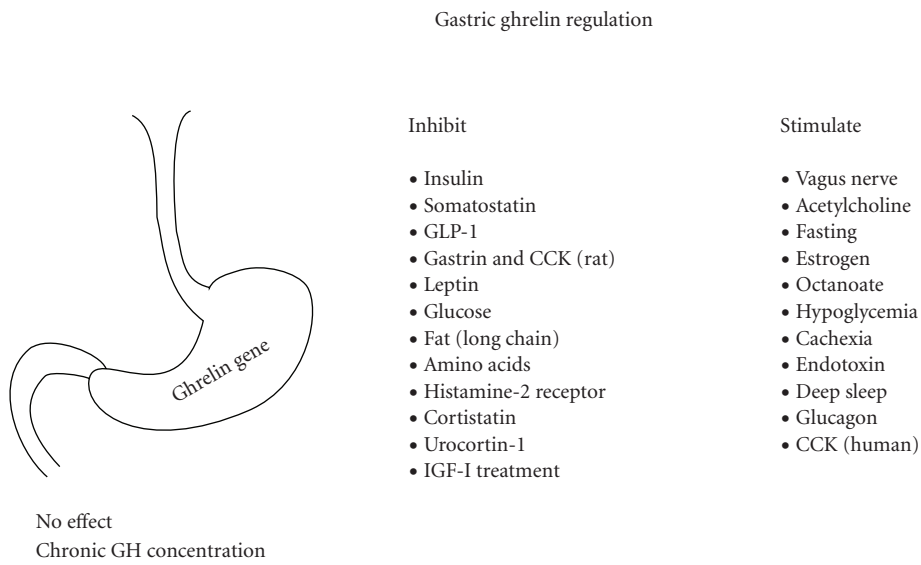

Figure 3: Key hormonal, gastrointestinal, nutritional, stress-related, infectious, and physiological regulators of gastric ghrelin secretion inferred in mammalian species.

not another study [92, 102]. Conversely, ghrelin levels fall in the high-testosterone milieu of male puberty [93]. Gastric ghrelin-secreting cells express estrogen receptor-alpha [103], thus potentially mediating some sex-steroid effects. Indeed, gonadal downregulation in girls and parenteral (but not transdermal) testosterone administration in boys diminish plasma ghrelin concentrations [104, 105]. In addition, clinical investigations indicate that E_2 or T supplementation can potentiate GH secretion stimulated by a fixed submaximal dose of GHS/ghrelin [106–112]. On the other hand, low-dose transdermal E_2 administration did not augment maximal GH stimulation by ghrelin in postmenopausal women [113], and the potentiating action of T administration on GHS was not evident in the dog, rat, or older men [114, 115]. Thus, more data are needed on the developmental dependence of sex-steroidal facilitation of GHS action. The differences before and after puberty do not seem to be due to feedback by differing GH levels, since acute infusion of GH or a GH-receptor antagonist does not feed back onto ghrelin secretion [116, 117]. Chronic GH excess or deficiency and exercise also do not consistently modify ghrelin production in humans or animals [118–120].

Food intake, especially amino acids, glucose but not fructose, dodecanoate, and other long-chain fatty acids, T administration, euglycemic hyperinsulinemia, impaired glucose tolerance, infusion of free fatty acids, somatostatin, cortistatin or urocortin, obesity (elevated body-mass index and increased total, subcutaneous or visceral fat), weight gain, hyperthyroidism, and leptin injection suppress ghrelin concentrations [86, 97, 116, 117, 128–144]. A possible intracellular mediator of fasting and feeding's reciprocal control of ghrelin secretion is the mammalian target of rapamycin [145], which is suppressed by fasting [145]. In general, total ghrelin concentrations nearly double before meals and fall to a nadir about one hour thereafter [146]. Protein (more than lipid) ingestion strongly suppresses plasma acylghrelin, whereas carbohydrate initially suppresses and then elevates circulating ghrelin in humans [147]. Gastrectomy reduces total ghrelin concentrations by 65%–80% (to 20%–35% of baseline) [148], thereby verifying that the stomach is the major source of this hormone.

Acylated ghrelin typically changes in parallel with total ghrelin availability and in the fed state may rise recurrently before GH pulses [149]. Dissociations between acyl and desacyl-ghrelin levels occur after fiber versus total energy intake [150], intravenous glucose infusion [142], in renal failure [86], and in the portal vis-à-vis hepatic veins because of preferential liver extraction of the acylated moiety [142]. In clinical studies, meal-induced depression of ghrelin levels may be attenuated in children compared with adults, and in women with polycystic ovarian syndrome (PCOS) compared with healthy women, but accentuated in African-American compared with Caucasian women [151–154]. The antiandrogen, flutamide, increased ghrelin levels in patients with PCOS, suggesting suppression of ghrelin production via the androgen receptor in this hyperandrogenemic syndrome [152].

2.5. Metabolism of Ghrelin. The metabolic clearance rates of acyl and desacyl-ghrelin injected by bolus in humans differ by several-fold [91]: Figure 4(a). Active ghrelin (1 μg/kg) is removed more rapidly (half-life 21 minutes) than unmodified ghrelin peptide (36 minutes) and partitions into a larger but finite distribution volume consistent with greater lipophilicity. At a higher ghrelin dose (3 μg/kg), half-lives are 47 and 64 minutes for acylated and unacylated peptide. This concentration-dependence suggests interconversion of ghrelin isoforms and/or 2-compartment kinetics of

acylated peptide [155]. Figure 4(b) illustrates that steady-state bioactive-ghrelin concentrations are not saturable at metabolic clearance rates of up to 60 L/kg/day. Acylated peptide binds to plasma high-density lipoproteins containing paraoxonase (an esterase) [156], but the size and fate of this reservoir are not known. An additional gastric ghrelin deacylation enzyme has been identified, lysophospholipase I [58]. The exact degree to which this enzyme regulates ghrelin biosynthesis secretion is still unknown. Desacyl-ghrelin appears to undergo significant renal clearance [86], whereas acylghrelin is extracted substantially by the liver [142, 157].

3. Ghrelin (GHS) Receptor-1a

The ghrelin receptor exhibits high (about 50%) basal constitutive activity [158, 159] and responds to inverse agonists, partial agonists, and allosteric antagonists [160, 161]. In particular, inverse agonists repress basal receptor activity, as defined by inositol-triphosphate, Ca^{2+}, or diacylglycerol signal generation [159, 162]. Since blood ghrelin levels rise between meals and overnight, a ghrelin-receptor inverse agonist might be used to minimize hunger at these times and overnight [163]. In two families, short stature accompanied GHS-R1a mutations that reduced constitutive GHS-R1a activity [27], thereby implying biological relevance of basal receptor activity.

Multiple experimental strategies have been employed to test the biological impact of silencing ghrelin or GHS-R1a activity: Table 2. Consistent outcomes in animal models comprise loss of appetitive, locomotor, and somatotropic regulation by exogenous ghrelin; modest reduction of body weight, IGF-I concentrations, and GH pulses in the female animal; increased fat oxidation; a rise in mean arterial blood pressure; reduced obesity and improved glucose tolerance, but with a potentially higher risk of hypoglycemia during prolonged fasting; and decreased development of fatty diet-induced diabetes mellitus [164–168, 174–185]. Double transgenic knockout of ghrelin and cognate receptor is marked by diminished adult body weight, greater energy expenditure, and higher locomotor activity [169, 186]. Thus, GHS-R1a is a physiological mediator of ghrelin's stimulation of GH secretion, repression of oxygen consumption and locomotor activity, and enhancement of appetite. GHS receptor type 1b arises from a nonspliced transcript, whose product does not bind acylghrelin or confer known bioactivity [5, 187].

Unlike ghrelin, synthetic GHS's acting via GHS-R1a typically do not require acylation and are not known GOAT substrates. Moreover, multiple biological effects have been reported for desacyl-ghrelin, which essentially does not bind GHS-R1a. A partial registry of effects comprises differentiation of skeletal muscle; relaxation of vascular smooth muscle; suppression of proinflammatory cytokines; inhibition of apoptosis of cardiomyocytes, pancreatic beta cells, and endothelial cells; antagonism of ghrelin's stimulation of somatic growth, hepatic gluconeogenesis, and appetite; hypotensive effects; repression of fatty-acid oxidation; and stimulation of adipogenesis [22, 75, 188–201]: Table 3.

Table 2: Experimental strategies for verifying ghrelin action.

Genetic
(1) transgenic silencing of ghrelin gene
(2) transgenic silencing of ghrelin receptor
(3) double knockout
(4) antisense transgene to neuronal ghrelin receptor
(5) overexpression of ghrelin
Immunologic
(1) immunoneutralization
(2) catalytic antibodies
Antagonists
(1) peptides
(2) nonpeptides
(3) RNA Spiegelmer
Infusion of ghrelin or desacyl-ghrelin
(1) agonism and antagonism
Anatomic definition of ghrelin-neural network
(1) transgenic green fluorescent protein-linked ghrelin

Selected References: [11, 65, 164–173]

Although local acylation could explain certain actions of unacylated ghrelin, other effects that oppose or differ from those of acylated peptide raise the possibility of non-GHS-R1a mediation. Synthetic analogs of ghrelin likewise may exhibit partial agonism (e.g., stimulation of appetite but not GH release, and vice versa), antagonism, and inverse agonism despite similar GHS-R1a binding affinities [160, 202–207]. To date, no desacyl-ghrelin and no other GHS receptors have been cloned to explain such data [175].

Acylghrelin, unlike the naked peptide, is a consistently effective agonist of GH secretion in multiple species from fish to mammals [34, 65, 215–221]. Nonacylated synthetic GHS can stimulate GH secretion in occasional models [222–224]. In fish, desacyl-ghrelin may function as an inhibitor of ghrelin's stimulation of appetite and locomotion [225]. In mice, transgenic overexpression of desacyl-ghrelin decreases food intake, gastric emptying, GH (female animal) and IGF-I (both sexes) concentrations, body weight and length, and GH release induced by exogenous ghrelin [75]. CNS delivery of desacyl-ghrelin in the rat likewise impedes food intake and gastric emptying [190, 196]. In humans, exogenous desacyl-ghrelin does not restrict ghrelin-induced GH secretion but disinhibits ghrelin's repression of insulin secretion [210, 221, 226]. Acylated ghrelin, unlike GHRH, does not induce GH-gene transcription. Exceptions include the pituitary glands of embryonic fish and prepubertal rats in vivo and ovine somatotropes in vitro [227–229]. Both GH and IGF-I can feed back to inhibit hypothalamopituitary stimulation by ghrelin/GHS, but feedback inhibition is less marked on GHS than GHRH stimulation [111]. Feedback appears to involve induction of periventricular hypothalamic somatostatin outflow [230], which quenches both GHRH and GH secretion [4].

Table 3: Reported actions of desacyl-ghrelin.

Effect of desacyl-ghrelin	Compared with acylghrelin shared or opposite	Reference
Adipocytes		
(1) ↓ fat oxidation		
(2) ↑ glucose uptake		
(3) ↑ differentiation	shared	[195, 199]
(4) ↑ hypertrophy		
(5) ↓ lipolysis		
anorexigenic	opposite	[190, 196]
Antiapoptotic		
(1) islet beta cells	shared	[22, 194]
(2) cardiomyocytes		
antiinflammatory	shared and unshared	[188, 200]
body weight	opposite	[75]
cardioprotection	shared	[194, 201]
gastric motility	opposite	[196, 208]
↓ hepatic gluconeogenesis	opposite	[198]
hypotension	shared	[197]
locomotion	unknown	[209]
↑ insulin sensitivity	opposite	[210]
neurogenesis	shared	[211–214]
skeletal-muscle differentiation	shared	[193]
↓ somatic growth	opposite	[75]
vascular smooth-muscle relaxation	similar	[197]

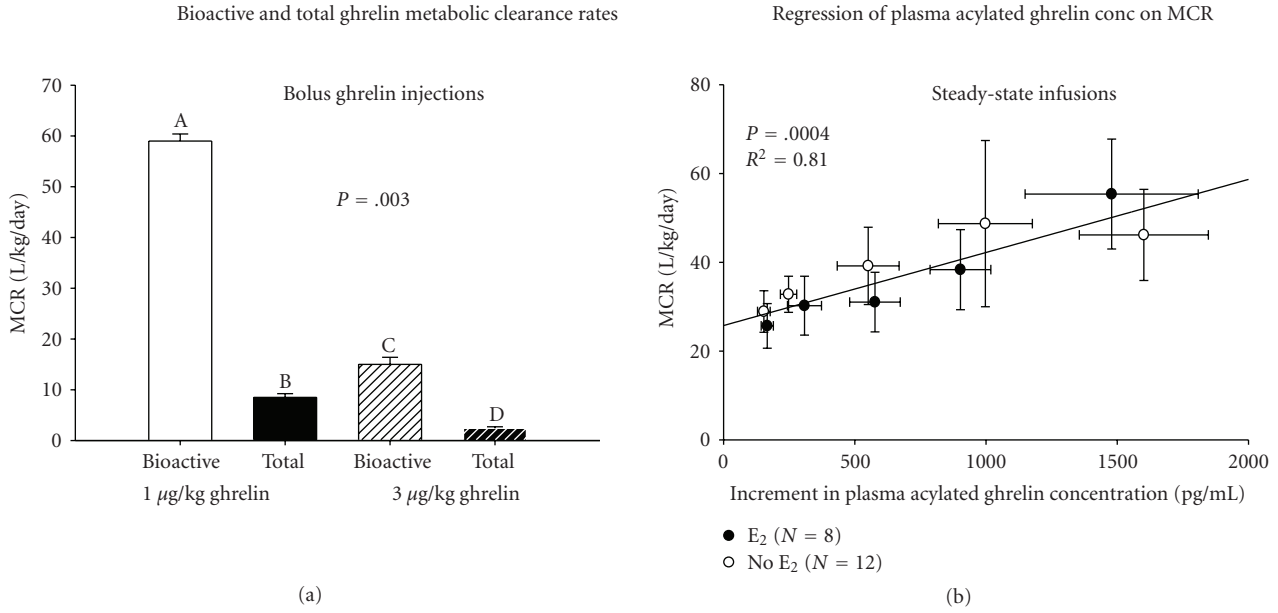

Figure 4: Strong impact of bolus ghrelin dose and ghrelin isotype (acylated [bioactive] or total ghrelin) on the metabolic clearance rate (MCR) of ghrelin in postmenopausal women. Means with different superscripts differ significantly by *post hoc* analysis after ANOVA ($P = .003$) (a). Linear relationship of steady-state MCR of acylated ghrelin to plasma acylghrelin concentration during constant ghrelin infusion (b). Adapted from [91] with permission.

4. Ghrelin and GHRH Synergy

Active ghrelin acts as an amplifier of GHRH, the primary agonist of GH secretion [4]. Human and murine hypothalami contain a wide network of ghrelin-expressing neurons, which extends across the arcuate, ventromedial, paraventricular and multiple other nuclei [8–11, 231–235]. Antisense RNA-mediated silencing of the murine GHS-1a receptor localized to GHRH neurons resulted in reduced adult weight, fat mass, pulsatile GH secretion, and IGF-I production in the adult female only [165]. Arcuate-nucleus GHRH-gene expression also declined in these animals [236], indicating that GHS-R1a not only transduces GHRH release but also maintains GHRH gene transcription [237, 238]. Consistent with this inference, GHS' stimulation of maximal (5-35-fold) GH release requires an intact hypothalamopituitary unit allowing GHRH outflow to the pituitary [239–242]. Accordingly, GHRH (−/−) mice, GHRH-receptor (−/−) mice, and rare patients with inactivating mutations of the GHRH receptor or congenital aplasia of the pituitary stalk are hyposomatotropic, and respond sparingly (<3-fold) to ghrelin/GHS [241, 243–246]. Immunoneutralization of GHRH and antagonists of GHRH also restrict GHS's effects markedly, resulting in responses of somatotrope cells to GHS similar in magnitude to those in inferred directly in vitro [1, 247].

Ghrelin or synthetic GHS achieves synergy with GHRH (supraadditive stimulation of GH secretion) when a near-threshold dose of GHS is combined with a maximally effective dose of GHRH in the human, rat, pig, cow, and dog in vivo [248–255]. Synergism is not observed after combined stimulation with either maximal GHRH and maximal GHS or submaximal GHRH and maximal GHS stimulation [65, 254]. Synergistic stimulation of GH release is absent in pituitary cells cultured in vitro and in patients with destructive lesions that separate the hypothalamus and pituitary gland [65, 216, 219, 242, 250, 256, 257], thus defining a critical role for joint hypothalamopituitary effects. Nonetheless, precise mechanisms subserving synergy are not established. Proposed mechanisms include ghrelin-mediated (a) opposition to hypothalamopituitary actions of SS and/or (b) stimulation of an unknown ("U") synergy factor [4, 29, 65, 151, 255, 258–260]. A substance like galanin or an endogenous opiatergic peptide might represent such a factor, since both peptides release and synergize with GHRH and their neurons innervate periventricular SS neurons [261–267]. Reduced pituitary action of SS is unlikely to be the sole potentiating mechanism subserving GHS action, since GHS and GHRH synergize even after immunoneutralization of SS [268]. Although GHRH can induce the pituitary ghrelin and GHS-R1a genes [42], the physiological impact of this potentially amplifying mechanism is not known.

Since highly selective GHS-R1a antagonists are not available for clinical investigation, determining the exact extent to which endogenous ghrelin participates in amplifying GH secretion in human physiology, including *in utero*, in infancy, childhood, puberty, adulthood, and senescence remains difficult [4, 65, 250]. Nonetheless, mutational reduction of constitutive ghrelin-receptor activity in humans is associated with short stature [27]. Conversely, overexpression of neuronal GHS-R1a in female mice elevates GH and GHRH gene expression [28]. In addition, prolonged administration of ghrelin or synthetic GHS in humans augments GH, IGF-I and IGFBP-3 concentrations and lean-body mass, and diminishes total-body fat, while eliciting transient secretion of adenocorticotropin hormone (ACTH), cortisol, and prolactin [64, 269–273]. The last observation is significant, because higher doses of ghrelin/GHS evoke ACTH, cortisol, and prolactin secretion acutely in humans and animals, putatively by inducing hypothalamic secretion of one or both primary ACTH-releasing peptides, corticotrophin-releasing hormone (CRH), and arginine vasopressin (AVP) [272, 274–278]. How GHS induces prolactin release is not clear. Doubling or tripling plasma ghrelin concentrations evokes GH secretion in humans without measurably altering ACTH, prolactin, insulin, or free fatty-acid concentrations [279]. The basis for the dose-response difference and tachyphylaxis of the corticotropic, but not the GH-releasing, effect of GHS is not yet evident [280]. Likewise, why age and obesity impair GHS-induced secretion of GH but not of ACTH and prolactin is not known [113, 281].

Depending upon chemical structure and dose, synthetic GHS can stimulate GH secretion after intranasal, oral, s.c., or i.v. administration [4, 65, 250, 272, 282, 283]. Entry of acylghrelin (but not desacyl-ghrelin) into and exit of the same from the brain is via structurally selective saturable transport [284–286]. Since hypothalamic GHRH release is prerequisite to maximal GH stimulation, GHSs have clinical therapeutic potential in treating: (i) idiopathic short stature in patients with preserved CNS outflow of GHRH [64, 287–290]; (ii) hyposomatotropism in aging, wherein GHRH release may be diminished but is not absent [271, 291, 292]; (iii) hyposomatotropism associated with visceral adiposity, including the HIV lipodystrophy syndrome [167, 179, 190, 293–295]; and (iv) conditions of partial GH resistance accompanying anorexia, cachexia, or heightened catabolism, such as metastatic cancer, hepatic or renal failure, chronic obstructive pulmonary disease, systemic inflammation, and advanced heart failure [133, 250, 296–298]. Preclinical data in these areas and clinical data in healthy subjects suggest the merit and feasibility of more comprehensive investigations [270, 271, 299–301], since prospective, double-blind, placebo-controlled trials are lacking. Critical issues to be resolved include long-term safety, duration, efficacy, and indications for GHS administration in selected GH-deficient states.

5. Multifaceted Actions

Short-term ghrelin/GHS administration stimulates GH secretion, locomotor activity, and appetite, increases plasma free fatty acids, imposes mild peripheral (muscle) insulin resistance, suppresses insulin secretion, inhibits fat oxidation, and promotes adipocyte glucose metabolism [4, 65, 170, 209, 210, 302–307]. Moreover, GHS is both antiproliferative and proliferative depending upon cell type [308–310].

These and other multifaceted actions of ghrelin are discussed next.

5.1. Appetitive Effects of Ghrelin. Ghrelin consistently enhances appetite by 25%–30% in fasting humans and animals with the possible exception of chicken, quail, and sheep [37, 39, 295, 311–315]. Endogenous ghrelin may enhance anticipatory motor activity, before scheduled meals [316]. Active ghrelin stimulates and suppresses glucose-sensitive neurons in the lateral and ventromedial hypothalamus, respectively, stimulating hunger and inhibiting satiety [317]. The orexigenic effect arises by combined activation of neuropeptide Y/agouti-related peptide (NPY/AGRP) and orexin A neurons [314, 318–322]. Concomitantly, acylghrelin antagonizes satiety-promoting and anorexigenic signals, such as leptin [38, 323], corticotropin-releasing hormone (CRH), cocaine and amphetamine-regulated transcript (CART), proopiomelanocortin (POMC), and alpha-melanocyte-stimulating hormone (alpha-MSH) [324–326]: Figure 5. Obestatin is not pictured because of its controversial role in appetite. AGRP is an endogenous inhibitor of alpha-MSH, thereby promoting positive energy balance. Ghrelin may act in part by inducing the intracellular signal mammalian target of rapamycin in AGRP neurons, a messenger which promotes protein synthesis and limits apoptosis [327]. Both NPY and AGRP participate in the orexigenic action of ghrelin, since transgenic disruption of both mediators is needed to quench appetite [303, 321]. The potent orexigen, orexin (hypocretin), which is a key peptide in maintaining wakefulness [328], is also involved in the appetitive action of ghrelin, in that orexin-A immunoneutralization or receptor silencing attenuates ghrelin's orexigenic action [329]. A key neuronal biochemical mediator of energy-depletion mediated appetitive drive may be the low-ATP sensing protein kinase, AMP kinase, which is stimulated by ghrelin and cannabinoids [330]. Desacyl-ghrelin exerts opposite effects, namely. decreases food intake, fat mass, and gastric emptying [190, 293]. Ghrelin also suppresses pancreatic beta-cell insulin secretion, which may contribute to orexigenesis, given that insulin can function as a CNS satiety signal [331].

The peripheral vagus nerve and its dorsal motor nucleus in the brainstem and ventral tegmental neurons mediate some of systemic ghrelin's appetitive effects [332–336]. Hunger-suppressing vagal afferent impulses from the stomach are subdued by ghrelin and by other orexigenic hormones, acting via the GHS-R1a, cannabinoid-1 (CB-1), and melanin-concentrating hormone-1 (MCH-1) and activated by anorexigenic receptors, like cholecystokinin-A (CCK-A), peptide YY (3–36), and glucagon-like peptide-1 [334, 337]: Figure 6. In fact, a CB-1 antagonist impedes ghrelin's appetitive effect, illustrating key facilitative interactions [338]. In addition, a CCK antagonist abolishes the capability of intraduodenal fat to suppress gastric ghrelin secretion [339], consistent with gastroduodenal feedback [340]. The dorsal motor nucleus of the vagus expresses GHS-R1a, which modulates limbic-system dopamine- and brainstem gaba, glutamine, and noradrenergic transmission to hypothalamic appetitive and satiety centers [335, 341–343]. Dorsal vagal neuronal GHS-R1a levels may decline with aging in the rat [344]. Ghrelin crosses the blood-brain barrier and directly inhibits leptin neurons and stimulates NPY/AGRP and orexin neurons [333], which are located, respectively, in the arcuate nucleu and lateral hypothalamus. MCH inhibits lateral hypothalamic neurons, providing additional feedback control [345]. Inhibition of brain (rather than exclusively peripheral vagal) GHS-R1a seems to explain the anorexigenic properties of ghrelin antagonists [346, 347].

Although genetic disruption of GHS-R1a abrogates orexigenic stimulation by ghrelin, cachexia does not develop in the transgenic animals, including those with combined ghrelin and GHS-R1a knockout, putatively reflecting signal redundancy within nutritional networks [167, 353–355]. In particular, pathways mediating orexigenesis and satiety are convergent, oppositional, adaptive, and complex [186, 295, 319, 356–358], involving hormones from endocrine glands as well as gut mucosa and vagal afferent signals [35, 38, 359–362]. For example, intestinal mucosal L-cell-derived oxyntomodulin, a satiety signal, acts via the hypothalamus to inhibit vagally driven gastric secretion of acylghrelin, thus quelling hunger [363]. Plasma leptin and ghrelin concentrations tend to vary inversely in various clinical settings [364], and CNS leptin is a strong ghrelin antagonist [35], possibly by modulating NPY-Y1 or Y5 signaling [8, 38]. GHS-R1a is subject to systemic modulation, including upregulation by thyroxine and E_2 and downregulation by glucocorticoids and GH [122]. In addition, E_2 inhibits the acute orexigenic effect of exogenous ghrelin in the male and female rat [123]. Accordingly, the ghrelin system is a pivotal but nonexclusive member of a robust nutritional network in mammals [10, 294, 314, 319, 365–368]. However, ghrelin reduces food intake in neonatal chickens, possibly by elevating brain fatty-acid synthesis [369].

GHS receptors are located in the stomach, gastrointestinal myenteric plexus, vagal nodose ganglion, dorsal motor nucleus of the vagus, arcuate-nucleus orexigenic neurons, such as NPY/AGRP and orexin A-expressing cells, anorexigenic neurons that are either leptin-sensing or leptin-producing, and multiple other unidentified neurons [314, 370, 371]. Silencing any one of NPY, AGRP or orexin-A-receptor genes limits but does not abolish ghrelin's orexigenic effect [329, 372–374]. Triple negation of NPY, AGRP, and orexin is necessary to eliminate ghrelin's appetitive effect [375]. Small interfering DNA-mediated neutralization of paraventricular neuronal GHS-R1a in rats imposes weight loss and reduces serum ghrelin without altering food intake, suggesting effects on energy expenditure as well [376]. Network robustness is conferred by the facts that ghrelin activates whereas leptin represses both NPY/AGRP, and orexin neurons; orexin A potentiates (feeds forward onto) NPY release via the orexin-1 receptor; NPY represses orexin release via NPY-Y1 receptors; neuropeptide W and galanin-like peptide further modulate orexin neurons [377, 378] NPY and orexin, respectively, suppress (feed back) and induce (feed forward onto) the potent anorexigens, leptin, and proopiomelanocortin, which in turn feed back on ghrelinergic cells [186, 235, 379–387]. Moreover, NPY and ghrelin

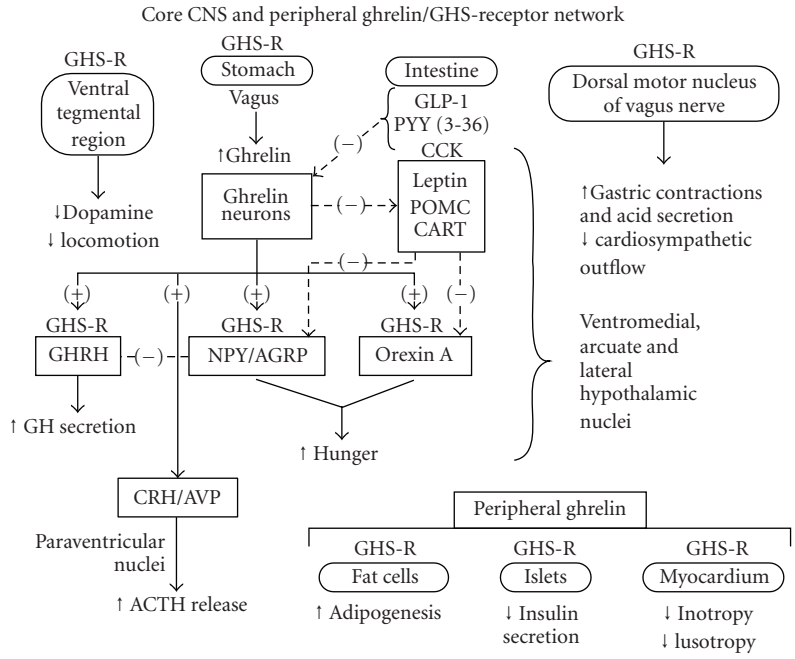

Figure 5: Basic ghrelin network influencing locomotion (*left upper quadrant*), gastrointestinal signals to appetitive and anorexigenic centers (*middle section*), gastric motility (*right upper quadrant*) or peripheral target tissues (*right lower segment*). Unpublished schema.

neurons synapse on themselves, suggesting autofeedback-dependent regulation [235]. Collective feedforward/feedback circuits presumably subserve enhancement and suppression of food intake in a manner defined by sex, species, age, physical activity, ultradian rhythmicity, and sleep-wake cycles [35, 231, 295, 328, 388]. For example, sleep curtailment may augment appetite by simultaneously lowering circulating leptin and elevating ghrelin levels. The latter correlates with meal initiation [389]. These and other experiments illustrate the complex adaptability of nutritionally regulated neural networks [234, 278, 353, 362, 390].

A 23-amino-acid C-peptidyl fragment of preproghrelin, named *obestatin*, has not fulfilled its original nomenclature as an antagonist of ghrelin's orexigenic effects [394–399]: Figure 7. In addition, transgenic knockout of the proposed obestatin receptor, GPR39, does not affect food intake [394, 396, 400, 401]. Whereas obestatin (probably better termed, ghrelin-associated peptide) does not alter pituitary hormone secretion [402], this peptide may suppress thirst, promote beta-cell survival and regeneration, activate urocortin-2 pathways and thereby inhibit gastroduodenal peristalsis, and induce early-response genes in gastric mucosa and preadipocytes [403–410]. A physiological distinction pertinent to CNS actions of ghrelin, but not obestatin, is that only ghrelin exhibits specific saturable binding to and endocytosis by cerebral microvessel endothelial cells, thereby allowing measurable permeation of the blood-brain barrier [284, 286]. However, obestatin attenuates the hypothermic effect of preproghrelin gene deletion [411].

Transgenic overexpression of CNS and gastric ghrelin causes hyperphagia, increases energy expenditure, and decreases sensitivity to insulin and leptin in mice [170]. The orexigenic action of ghrelin does not seem to wane in this model, since adult transgenic animals continue to eat excessively after fat depots reach a maximum [412]. In a second model, 8 weeks of GHS treatment maintained orexigenesis in the rat [413]. In a third model, overexpression of GHS-R1a in GHRH neurons increased organ and muscle weight, while decreasing body fat in female animals [28]. Conversely, in a fourth model, transgenic reduction of GHS-R1a expression on GHRH neurons suppressed both GHRH and NPY gene expression, thereby diminishing GH and fat mass in the female [236]. In patients with renal or cardiac failure or chronic lung disease and cachexia, short-term (up to 3 weeks) administration of ghrelin once or twice daily may stimulate appetite and weight gain as assessed in uncontrolled studies [298–300, 414, 415]. However, in the perioperative orthopedic setting, ghrelin infusions for 3 weeks did not improve overall functional physical performance [416]. Nonetheless, the precise extent to which GHS agonists and antagonists will alter hunger or satiety over the longterm remains difficult to forecast [74, 232]. This issue is important since studies with leptin, an anorexigenic adipokine that antagonizes ghrelin, revealed tachyphylaxis to satiety-promoting effects [186, 417].

Ghrelin itself undergoes tissue-specific regulation by short-term fasting, hypoglycemia, and nutrient deprivation. These factors depress brain ghrelin expression, while promoting gastric ghrelin secretion and amplifying CNS responses to ghrelin [9, 418, 419]. In species in which desacyl-ghrelin can antagonize the orexigenic effects of acylghrelin [293], regulation of GOAT activity in both

Table 4: Cardiovascular actions of GHS.

↓ mean arterial pressure
↑ inotropy (myocardial tension generation)
↓ lusitrophy (tension relaxation)
↓ ventricular end-systolic pressure
↓ cardiomyocyte apoptosis
↓ endothelial apoptosis
↓ pulmonary hypertension
↑ renal perfusion
↑ coronary perfusion pressure
↑ left-ventricular ejection fraction
↓ oxygen consumption
↓ cardiac sympathetic drive

Selected References: [15, 22, 194, 197, 201].

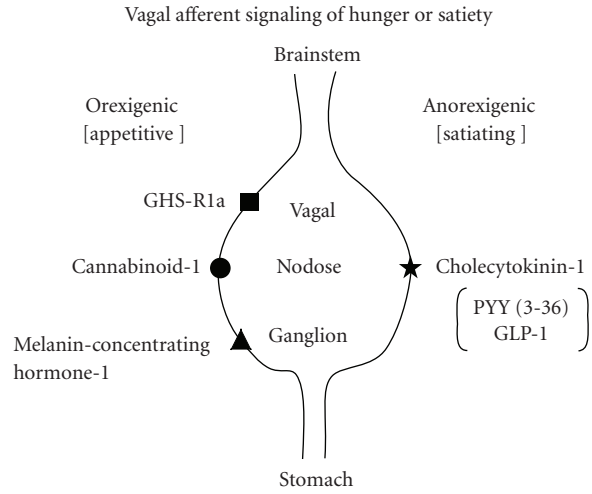

Figure 6: Complementarity of ghrelin's vagal-nerve signaling via GHS-R1a with that of other orexigenic (*left*) or anorexigenic (*right*) peptides. PYY: polypeptide YY; GLP-1: glucagon-like peptide. Unpublished sketch.

the stomach and brain may be important to appetitive homeostasis [191, 196]. GHS analogs that do not require acylation have the merit of bypassing GOAT [420].

Gastric-bypass procedures, especially when combined with truncal vagotomy, often reduce serum total ghrelin concentrations initially [426, 427]. In principle, reduced ghrelin availability would further attenuate adipogenesis [191, 195, 199]. However, in some studies prolonged weight loss after gastric bypass had no effect on fasting ghrelin levels [250, 428, 429] or reduced only acylated ghrelin [430]. In rodents, vagotomy impedes both appetitive and GH-stimulating effects of ghrelin [334, 336, 431, 432]. In humans, pharmacologic or surgical interruption of vagal cholinergic traffic lowers ghrelin concentrations and blunts appetitive stimulation but does not block GHS-induced GH release [336, 431, 433].

5.2. Insulinostasis. Ghrelin inhibits insulin (beta cells) and somatostatin (delta cells) but stimulates glucagon (alpha cells), secretion in vivo and in isolated pancreatic islets in vitro [304, 434–439]. Some early studies reported that GHS induces insulin secretion [440]. Inconsistencies may reflect the capabilities of desacyl-ghrelin to suppress hepatic glucose production and/or block acylghrelin's inhibition of insulin secretion [198, 221, 434, 441]. Other studies did not control for the fact that acutely elevated GH and glucose concentrations (induced by GHS) stimulate insulin secretion acutely. GHS receptors are expressed by both beta and alpha cells [442]. Ghrelin's inhibition requires Gi, which activates outward K^+ currents and disables inward Ca^{2+} flux [443, 444]. These effects particularly impede rapid first-phase insulin release [444]. Moreover, acylghrelin antagonizes peripheral insulin action (muscle > liver), which effect is reversed by desacyl-ghrelin in GH-deficient adults [210]. However, desacyl-ghrelin has little if any effect on glucose, insulin, free fatty acid, or GH releases in (obese) humans [445].

From a reciprocal perspective, insulin and somatostatin repress, whereas glucagon induces the gastric ghrelin gene in the rat [88, 124, 130]. In humans, glucagon injection inhibits ghrelin secretion [121], but via uncertain pathways. In rodents, insulin represses not only gastric but also CNS preproghrelin expression [446]. In accordance with insulin's negative effects, hyperinsulinemia associated with hepatic or skeletal-muscle insulin resistance and/or obesity correlates inversely with fasting ghrelin concentrations [71, 447, 448]. An exception is the genetic Prader-Willi syndrome marked by obesity, mild insulin resistance, and unexplained hyperghrelinemia with increased numbers of gastric ghrelin-expressing cells [449–451].

Adult pancreatic islets express relatively little ghrelin protein and few GHS receptors, but fetal and neonatal islets exhibit abundant ghrelin gene transcripts and peptide in epsilon cells [452–454]. Although both acyl and desacyl-ghrelin promote beta-cell regeneration [408, 455], endogenous pancreatic ghrelin inhibits beta-cell function, since perfusion of the pancreas with ghrelin antiserum augments insulin secretion [408, 438, 444, 456]. In addition, genetic knockout of ghrelin and administration of GHS-receptor antagonists diminish fasting hyperinsulinemia and enhance glucose tolerance in rodent models [205, 439, 443, 444]. Thus, in knockout ghrelin mice, increased constitutive ghrelin-receptor activity may result in increased insulin secretion, which would be susceptible to inhibition by a GHS receptor antagonist with inverse agonist activity. Further investigations are required to appraise the influence of diet, age, gender, species, and obesity on pancreatic actions of ghrelin, given the high importance of drug development in this area. A desirable ghrelin antagonist would limit appetite, disinhibit insulin secretion, and minimize adiposity (by favoring fat oxidation) in patients at risk for the metabolic syndrome or type II diabetes mellitus [84, 205, 223, 457, 458]. Table 4 highlights some of these issues and identifies potential adverse consequences of prolonged GHS-receptor blockade.

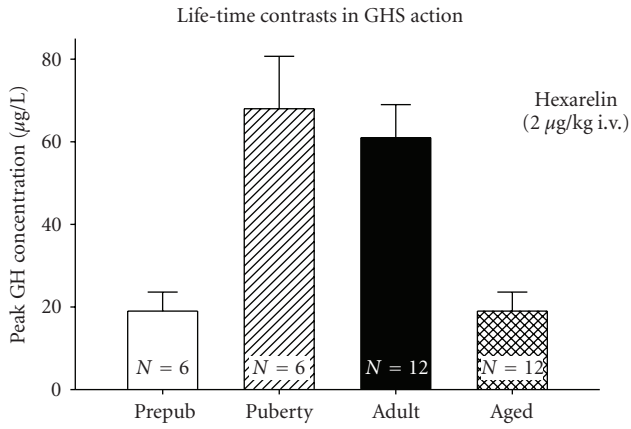

Figure 7: Lifetime variations in GHS (hexarelin) action to induce GH secretion in prepubertal, pubertal, adult, and aged humans (redrawn with permission from [488]).

Table 5: Reported modulatory messengers of ghrelin.

Messenger/mediator	Site/mechanism
(1) phospholipase C	GHS receptor-transfected cells
(2) protein kinase C	neurons
(3) cAMP potentiation	pituitary
(4) nitric oxide	vasculature
(5) urocortin-2 receptor	pancreas, stomach
(6) mitogen-activated protein kinase	cardiomyocytes
(7) extracellular-regulated kinase	cardiomyocytes
(8) potassium and calcium channels	islets, somatotropes
(9) AMP kinase	neurons, gastric mucosa

Selected References: [21, 22, 208, 238, 294, 348–352].

5.3. Cardiovascular Effects. At doses that induce maximal GH secretion, ghrelin and GHS can exert direct vasodilatory, cardiotropic, and brainstem-mediated hypotensive effects, typified by a 10%–20% decrease in mean arterial blood pressure and an increase in left-ventricular ejection fraction [14, 183, 201, 459–464]. Desacyl-ghrelin also causes vasodilation and opposes cardiomyocyte and endothelial apoptosis [194, 465]. Conversely, a ghrelin antagonist elevates heart rate and arterial pressure in the conscious rat [183]. The mechanisms of ghrelin's effects include central suppression of sympathetic cardiac drive, systemic vasodilation, antagonism of angiotensin II and endothelin-induced vasoconstriction including of the pulmonary arteries, and potentiation of nitric oxide-mediated relaxation of vascular smooth muscle [14, 20, 197, 465–471]: Table 4. GHS receptors exist on endothelium, vascular smooth-muscle cells, and cardiomyocytes [13, 201, 472, 473]. Furthermore, ghrelin transcripts and protein are expressed in endothelial cells and cardiac muscle [466, 472]. Both ghrelin and desacyl-ghrelin reduce the rate of myocardial tension generation (negative inotropic effect) and the rate of tension relaxation (negative lusitropic effect), possibly in part independently of the GHS-1a receptor [13, 201, 330, 474]. Obestatin does not initiate these responses [475]. The membrane glycoprotein, CD36 initially recognized as a macrophage type B LDL-scavenger receptor and recently as a fatty-acid binding protein, may transduce or modulate certain cardiovascular and anti-inflammatory effects of ghrelin [188, 207, 474].

The capability of ghrelin to limit apoptosis of endothelial cells and cardiomyocytes in vitro introduces the possibility of reducing endothelial dysfunction associated with atherosclerotic risk [476–478] and salvaging myocardium in zones of marginal chemotoxicity [479] or ischemia [22, 194, 461, 480]. Desacyl-ghrelin shares cardioprotective effects, but the mechanism is not known [194]. Increased coronary-artery perfusion pressure, reduced cardiac sympathetic activity, antagonism of L-type Ca^{2+} channel-mediated contractility, and activation of low-energy AMP-kinase may contribute to cardiotropism [461, 481, 482]. Resultant effects are reduced left-ventricular endsystolic volume and increased cardiac output [414, 460]: Table 5. How much of this benefit is due to stimulated secretion of GH, which exerts cardiotropic effects, is not known [483].

Elevated ghrelin concentrations in patients with chronic heart failure may indicate a compensatory mechanism in particular [484] or reflect partial tissue resistance to ghrelin in cachetic states more generally [83, 485]. Three- and four-week pilot studies of exogenous ghrelin's anabolic and anticatabolic effects in chronic cardiac cachexia in the rat and human seem favorable [194, 301, 461, 486], although parallel-cohort prospectively randomized double-blind controlled clinical studies are not available [414]. Collective data invite more rigorous interventional studies in experimental models of both early and advanced myocardial injury and failure. In addition, further investigations are necessary to assess how GHS affects perfusion of the brain, skeletal muscle, kidney, liver, and intestine [296, 487].

5.4. Gastric Motility. Ghrelin is expressed in granules in gastric X/A-like oxyntic cells [489, 490]. The same granules contain immunoreactive motilin, a comparably strong but distinct prokinetic peptide [491, 492]. Obestatin is additionally present in many of the same cells, but its gastrointestinal function is not known [189, 404]. In one report, obestatin was able to elicit pancreatic zymogen secretion [493]. In other contexts, desacyl-ghrelin and obestatin, unlike ghrelin, inhibit gastric antral contractions [189]. Ingestion of medium-chain triglycerides in the infant or adult enhances gastric acylghrelin concentrations [494, 495]. Inactivation of prohormone convertase 1/3 (a preproghrelin endopeptidase) also increases ghrelin gene transcripts, suggesting negative feedback by ghrelin on its own transcription [496]. Fasting induces the preproghrelin gene in fish and mammals via efferent vagal cholinergic signals [497–499]. The cephalic (meal-visualization) phase of digestion in humans clearly depends on vagal efferents, which transduce prandial inhibition of ghrelin secretion [500]. In sheep, cholinergic blockade stimulates stomach ghrelin output [311], suggesting baseline cephalic phase-like inhibition in this species.

Motilin and ghrelin receptors are 36% homologous in peptide sequences; yet each is activated principally by the homologous agonist at physiological concentrations [492, 501, 502]. Further unlike GHS-R1a, the motilin receptor exhibits little constitutive activity. The GHS-R1a gene is conserved with 58% nucleotide homology in puffer fish and humans [63]. The receptor is present in the myenteric (neuronal) plexus, gastroduodenal mucosa, smooth muscle, and vagal nodose ganglia [503, 504]. Ghrelin stimulates smooth-muscle contractions and type III primary migrating-motor complexes in the stomach and proximal duodenum of the eel, rat, rabbit, mouse, guinea pig, and human; inhibits postprandial contractions (dog) and gastric accommodation (decreases residual stomach volume); and promotes gastric-acid secretion, which facilitates protein hydrolysis and Ca^{2+} absorption [505–511]. Current promotility drugs for the treatment of idiopathic, postvagotomy and diabetic gastric atony, and morphine-induced postoperative ileus include serotoninergic-3 agonists, dopamine antagonists, and motilin-receptor agonists, like erythromycin [492, 512–514]. Whereas desacyl-ghrelin may block certain promotility effects of ghrelin [208], ghrelin is unique in acting on all four of gastroduodenal myenteric neurons, smooth-muscle cells, the vagus nerve, and brain GHS-1a receptors to augment gastric emptying and lower pH [333, 512]. An additional effect is gastroprotection against alcohol and ischemic mucosal stress [515]. Histamine-2 agonists and gastrin synergize in promoting preprandial acid secretion [516, 517], thus illustrating intragastric interactions with ghrelin.

Preclinical and clinical data indicate that GHS-receptor agonists can accelerate gastric emptying even in the presence of autonomic denervation, and reduce postoperative gastrointestinal ileus [492, 506, 509, 511, 512, 518–522]. Patients with diabetic gastroparesis may have low plasma total ghrelin levels, possibly due to vagal denervation and/or chronic glucagon excess [121, 523, 524]. What is unclear is the risk/benefit ratio of systemic GHS treatment in patients with GI bleeding, autonomic neuropathy, and/or concurrent postoperative needs for narcotics, anticholinergics, and other drugs. Concerns would include possible hypotension due to GHS-mediated relaxation of vascular smooth muscle and negative cardiac inotropy and possible deterioration of glucose tolerance due to suppression of insulin secretion and augmentation of hepatic glucose output [198]. Thus, high agonist selectivity will be necessary to ensure clinical safety.

5.5. Sleep-Wake Regulation. Ghrelin concentrations rise during the first four hours of normal sleep in humans [525]. The mechanism is not known. Viewed conversely, ghrelin administration enhances slow-wave (non-REM) sleep including in young and older men but not women [526–530]. In one clinical study, the GHS peptide, hexarelin, reduced non-REM sleep whereas GHRP-6 increased the same, suggesting GHS-receptor pleiotropy or multiplicity [531]. How ghrelin can enhance deep sleep and yet activate orexin-A neurons [328], which are coupled to arousal-wakefulness and appetite, is not yet clear [328, 532]. In addition, why patients with obstructive sleep apnea, albeit obese, have elevated acylghrelin concentrations has not been elucidated [533]. However, reduction of hypoxic episodes decreased ghrelin, suggesting that ghrelin is a stress-responsive hormone.

5.6. Cell Survival and Cachexia. Ghrelin and GHS analogs exert differentiative as well as proliferative and antiproliferative effects in vitro and in vivo [193, 211, 455, 534]. Examples include mitogenic and antiapoptic actions on pancreatic islets, spinal-cord, cortical and brainstem neurons, osteoblasts, fetal lung branches, endothelium, cardiomyocytes, and adipocytes on the one hand, and apoptotic effects in certain adrenal, lung, and prostate cancer cell lines on the other hand [15, 21, 22, 192, 211–213, 309, 391, 480, 535–541]. Several cell-cycle effects can be induced by incubation with either acyl- or desacyl-ghrelin, raising the possibility of involvement of both GHS-R1a and non-GHS-R1a receptor pathways [58, 537]. This reflects the fact that GHS-1a is essentially unresponsive to desacyl-ghrelin [22, 177]. Short-term ghrelin/GHS administration to enhance neuronal regeneration after ischemic or toxic insults thus represents another major point of potential therapeutic focus [214, 542, 543]. Because ghrelin can also stimulate proliferation of certain carcinoma cell lines in vitro [308, 309, 544], studies to exclude longterm oncogenic effects are needed.

Inasmuch as appetite wanes in the cachectic stage of carcinomatosis, ghrelin is being evaluated as an anticachectic agent. Studies in the rat, mouse, and human indicate that ghrelin/GHS administration can dose-dependently enhance appetite, body weight, and fat mass in several short-term models of disseminated neoplasia [545–549]. Caveats are that rigorous controls and blinding are often lacking, and evaluations are necessarily of short duration initially. In addition, some studies do not show orexigenic benefits [550, 551]. A preliminary study in 12 cachectic patients with dialysis-dependent kidney failure showed appetitive enhancement by ghrelin compared with randomized double-blind placebo injection over a 1-week interval [415].

5.7. Adipogenesis. Prominent effects of ghrelin/GHS include direct promotion of preadipocyte proliferation and adipocytic differentiation and hypertrophy [76, 552]: Table 6. GH-independent CNS actions may participate in lipogenesis [553]. Desacyl-ghrelin shares several of these actions [191, 199]. Relevant mechanisms embrace repression of insulin-sensitizing genes, such as adiponectin, and induction of adipocyte leptin and peroxisome-proliferating activator receptor- (PPAR-) gamma genes; stimulation of endothelial lipoprotein lipase, adipocyte fatty acid synthase, acetyl CoA carboxylase, and fat-cell glucose uptake; and inhibition of fat oxidation via rate-limiting carnitine-palmitoyl transferase [167, 171, 172, 191, 554, 555]. As a more general metabolic mechanism, ghrelin mediates inhibition of sympathetic outflow to thermogenic fat depots, reduction of uncoupling protein-1 expression, and thereby decreased resting energy expenditure [556, 557]. Species appears to influence some mechanisms. For example, in

Table 6: Adipogenic effects of ghrelin.

(1) decrease fat-cell lipid export
(2) enhance lipoprotein lipase
(3) reduce insulin sensitivity
(4) stimulate preadipocyte proliferation
(5) promote adipocyte differentiation
(6) inhibit 5′-adenosine monophosphate protein kinase (AMP-kinase)
(7) augment hepatic glucose output and triacylglyceride content
(8) activate acetyl CoA carboxylase
(9) inhibit fatty acid oxidation
(10) induce leptin, sterol-response element binding protein-1c and (11) PPAR-gamma
(12) suppress adiponectin
(13) increase appetite

Selected References: [44, 195, 198, 199, 210, 276, 293, 391–393].

Table 7: Key regulators of GHS-1a receptor.

Downregulation	Stimulation
glucocorticoids (rodent)	estrogen (VMN, stomach)
ghrelin (fish)	thyroxine (rat)
certain promoter hyplotypes	lactation (rat hypothalamus/pituitary)
age (human brain)	puberty (rat, pituitary)
GH (arcuate nucleus)	GHRH (pituitary)
	atherosclerosis (human)
	ghrelin-responsive corticotropinoma (human)

Selected References: [122, 187, 393, 421–425].

sheep, ghrelin potentiates glucose-induced insulin secretion, which is antilipoytic [558]. In rodents, unacylated ghrelin can elevate insulin concentrations, which is adipogenic [441]. Neither is the case in humans [210].

CNS pathways may participate in stimulating adipocyte hypertrophy [171] and increasing the respiratory coefficient (increased ratio of glucose/fatty-acid oxidation) [234, 392, 555]. In particular, ghrelin acts in part by repressing leptin, CRH, and histamine (anorexigenic) and activating orexin and NPY (appetitive) signaling [184, 559–561]. Orexin A in turn inhibits GHRH gene expression, thus limiting GH-dependent lipolysis [562]. Thus, orexin contributes to fat accumulation by augmenting appetite, attenuating satiation, and restricting GH secretion [38, 171, 443].

Despite ghrelin's strong adipogenic effects, subcutaneous, visceral, and total adiposity correlate negatively with blood ghrelin concentrations [116, 563]. This may be because a significant longterm effect of ghrelin is to drive pulsatile GH secretion, which is strongly lipolytic [4]. Animal models indicate that ghrelin antagonists are able to reduce hyperglycemia and adipogenesis, enhance energy expenditure and fat oxidation, stimulate insulin secretion and action, and heighten resistance to diet-induced obesity [167, 168, 179, 182, 184, 205, 564]. A potential risk of prolonged antagonist administration in the fasting state could be endogenous insulin-induced hypoglycemia [166], although this adverse event has not been observed in humans.

5.8. Bone Formation. GHS-R1a and ghrelin peptide are synthesized in bone cells, such as chondrocytes and osteoblasts [12, 565]. Albeit moderately well understood in other tissues, mechanisms mediating regulation of GHS-R1a in bone remain unclear: Table 7. Acylated and unacylated ghrelin stimulate osteoblast proliferation and differentiation, increase osteoblastic markers like osteocalcin and bone alkaline phosphatase, and repress osteoblast apoptosis putatively via phosphatidylinositol 3-kinase and mitogen-activated protein kinase signaling [566]. Moreover, in GH-deficient rodents, ghrelin administration augments Ca^{2+} retention in the skeleton, thereby elevating bone-mineral density [565]. The clinical impact of these effects is not yet established.

5.9. Stress Adaptations. Recent studies point to a role for ghrelin in modifying pathophysiological adaptations to stress [275, 567, 568]. For example, ghrelin concentrations rise acutely after major surgery along with inflammatory cytokines like tumor-necrosis factor-alpha and interleukin (IL)-6 [569]. In mice, transgenic knockdown of the ghrelin receptor accentuates adverse effects of chronic social-defeat stress [570]. Analogously, ghrelin may permit adaptation to pain, since it blocks spinal-cord nociceptive signals [571]. Lipopolysaccharide endotoxin stress lowers blood ghrelin levels, causing an associated reduction in gastric emptying [572]. The latter is substantially relieved by ghrelin infusion. Under some conditions, desacyl-ghrelin inhibits gastric contractions by activating stress-adaptive CRH receptor-2, for which urocortin is the natural ligand [208, 348]. Ghrelin also induces the anorexigenic hypothalamic CRH gene [567], which normally stimulates ACTH release. Whether CNS-mediated inhibition of food intake by desacyl-ghrelin proceeds by activating the CRH pathway is not known [196].

Acute vascular-endothelial biochemical stress responses are modified by ghrelin. This peptide induces nitric oxide synthase (NOS) and represses the generation of reactive oxygen species, thereby attenuating endothelial injury [20, 573]. The protective effects require GHS-R1a, phosphotidylinositol-3 kinase, and Akt/protein kinase B [20]. A long-term action of ghrelin may be to retard endothelial apoptosis [574]. Ghrelin can also directly stimulate human vascular endothelial-cell migration, but the impact of this effect is not so clear [575]. Substantial additional work is needed to extend understanding of these aspects of ghrelin pathophysiology.

5.10. Immune Modulation. Ghrelin, GHS receptors, GH, and GH receptors are expressed in human monocytes and B and T lymphocytes [576]. In general, ghrelin's effects are antiinflammatory and immune-enhancing, for example, diminution of monocytic, bacterial and endothelial inflammatory factors driven by endotoxin exposure, sepsis, arthritis, interleukin 1 and 6, tumor necrosis factor-alpha, and nuclear-factor kappa B [200, 577–579], and

stimulation of proliferation of thymic epithelial cells and T lymphocytes [580, 581]. Ghrelin also suppresses neutrophil and macrophage migration, caspase activation, reactive oxygen-species generation, and endoplasmic-reticular stress activation [582–584]. The combined impact may be to restrict lethal hepatic and pulmonary microvascular injury in sepsis [515, 585–587]. Ghrelin concurrently induces antiinflammatory cytokines, like interleukin-10 [578], and augments organ perfusion pressure in sepsis [515, 588]. Sepsis-associated gastric mucosal injury, an additional cause of mortality due to hemorrhage, is prevented in some models [589]. Certain of these effects may require the vagus nerve [590]. In acute renal failure, ghrelin treatment can repress inflammatory cytokines in the blood and brain, limit protein catabolism, enhance food intake, and attenuate renal injury due to endotoxemia and ischemia [415, 591–594]. Total but not acylghrelin concentrations are elevated in endstage renal failure [595] and normalize following kidney transplantation.

Long-term surveillance of ghrelin and GHS receptor-deficient and ghrelin-overexpressing transgenic animals will be needed to assess whether prolonged changes in ghrelin availability influence immune function or inflammatory disease.

5.11. GHS Administration in Protracted Critical Illness. Although acute stress elevates GH secretion [596], extended critical illness suppresses all three of GH, IGF-I, and IGFBP-3 concentrations and inhibits tissue actions of GH. In unfed patients with multiorgan failure, total ghrelin levels rise [86, 131]. Infusions of GHS alone or combined with GHRH substantially reverse biochemical markers of hypo-somatotropism in this setting [597, 598]. However, recovery from multiorgan failure and inflammation is necessary to alleviate tissue resistance to GH [4]. In animal models, ghrelin administration reduces sympathetic outflow, acute renal failure, inflammation in the lung, stomach and liver, and lethality of sepsis [200, 344, 515, 586–590, 599–601]. Contrastingly, glucocorticoid deficiency, and glucocorticoid excess alter the GHS axis by, respectively, diminishing blood ghrelin and brain GHS-R1a levels [421, 602, 603]. Further investigations are needed in this meritorious area.

5.12. Ghrelin in Pregnancy and Lactation. Ghrelin is expressed abundantly by the placenta [604], and ghrelin can stimulate GH release by human fetal pituitary cells in vitro [605]. Immunoneutralization of maternal ghrelin in the rat diminishes fetal body weight, raising the possibility of maternal-fetal exchange of ghrelin [606]. Maternal ghrelin and placentally derived variant-GH concentrations in the human peak at about 18 and 34 weeks gestation, respectively, fall thereafter, and reach a nadir approximately 3 days postpartum [607–609]. The decline in ghrelin in the third trimester of pregnancy is inversely related to blood pressure, resistin, and TNF-alpha levels [95, 610]. Although human umbilical-cord acylghrelin levels exceed those in the mother, the role of placental ghrelin is not well understood [611]. Placental insufficiency, low birth weight, and maternal fasting elevate fetal ghrelin levels [611, 612]. Postpartum acylghrelin concentrations increase by several-fold over midpregnancy values [613], but GH responses to GHS are reduced in breastfeeding women, especially in the hyperprolactinemic setting [614]. The basis may involve pregnancy-associated suppression of hypothalamic GHRH and GHS-R1a with reciprocal induction of the SS gene due to feedback by high placental somatomammotropin (GH isotype V) and maternal IGF-I concentrations [422].

In the rat, lactation induces both hypothalamic and pituitary GHS receptors [615]. Pituitary GHS-R1a is maximal in the newborn pup and pubertal animal [423]: Table 7. Estradiol, T4, and GHRH may contribute to these maxima, since the GHS-R1a promoter is responsive to estrogen and cyclic AMP [122, 187]. Ghrelin is secreted into colostrum and milk, but its effect on the suckling infant is not well delineated [616]. In one preclinical study, treating rat pups with ghrelin reduced pancreatic exocrine development before weaning and exerted the opposite effect after weaning [617].

5.13. Antireproductive Effects of GHS. Exogenous ghrelin reduces LH pulse frequency in the adult male rat, cyclic or gonadectomized female rat, and ovariectomized monkey [618–622]. Ghrelin may also inhibit embryo development, decrease litter size, and delay pubertal onset in the male rat [623, 624]. The mechanism may involve neuropeptide Y (Y1 or Y5) or CRH-dependent inhibition of kisspeptin, which together supervise gonadotropin-releasing hormone secretion, and thereby pulsatile LH secretion [622, 624–627]. Other sites of inhibition may include uterine epithelium (by inducing IGFBP-1 and lowering free IGF-I), Leydig cells in the testis, and granulosa-luteal cells in the ovary (by blocking steroidogenesis) [624, 628–633]. Although physiological effects are not clear, ghrelin is expressed in sheep oocytes [634], and GHS-R1a in Sertoli (nurse) cells in spermatogenic tubules [623]. Further study is required to verify these inferences and evaluate clinical relevance [623]. For example, ghrelin infusion had no demonstrable effects on LH secretion in the early follicular phase of the menstrual cycle [635].

5.14. Neuroendocrine Tumors. Both ghrelin and obestatin are detected in various neoplasms, such as enterochromaffin tumors (e.g., carcinoids), pituitary adenomas, and carcinoma of the pancreas, lung, and breast [406, 636, 637]. The amount of peptide secreted is usually insufficient to serve as a tumor marker or to elicit clinical symptoms or signs.

5.15. Body-Composition Effects. Clinical studies in older individuals indicate that prolonged (up to 1 year) administration of GHS orally can increase lean-body mass, decrease abdominal visceral fat, and possibly improve certain performance measures, such as stair climbing and timed walking [548, 638]. In analyses comprising 1-to-30 days of parenteral GHS delivery, IGF-I concentrations also rise, but cortisol and prolactin do not [270–272]: Figure 8. Adverse events included insomnia, fatigue, small increases in fasting glucose

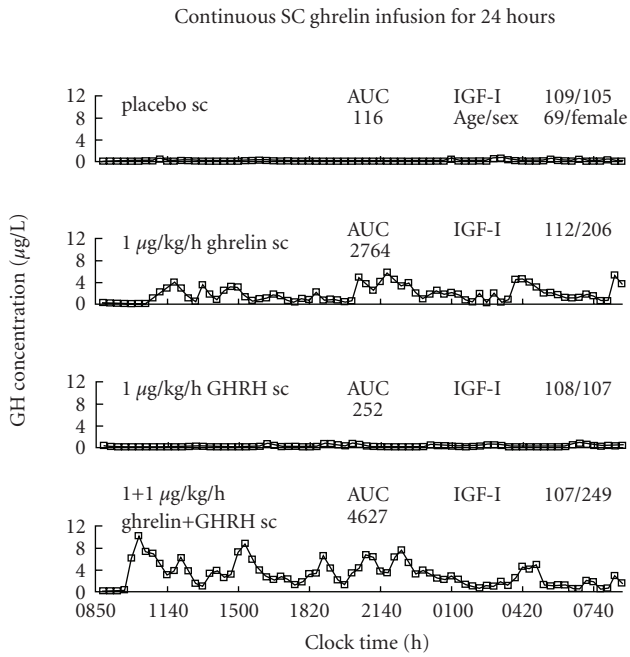

Figure 8: Continuous subcutaneous (SC) infusion of saline, GHRH or ghrelin, or both (1 μg/kg/hour) for 24 hours in a normal 69-year-old woman. Data are 20-minutes GH concentrations (y-axis) plotted against time (x-axis). AUC: area under the GH versus time curve. IGF-I concentrations at the start and end of each infusion are stated in the upper-right corner of each panel in units of μg/L.

or glycated hemoglobin, and mild insulin resistance [638]. An important consideration is that acute diabetogenic effects of GHS may be attenuated by its longterm antidiabetic effects: Table 8. Insomnia could reflect GHS's stimulation of orexin pathways, whereas glucose intolerance could reflect insulinostasis and insulin resistance due to acute free fatty-acid release in humans [306]. Short-term use of ghrelin in severe cachexia enhanced appetite and/or weight gain in some studies [298, 300]. Whereas both glucocorticoid deficiency and excess in chronic diseases can reduce GH response to GHS [4, 421], combined GHRH and ghrelin infusion is more effective at driving GH secretion than ghrelin alone [270]: Figure 8. Viral vector delivery of ghrelin augmented weight in the rat [639], introducing an additional potential avenue of treatment beyond subcutaneous, intravenous, oral, or intranasal administration [282].

A small percentage of GH-deficient adults (10%) also respond acutely to GHS, suggesting some preservation of somatotrope function and GHRH availability [640]. In other settings, injected GHS showed high specificity (95%) but low sensitivity (80%) in detecting GH deficiency [641]. Combining GHS with GHRH and/or L-arginine improves test sensitivity [4, 163].

5.16. Species Differences. Species differences in ghrelin structure, and to a lesser degree ghrelin action, have been articulated [2, 26, 62, 276, 315, 642]. In the eel, ghrelin-21 predominates instead of ghrelin-28 [643]. In fish, decanoyl

Table 8: Diabetogenic and antidiabetogenic actions of ghrelin.

Prodiabetic effects	Antidiabetic effects
stimulation of hepatic glucose output	chronic ↑ GH (lipolysis)
adipogenesis*	increase lean-body mass (chronic)
inhibition of insulin secretion	decrease oxygen consumption
appetite enhancement	increase uncoupling protein-1
acute free-fatty acid release* (human)	
Antithermogenesis	
decreased sympathetic outflow	

*reduces tissue insulin action.
See Tables 3 and 6 for selected references.

rather than octanoyl ghrelin stimulates food consumption and increases liver and fat mass [644].

5.17. Genetic Considerations. Epidemiological studies have not identified common genetic haplotypes of GHS-R1a, which predispose to obesity or short stature [645]. Two-weak associations of GHS-R1a polymorphisms with metabolic syndrome or cardiovascular risk require confirmation [646, 647]. Similar preliminary data apply to polymorphisms of the ghrelin gene [393, 648].

6. Summary

In addition to increasing GH, as the term *ghrelin* implies, GHS regulates inflammation, cellular proliferation, apoptosis, differentiation, and hormone secretion via receptors located in the brain, stomach, intestine, heart, arterial wall, bone, fat cells, and pancreas (exocrine and endocrine). Acylated and unacylated ghrelin can exert both similar (antiapoptotic) and opposite (appetitive) effects. Promising clinical applications of ghrelin agonists and antagonists arise in relation to metabolic, gastric, GH-stimulating, anti-inflammatory, and cardiotropic effects. Nonetheless, there are both desirable and potentially undesirable aspects of chronic administration of ghrelin agonists or antagonists: Table 9. Accordingly, substantial further advances in ghrelin biology will be important.

7. Speculations

A saga is evolving within the ghrelin system due to the recent identification of the ghrelin O-acyl transferase (GOAT) enzyme [47, 48]. The octanoyl addition is of major biochemical and functional significance. Not only is ghrelin the first natural hormone with a fatty-acid addition, but also octanoylation is essential for binding and activating the receptor, GHS-R1a, probably by determining the active receptor-specific conformation of the ghrelin 1–28 molecule. Final isolation of the GOAT enzyme may not be so easy, since

Table 9: Issues concerning longterm administration of ghrelin antagonists.

Desirable
↓ hyperglycemia
↑ insulin secretion
↓ appetite and food intake
↑ fat oxidation
↑ LH secretion?
Undesirable
↓ gastric emptying
↑ blood pressure (vasoconstrict)
↑ cardiac oxygen consumption?
↓ neoplastic apoptosis?
↑ inflammatory mediators?
↓ bone growth?
↑ gastric alkalinity and mucosal permeability?
↓ GH secretion (female)?
↓ neurogenesis (brainstem, cortex)?
↑ hypoglycemia during prolonged fast?

it is tightly bound by 12 transmembrane domains spanning the endoplasmic reticulum (ER) [47].

Important issues arise regarding the chemistry and biology of posttranslational octanoylation of ghrelin hormone. The current concept is that preproghrelin 1–117 or proghrelin 1–94 is octanoylated for fundamental biological reasons. In consonance with this postulate, production of octanoylated ghrelin 1–28 is restricted to the conjoint anatomical and intracellular sites of GOAT and preproghrelin 1–117 biosynthesis. Furthermore, if the desacylated ghrelin 1–28 is not directly octanoylated by GOAT, the specificity of biological regulation is made even more precise. Available evidence indirectly suggests that GOAT first octanoylates the preproghrelin 1–117 or the proghrelin 1–94 molecule. In the former case, octanoylation would occur cotranslationally rather than posttranslationally. This would require an integrated cleavage of the signal peptide. Subsequently, several prohormone convertases (PC1/3, PC2, and furin) permit sequential formation of octanoylated Ser^3 proghrelin 1–94 and octanoylated Ser^3 ghrelin 1–28 [496, 649, 650].

Before the identification of GOAT, Zhu, Cao, Voogd, and Steiner published the finding that 1–94 proghrelin was octanoylated, whereas ghrelin 1–28 remained unoctanoylated [496]. These results support the inference that octanoylated Ser^3 ghrelin 1–28 is derived from octanoylated proghrelin 1–94. The specific actions of convertases are now being resolved. Zhu et al. demonstrated that PC1 must be the enzyme that cleaves proghrelin in vivo, because only proghrelin and not processed ghrelin is made in PC1-knockout mice [496]. In addition, furin can cleave proghrelin in vitro as described in the laboratory of Lindberg and, more recently, that of Kojima in transfection studies [72, 649]. Why furin does not suffice in the PC1 gene-deletion model is not clear.

In addition to GOAT, another human ER oxyesterase has been purified and characterized from a stable human erythroleukemia cell line by Ozawa, Speaker, and Lindberg [56]. The acronym of this additional oxyesterase is ERAT for endoplasmic reticulum O-acyl transferase. ERAT will esterify modified proghrelin having an N-terminal tripeptide extension, but not the proghrelin 1–94 molecule. Notably ERAT octanoylation is limited to the Ser^2 amino-acid residue, while Ser^3, Ser^6, or Ser^{18} of ghrelin 1–28 are not octanoylated. In addition, ERAT may transfer an array of long-chain natural fatty acids, not just octanoic, to as yet unknown substrates as well as to ghrelin 1–28, when the latter contains an N-terminal tripeptide extension. This specificity is reminiscent of N-myristoyltransferase. Furthermore, ERAT is a soluble enzyme, but firmly bound to the ER membrane, whereas GOAT is an insoluble integral ER membrane-spanning enzyme. If ERAT and GOAT were colocalized in the human stomach, one could envision both independent and interdependent roles of ERAT and GOAT. Although mammalian GOAT was initially considered to only add octanoic or decanoic fatty acids to Ser^3, the laboratory of Kojima reported that GOAT effectively acylates truncated ghrelin peptides with n-hexanoic acid in cultured cells [134]. ERAT might interact by limiting in situ availability of octanoic acid to GOAT. A significant analytical point is that Ser^2 versus Ser^3 octanoylated ghrelin would not be distinguishable by mass spectrometry, HPLC, or probably immunologically, requiring instead mutagenesis studies, amino-acid sequencing, binding, and biological activity studies.

Whether ERAT acylates proghrelin in vivo is not known. Albeit low in potency, $Ser^{2,3}$ dioctanoylated ghrelin 1–28 can release GH in vivo in rats [125]. In addition, scientists at Merck laboratories demonstrated that Ser^2 monooctanoylated ghrelin 1–28 binds to GHS-R1a in vitro. Since octanoylated ghrelin 1–28 may exert endproduct repression of GOAT acyltransferase activity and since binding sites for GOAT, ERAT, and GHS-R1a may overlap, small amounts of Ser^2 or $Ser^{2,3}$ octanoylated ghrelin 1–28 might have GOAT inhibitory activity. The proposition would be that endogenous ERAT-derived ghrelin peptides may act on GOAT only at intracellular sites.

Notable are two ghrelin deacylation enzymes, plasma paraoxanase and gastric lysophospholipase I [156, 651]. Plasma paraoxanase is bound to high-density lipoproteins and may especially determine physiological plasma ghrelin levels. An interesting question is whether a major role of blood-borne paroxanase is to minimize the actions of octanoylated ghrelin or to maximize the actions of desacylated ghrelin. On the other hand, gastric lysophospholipase I may primarily regulate tissue ghrelin bioactivity and subsequent vagal-afferent activity as a function of the amount and type of oral fatty-acid intake. In this regard, oral octanoate increases, but oral dodecanoate decreases, plasma octanoylated ghrelin levels [134]. Accordingly, the activity of the two deacylases could be a function of amount and type of substrate as well as factors that modify synthesis of and catalysis by paroxanase and lysophospholipase I.

The multiple influences of the ghrelin system depend upon local and systemic posttranslational regulatory mechanisms and pleiotropic receptor-signaling in diverse target tissues [518]. Moreover, gastric ghrelin is subject to food-entrainable and circadian-clock inputs [652]. There is an additional possibility that partial overlap of ghrelin-binding properties of GHS-R1a, GOAT, and ERAT could contribute to differences and similarities between ghrelin and GHS mimetics as well as competition between full and partial GHS-R1a agonists [653]. Brown and Goldstein demonstrated that truncated ghrelin peptide analogs inhibit activity of GOAT in vitro [46]. They proposed that native octanoyl ghrelin 1–28 in higher concentrations may also inhibit GOAT. If so, one could envision that certain GHS mimetics might decrease or increase the activity of GOAT and ERAT as well as that of GHS-R1a. For example, at a low dosage Dap^3 octanoylated ghrelin 1–28 inhibits octanoyl transferase activity in vitro indicating that GOAT is a significant site of action, whereas this analog stimulates both GH release and food intake in vivo, thus defining a GHS-R1a (ghrelin receptor) site of action [46, 654]. To assess the biochemistry, physiology, endocrinology, and therapeutic implications of peptide/non-peptide GHS mimetics and ghrelin will require combined in vitro and in vivo structure-function and dose-response analyses.

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Review Article

Ghrelin Cells in the Gastrointestinal Tract

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Ghrelin is 28-amino-acid peptide that was discovered from the rat and human stomach in 1999. Since the discovery of ghrelin, various functions of ghrelin, including growth hormone release, feeding behavior, glucose metabolism, memory, and also antidepressant effects, have been studied. It has also been reported that ghrelin in the gastrointestinal tract has an important physiological effect on gastric acid secretion and gastrointestinal motility. Ghrelin has a unique structure that is modified by *O*-acylation with n-octanoic acid at third serine residues, and this modification enzyme has recently been identified and named ghrelin *O*-acyl transferase (GOAT). Ghrelin is considered to be a gut-brain peptide and is abundantly produced from endocrine cells in the gastrointestinal mucosa. In the gastrointestinal tract, ghrelin cells are most abundant in the stomach and are localized in gastric mucosal layers. Ghrelin cells are also widely distributed throughout the gastrointestinal tract. In addition, abundance of ghrelin cells in the gastric mucosa is evolutionally conserved from mammals to lower vertebrates, indicating that gastric ghrelin plays important roles for fundamental physiological functions. Ghrelin cells in the gastrointestinal tract are a major source of circulating plasma ghrelin, and thus understanding the physiology of these cells would reveal the biological significance of ghrelin.

1. Introduction

Several distinct types of endocrine cells, including histamine-producing ECL cells, somatostatin-producing D cells, gastrin-producing G cells, and serotonin-producing EC cells, have been shown to be present in the mammalian stomach. Before the discovery of ghrelin, gastric ghrelin cells were classified as unknown endocrine cells by their round, compact, electron-dense secretory granules (P/D(1)-type in humans, A-like-type in rodents, and X-type in dogs) that distinguish them electron-microscopically from other previously characterized gastric endocrine cell types [1, 2]. Ghrelin was identified as an endogenous ligand for growth hormone secretagogue receptor (GHS-R) from the stomach in 1999 [3]. Ghrelin structurally resembles motilin and they share about 21% amino acid identity and their receptor also has structural similarity with 44% similarity in amino acids, indicating that they comprise a motilin-ghrelin family [4, 5]. Interestingly, ghrelin was found to be present as two molecular forms: acyl-ghrelin modified with medium chain fatty acids and des-acylated-ghrelin lacking side chain modification [3, 6]. Although acyl-ghrelin is only known to bind to GHS-R [7], accumulating results have shown that des-acylated-ghrelin has various physiological functions via unknown specific receptor, including involvement in cell death, feeding behavior, and energy and glucose homeostasis [8–13]. Moreover, in 2005, obestatin was identified as a peptide hormone derived from preproghrelin in the stomach [14] and obestatin has been shown to be involved in energy homeostasis, gastrointestinal motility, memory, sleep, and cell proliferation [13–18].

In this review, we focus on ghrelin cells in the gastrointestinal tract and describe the characteristic features, development, and regulation of ghrelin cells.

2. Distribution and Morphological Features of Ghrelin Cells in the Gastrointestinal Tract

The distribution of ghrelin-producing cells in the gastrointestinal tract has been studied by using immunohistochemical techniques and in situ hybridization [1, 2, 19, 20].

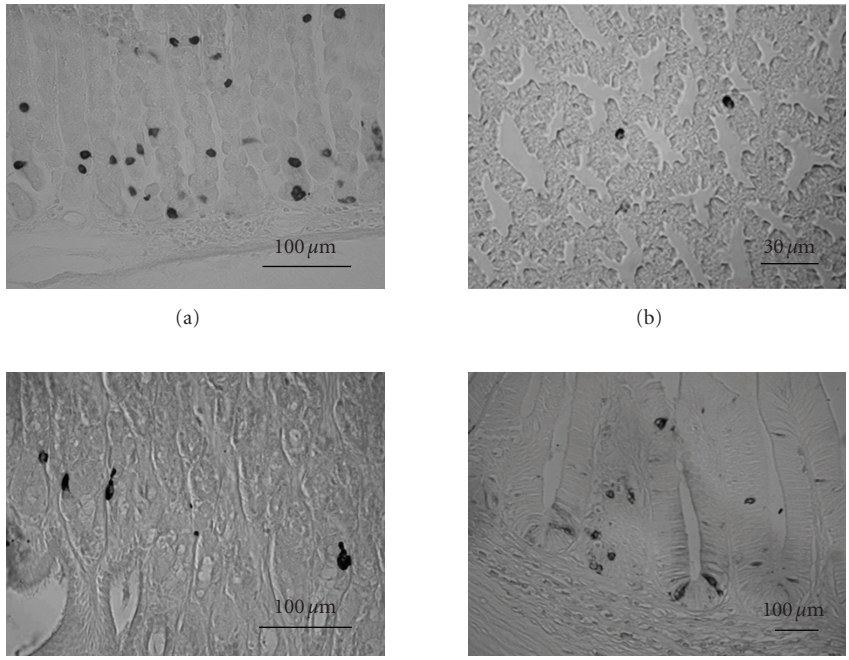

Figure 1: Localization of ghrelin-immunopositive cells in the stomach (rodents, avians, amphibians and fish). (a) Ghrelin cells in the rat stomach. Ghrelin cells are mainly observed from the glandular base to the body of the fundic gland, and gastric ghrelin cells in the rat have been clarified to be closed-type cells. Ghrelin cells are scattered throughout the mucosal layer in (b) the proventriculus of the chicken and (c) bullfrog stomach, and those ghrelin cells are also closed-type cells as rodents. (d) Ghrelin cells in the trout stomach are localized in the mucosal layer, and both closed- and opened-type cells are found in the trout. Scale bars = 100 μm in (a), (c), (d); 30 μm in (b).

In rodents, ghrelin-producing cells were observed in all regions of the gastrointestinal tract: gastric body, antrum, duodenum, ileum, cecum, and colon. Ghrelin-producing cells were most dense in the gastric body (Figure 1(a)) and were found in the mucosal layer but not in the myenteric plexus in all of the examined regions. In the stomach, most of the ghrelin cells were observed in the glandular base decreasing in amount as the gland extends toward the lumen with a few cells observed in the glandular neck. In the duodenum, ileum, cecum, and colon, ghrelin cells were scattered in the epithelia of crypts and villi. The densities of ghrelin cells were dramatically decreased toward the lower gastrointestinal tract [19]. The number of ghrelin-producing cells in each gastrointestinal tract corresponded well with the amount of peptide and mRNA expression levels [21]. In addition, plasma concentration of ghrelin in rats was decreased by 80% after gastrectomy [1], and also plasma ghrelin levels were reduced by 65% in human gastrectomised patients, strongly suggesting that major source of circulating plasma ghrelin is the gastric mucosa [22].

The morphological features of ghrelin-producing cells in the stomach differ from those of the intestinal tracts. In the stomach, ghrelin-producing cells were found to be small and round-shaped, so-called closed-type cells [1, 19, 23]. However, in the duodenum, ileum, cecum, and colon, two types of ghrelin cells were found; that is, closed-type cells with triangular or elongated shapes and opened-type cells with their apical cytoplasmic process contacting to the lumen. In general, opened-type cells are functionally regulated by receiving luminal information such as nutrients and pH, while closed-type cells are functionally modulated by hormones, neuronal stimulation, or mechanical distension [24]. Thus, the regulatory mechanisms of ghrelin release would be different in the stomach and other parts of the gastrointestinal tract. By electron microscopic observation, several groups have shown that immunogold labeling for ghrelin was localized on the round and electron-dense granules in gastric mucosal cells [2, 25–27]. Yabuki et al. showed that the diameters of granules containing ghrelin in mice (277.7 ± 11.1 nm) and rats (268.8 ± 13.0 nm) were similar; however, those in hamsters (200.8 ± 8.8 nm) were significantly smaller than those in mice or rats [26]. Rindi et al. demonstrated that mouse and canine ghrelin-immunoreactive cells closely resembled those of the human stomach, though it has been shown that dog ghrelin cells have obviously larger granules (mean diameter of 273 ± 49 nm) than those of rats (mean diameter of 183 ± 37 nm) and humans (147 ± 30 nm) [27].

Localization of GOAT (Ghrelin *O*-Acyltransferase) and ghrelin was also recently studied by using in situ hybridization histochemistry and immunohistochemistry, and a high degree of colocalization of GOAT and ghrelin was observed in mouse gastric oxyntic mucosal cells [28]. On the other hand, ghrelin-GFP transgenic mice expressing humanized *Renilla reniformis* green fluorescent protein (hrGFP) under control of the mouse ghrelin promoter has recently been

generated and it has been confirmed that hrGFP expression was especially abundant in the gastric oxyntic mucosa, in a pattern mirroring that of ghrelin immunoreactivity and ghrelin mRNA [28].

3. Relationship of Ghrelin and Motilin Cells

Motilin was originally isolated from porcine intestinal mucosa in the 1970s [29, 30]. Motilin is 22-amino-acid peptide and it is known to play an important role for the interdigestive migrating motor complex (MMC) [31]. Ghrelin and motilin and their receptors have similar structures and these two peptides are known to form a motilin-ghrelin peptide family [5, 32]. It has been demonstrated that ghrelin stimulates gastrointestinal motility in rodents, suggesting that a part of the physiological effects of these two peptides is also overlapped. The distributions of ghrelin and motilin cells are separated; motilin is mainly produced in the upper intestine, but ghrelin cells are localized mainly in the stomach. Recently, Wierup et al. demonstrated that over 90% of ghrelin cells in the porcine duodenum coexpressed motilin, and they also observed by electron microscopic immunolabeling that they were localized on the same secretory granules, indicating that they were cosecreted with the same stimulation [33]. On the other hand, sequences of ghrelin and motilin in the house musk shrew were determined, and no coexistence of ghrelin and motilin was found in gastric and duodenal cells [34, 35]. Therefore, further studies are needed to elucidate the coexistence of ghrelin and motilin in one cell by using other animal models.

4. Development of Ghrelin Cells in the Gastrointestinal Tract

Ghrelin was found to be expressed in the fetal stomach from embryonic day 18 and the number of fetal gastric ghrelin cells increased as the stomach grew, with gastric ghrelin content also increasing with advance of age [36]. Detailed study showed that ghrelin-immunopositive cells appeared in the glandular base of the fundic gland at 1 week of age and they were found in the glandular base and the glandular neck at 3 weeks of age. Then the distribution of ghrelin cells was extended from the glandular base to the glandular neck during the postneonatal developmental period [37]. Walia et al. have also reported that ghrelin-immunoreactive cells were rare at embryonic day 21 and that their number increased progressively until weaning [38]. Gastric ghrelin mRNA levels also increased in an age-dependent manner similar to the number of ghrelin cells [36, 37, 39]. In addition, ghrelin cells in female rats differentiated at an earlier stage of development than that in male rats, and the density of ghrelin cells in female rats was also higher than that in male rats [37]. In humans, plasma ghrelin level was also higher in females than in males [40].

5. Distribution and Features of Ghrelin Cells in Nonmammalian Vertebrates

Ghrelin has been characterized not only in mammals but also, many other species, avians, amphibians, reptiles, and fish, and it has been shown that the structures of ghrelin peptide were well preserved from mammals to lower vertebrates [41]. The N-terminal regions of the first seven amino acids in lower vertebrates were well conserved, and the third serine or threonine residue is modified with medium chain fatty acid, mainly octanoic acid, as in mammals [41]. In nonmammalians, it has been confirmed that ghrelin mRNA is abundantly expressed in the stomach [42–48]. In avians, ghrelin-immunopositive cells were found in the mucosal layer of the proventriculus (Figure 1(b)) that corresponds to a first glandular part of the stomach in which digestive enzymes are mixed with food before the gizzard [49]. However, ghrelin immunoreactivity in avians was not located in the myenteric plexus, and many more ghrelin-immunopositive cells were found in the middle layer than in the base of the mucosal layer, with the majority of ghrelin cells being round-shaped and closed-type cells [49]. Features of gastric ghrelin cells in amphibians and reptiles are similar to mammals or avian ghrelin cells. Ghrelin cells in these animals were also found in the gastric mucosal layer but not in the myenteric plexus or muscle layers of the stomach, and the ghrelin cells are closed-type cells in frogs (Figure 1(c)) and turtles [45, 50]. Ghrelin cells in rainbow trout were found to be localized as closed-type cells and opened-type cells in the gastric mucosa (Figure 1(d)) [51].

6. Regulation of Ghrelin Cells in the Gastrointestinal Tract

6.1. Ghrelin Cells on Fasting. Ghrelin is a potent orexigenic peptide that stimulates food intake and body weight gain, suggesting that ghrelin plays an important role in energy homeostasis. Numerous data have shown that plasma ghrelin levels were elevated in a fasting state and returned to basal levels after refeeding [32, 52, 53]. On the other hand, peptide content of ghrelin in the stomach decreased after fasting [54], indicating that cytoplasmic ghrelin released from gastric ghrelin cells caused an increase in plasma ghrelin levels. Compared to biochemical analysis, studies on changes in the number and staining property of gastric ghrelin cells in a fasting state are quite restricted. For example, the number of ghrelin-immunopositive cells significantly increased in rats during fasting for 7 days, and then the cell number decreased to a normal level after refeeding [55]. In bullfrogs, no significant differences in numbers of ghrelin cells were observed during fasting for 20 days [50]. Moreover, in fish fasted for 72 hours, Govoni et al. found no clear differences in ghrelin cell numbers and their immunostaining intensity [18, 30, 56]. Therefore, further studies are needed to elucidate the number of ghrelin cells in a multiple time point study during a fasting period.

6.2. Ghrelin and Nutrients. As mentioned above, ghrelin is known to be a hunger signal from peripheral tissues, and plasma ghrelin levels drop to basal levels after a meal, suggesting that ghrelin cells are regulated by nutrient uptake. It has been reported that oral and intravenous glucose administration sharply reduced plasma ghrelin concentration in rodents [53, 57], and this effect of glucose on ghrelin

inhibition was similar to that found in human studies [58–61]. Williams et al. studied the effect of intragastric load of glucose on plasma ghrelin levels, and they found that plasma ghrelin level was reduced by approximately 50% under a normal gastric emptying condition [62]. On the other hand, plasma ghrelin levels were not changed in pyloric cuff-implanted rats, indicating that ghrelin release from gastric ghrelin cells is affected by glucose that is absorbed from the intestine [62]. In addition to glucose, duodenal and jejunal infusions of lipids or amino acids reduced ghrelin levels in rats [63]. These effects have been confirmed in human studies; free fatty acids or protein administration decreased plasma ghrelin levels [64, 65]. However, the mechanisms by which these nutrients regulate on gastric ghrelin release in the stomach remain unclear. Further studies are needed to elucidate the molecular pathway of ghrelin cell regulation by nutrients.

6.3. Hormonal Control of Gastric Ghrelin. Ghrelin is also regulated by circulating and gastric hormones. Ghrelin cells are located close to somatostatin-producing D cells [66], and somatostatin inhibits ghrelin secretion in rats and humans [66–69]. Intravenous administration of glucagon caused transient increases in both acyl- and des acyl-ghrelin levels. Moreover, ghrelin secretion from the perfused stomach was stimulated by glucagon treatment in a dose-dependent manner [70], and this effect was shown to be mediated by glucagon receptors on ghrelin cells [71]. de la Cour et al. performed a microdialysis study to find a possible ghrelin regulator in the stomach, and they found that epinephrine, norepinephrine, endothelin, and secretin stimulated ghrelin release [69].

Steroid hormone is also involved in ghrelin regulation. A role of estrogen in the regulation of gastric ghrelin has also been suggested by several studies. In humans, estrogen regulates plasma ghrelin concentration [72–75]. The levels of gastric ghrelin mRNA and plasma ghrelin and the number of ghrelin cells were found to be transiently increased by ovariectomy in female rats [76]. Treatment of gastric mucosal cells with estrogen showed that estrogen stimulated ghrelin expression and ghrelin secretion [77, 78]. These findings indicate that gastric estrogen plays an important role in the regulation of gastric ghrelin at the transcriptional level.

7. Ghrelin Cells in the Digestive Diseases

In addition to the physiological, biochemical, and structural studies of ghrelin, pathological aspects of ghrelin have been studied in various diseases, such as anorexia nervosa, Prader Willi Syndrome (PWS), Polycystic Syndrome (PCOS), and Celiac Disease (CD) [79–84]. The role of ghrelin in Helicobacter pylori (H. pylori) infections, gastric diseases such as chronic gastritis, gastric and duodenal ulcers, and gastric carcinomas, has been widely studied. While there are conflicting results, many papers have shown that plasma ghrelin concentrations in the H. pylori positive subjects were lower than in H. pylori negative subjects [85–87]. When correlated with the plasma ghrelin levels, the number of ghrelin immunopositive cells was significantly lower in H. pylori positive subjects than in negative subjects [85, 88]. Furthermore, ghrelin mRNA expression levels were also lower in H. pylori-infected human subjects [88]. Using Mongolian gerbils as an animal model of H. pylori infection, Suzuki et al. showed that ghrelin mRNA expression levels were significantly lower in animals with H. pylori, compared to control animals [89]. Furthermore, the number of ghrelin immunopositive cells decreased in H. pylori-infected groups 17 and 23 weeks after infection, while the total number of gastric mucosal cells remained unchanged. In addition, when compared to control animals, gastric ghrelin contents were significantly decreased in infected animals 17 and 23 weeks after H. pylori inoculation, although no differences were detected after only four weeks. Notably, ghrelin cells in the gastric mucosa are located close to the parietal cells, and some of these cells are found to be in contact with each other [77, 89], and it has been reported that H. pylori affected the function of parietal cells [90, 91]. Although further studies are needed to elucidate the biological mechanisms behind the regulation of gastric ghrelin after H. pylori infection, it is possible that gastric estrogen, which is produced in parietal cells, could affect ghrelin cells in a paracrine manner. Alternatively, inflammatory factors may act directly on ghrelin cells to decrease their activity.

8. Conclusions

Ghrelin is predominantly produced in the stomach, and gastric ghrelin is thought to play important multiple physiological roles in peripheral signaling. Results of the analysis of ghrelin cells in the gastrointestinal tract have been accumulating. Many factors including nutrients as well as circulating and gastric hormones were found to be involved in the regulation of mRNA and release of ghrelin, indicating that ghrelin cells are under the control of complicated mechanisms, which are still obscure. In addition, characteristics of ghrelin cells in the stomach and other parts of the gastrointestinal tract are different; closed-type ghrelin cells are localized in the stomach and many opened-type ghrelin cells are localized throughout the small and large intestines. Since the length of the gastrointestinal tract is long, considerable amounts of plasma ghrelin can be assumed to be derived from ghrelin cells in small and large intestines. Further studies are needed to understand the physiological and pathological roles of ghrelin cells in both small and large intestines. Moreover, posttranscriptional modifications are very important for physiological active ghrelin, necessitating the need to understand how GOAT is involved in the regulation of ghrelin biosynthesis and consequently may be important for controlling ghrelin cells in some physiological states.

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Review Article

Differential Roles for Octanoylated and Decanoylated Ghrelins in Regulating Appetite and Metabolism

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Since its identification in 1999, ghrelin has been identified in all vertebrate groups. The "active core" of ghrelin is highly conserved among vertebrates, suggesting its biological activity to be also conserved. In fish, both acylated forms of ghrelin have been identified; however, the ratio of the ghrelin-C8 to ghrelin-C10 is not as great as observed in mammals. In the tilapia (*Oreochromis mossambicus*), ghrelin-C10 is the major form of ghrelin. Since fish are known to inhabit every ecological niche on earth, studies on fish have provided valuable insight into vertebrate physiology in general; it is likely that understanding the role of both acylated forms of ghrelin, in more detail, in fish will result into novel insights in the biology of ghrelin within vertebrates. In this paper we discuss ghrelin's role in regulating appetite and metabolism in fish, in general, and provide evidence that the two tilapia ghrelins exhibit different biological roles.

1. Introduction

The discovery of ghrelin in 1999 [1] broadened our understanding of energy metabolism in vertebrates, resulting in a shift in our approach to investigat the regulation of energy homeostasis in vertebrates. In mammals, two major forms of ghrelin are found in circulation: octanoylated ghrelin at Ser-3 and des-acyl ghrelin [2]. The acyl modification is essential for biological activity [1]; however, some findings provide evidence that des-acyl ghrelin exhibits some biological action [3–7]. Ghrelin has also been identified in all vertebrate classes including sharks [8]. As seen in mammals, the ghrelins identified in other vertebrates are uniquely acylated by either octanoic or decanoic acid on the third amino acid residue from the N-terminus. Indeed, the first seven amino acids of N-terminal region—"active core"—in all vertebrate ghrelins display high sequence homology [8], suggesting that the biological actions of ghrelin are highly conserved across vertebrates. Interestingly, fish ghrelins possess an amide structure on the C-terminus which is not found in tetrapod and shark ghrelins [8]. In the Mozambique tilapia (*Oreochromis mossambicus*), a warm water teleost (fish), we have identified two forms of ghrelin, with identical amino acid sequences, acylated by octanoic or decanoic acid, ti-ghrelin-C8 and ti-ghrelin-C10, respectively [9]. It appears that ti-ghrelin-C10 is the primary form of ghrelin in tilapia. A recent report in goldfish identified 11 different forms of ghrelins; a 17-residue octanoylated form being the predominate form [10]. This finding in goldfish is similar to other vertebrates, with ghrelin-C8 being the major form of ghrelin. In humans, 25% of the ghrelin isolated from the stomach is ghrelin-C10 [11], in the bullfrog, ghrelin-C10 represents 33% of the total ghrelin [12], and in the Japanese eel, ghrelin-C10 represents 44% of the total ghrelin [13]. Due to the evolutionary diversity and breadth of ecological niches occupied by fish; studies using fish as a model have been a rich source of information on the mechanisms that regulate vertebrate growth, metabolism, and development [14–17]. Furthermore, the fact that the ghrelin gene and peptide exhibit high structural similarities and biological actions across vertebrates, suggest that ghrelin is an evolutionary conserved, essential hormone in vertebrates. However, our understanding of ghrelin's basal biological role in vertebrates is unclear. Therefore, studies on fish will provide an evolutionary role for ghrelin and provide insight into the basal function of ghrelin within all vertebrates. This paper will

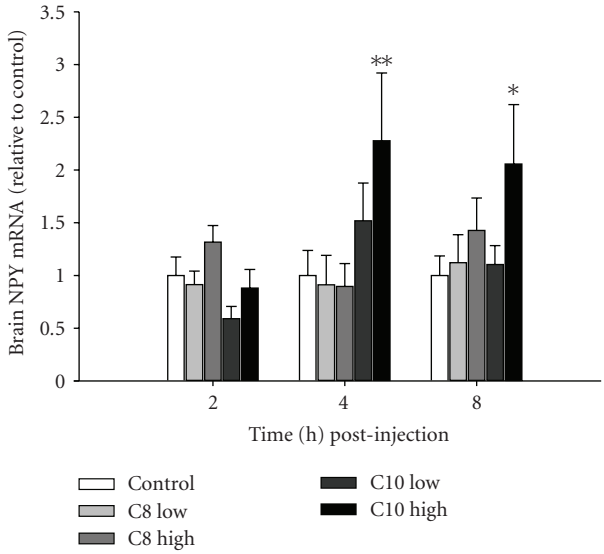

Figure 1: The effect of tilapia ghrelin-C8 and ghrelin-C10 on brain NPY mRNA levels. Two different doses of both ghrelins, low (1 ng/gm) and high (10 ng/gm), were administered by a single i.p. injection and samples were collected a 2, 4, and 8 h postinjection. mRNA levels were normalized to the house keeping gene, acidic ribosomal phosphoprotein P0 (ARP). Vertical bars represent mean ± SEM (n = 8–10). ∗, ∗∗ are significantly different from time-matched control at $P < .05$ and $< .01$, respectively (2-way ANOVA).

highlight our current understanding of ghrelin's role in food intake and metabolism in fish and provide evidence on how the decanoylated ghrelin plays a significant role in regulating overall energy homeostasis. The reader is directed to reviews focusing on ghrelin sequence identity and other biological actions in nonmammalian vertebrates [8, 18].

In mammals, ghrelin has been shown to exhibit a range of actions on cardiovascular, gastrointestinal, and pancreatic functions, as well as lipogenic and glucogenic actions [19]. In mammals, it is suggested that the main physiological function of ghrelin is to stimulate growth hormone release from the pituitary and increase food intake [20]. However, some reports demonstrate that ghrelin does not play a primary role in initiating feeding nor as a regulator of feeding patterns [21]. Indeed, accumulating data suggests that ghrelin's role may be directed to maintain overall energy homeostasis as observed in humans [22, 23] and in pigs [24].

Ghrelin's first reported action was as a potent growth hormone (GH) secretagogue [1]. Since then, similar findings have been reported in fish. We first reported in fish that rat ghrelin-C8 stimulated the release of GH from cultured tilapia pituitaries after 8 h of incubation [25]. Both eel and tilapia ghrelin-C8 stimulated the release of GH from static tilapia pituitary cultures after 2 h of incubation [9, 13]. Recently, we demonstrated that ti-ghrelin-C10 appears to be more effective than ti-ghrelin-C8 in elevating plasma GH levels and in stimulating GH release from tilapia pituitaries [26]. However, these responses occur 4-5 h after treatment. Unlike the delayed response observed in tilapia, intraperitoneal (i.p.) injections of homologous ghrelin-C8 in goldfish and rainbow trout significantly elevated plasma GH levels within 30 min [18, 27]. At least in fish, only the tilapia pituitary releases prolactin (PRL) after ghrelin treatment. Both eel and tilapia ghrelins stimulated the release of PRL from cultured tilapia pituitaries [9, 13, 25]. Similar findings were observed in the bullfrog [12], but not in dispersed rat pituitary cells [1]. These findings clearly suggest that the response to ghrelin is species specific, but what needs to be more clearly investigated in fish is; does ghrelin exhibit the same stimulatory effect on GH release during altered physiological states (i.e., fasting or stress). We have recently reported for the first time using the hybrid striped bass model that ghrelin was equally effective in stimulating GH release from pituitaries of fed and starved animals. Furthermore, both plasma levels of ghrelin and GH were significantly elevated in fasted cold-banked animals [28]. Suggesting that ghrelin is driving the elevation of plasma GH levels during fasting as proposed in mammals [29] or regulating energy partitioning during catabolic states [28].

As mentioned above, several reports in mammals have demonstrated that acute ghrelin treatment stimulates food intake [20]. However, in teleosts, ghrelin's orexigenic actions have not been well studied and appear not to be widespread. The only report of ghrelin exhibiting rapid orexigenic actions—as seen in mammals—is in goldfish [30, 31], whose actions have been shown to be mediated by neuropeptide Y (NPY) [32]. In tilapia, we have been unable to observe an acute increase in food intake following ti-ghrelin-C8 or ti-ghrelin-C10 treatment (unpublished observations). However, we have observed in tilapia given a single i.p. injection of ti-ghrelin-C10 (10 ng/gm BW) a significant increase in brain NPY mRNA levels 4 and 8 h ($P < .01$ and $P < .05$, resp.) postinjection was observed, whereas ti-ghrelin-C8 did not alter NPY mRNA levels (Figure 1). The inability of ti-ghrelin to stimulate acute food intake may likely be a result from the site of treatment. Centrally administered ghrelin is very potent in stimulating food intake in mammals [30, 33] and goldfish [10, 30], whereas peripherally injection of ghrelin is less effective in stimulating food intake [33]. It may be likely that orexin, which has not yet been identified in tilapia, is mediating acute food intake in tilapia. In goldfish, it has been shown that ghrelin and orexin interact to stimulate feeding [34]. It is of interest to identify orexin in tilapia and investigate its action on food intake. We have shown previously that 21 days of ti-ghrelin-C10 (ti-ghrelin-C8 had no effect) treatment significantly increased food intake and adiposity in liver and muscle tissue in tilapia [35]. Similar findings have been observed in rodent models [33]. In addition, 21 days of ti-ghrelin-C10 treatment did not alter plasma GH levels, but plasma levels of insulin-like growth factor-I (IGF-I) were significantly reduced, suggesting that ti-ghrelin-C10 is inhibiting growth in favor of storing metabolic energy as fat; generating a positive energy balance. Interestingly, ti-ghrelin-C8 treatment significantly increased pituitary GH mRNA levels [35]. In rats receiving a continuous i.c.v. infusion of ghrelin for 12 days, plasma GH levels were not altered [36]. Furthermore, in rainbow trout, a single i.p. injection of ghrelin failed to stimulate appetite, however, plasma ghrelin levels were positively correlated with

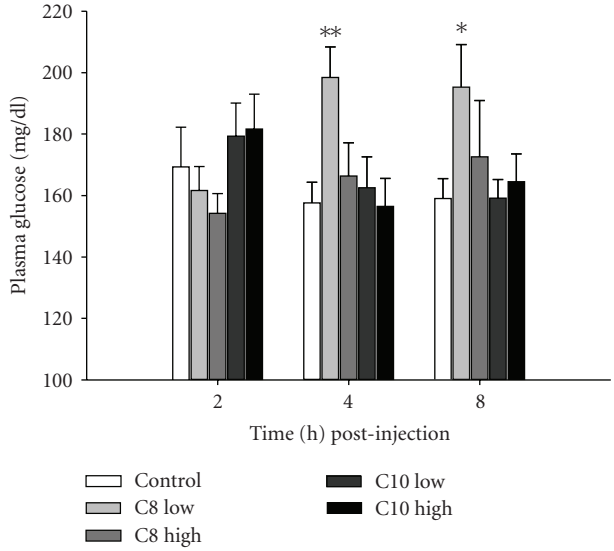

Figure 2: The effect of tilapia ghrelin-C8 and ghrelin-C10 on plasma glucose levels. Two different doses of both ghrelins, low (1 ng/gm) and high (10 ng/gm), were administered by i.p. injection and samples were collected a 2, 4, and 8 h postinjection. Vertical bars represent mean ± SEM ($n = 8$–10). ∗, ∗∗ are significantly different from time-matched control at $P < .05$ and $< .01$, respectively (2-way ANOVA).

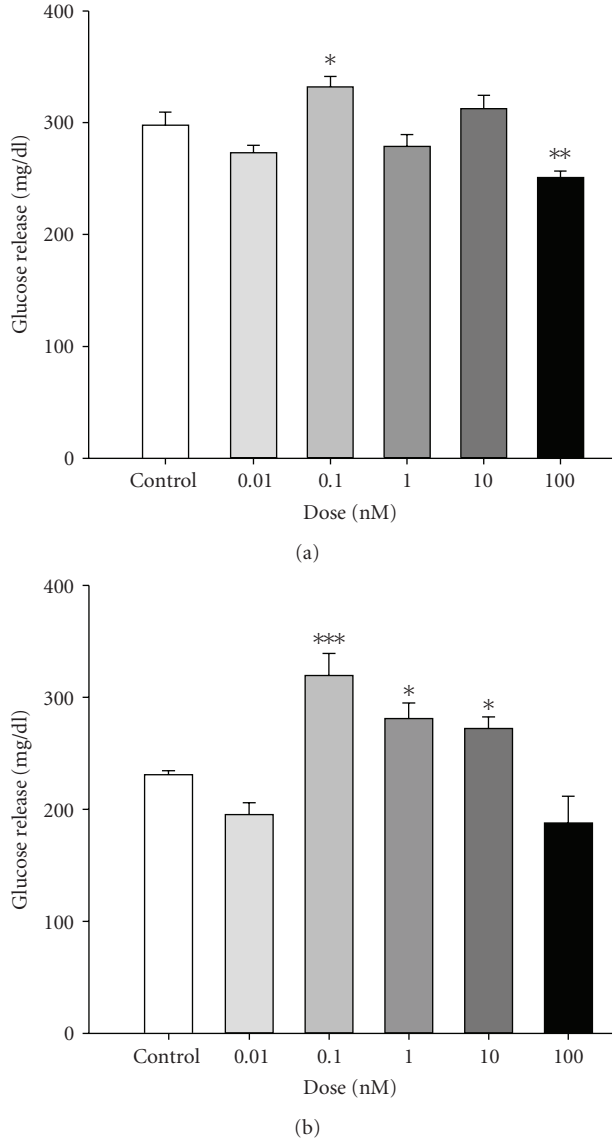

Figure 3: The effect of tilapia ghrelin-C8 (a) and ghrelin-C10 (b) on glucose release from cultured tilapia hepatocytes. Hepatocytes were exposed to ti-ghrelins for 6 h at that time culture media was collected and analyzed for glucose content. Vertical bars represent mean ± SEM. ∗, ∗∗ are significantly different from control at $P < .05$ and $< .01$, respectively (1-way ANOVA). $n = 8$–10.

growth rate and negatively correlated with plasma GH and IGF-I, suggesting that ghrelin may be linked to growth and metabolism and not appetite [37]. It would be of interest to see if the two acylated forms of ghrelin identified in other vertebrates exhibit different biological effects as we have observed in tilapia. What is interesting in tilapia is that we have shown that the GHS-R antagonist, [D-Lys^3]-GHRP-6, completely abolished the stimulation of GH release by both forms of tilapia ghrelin from dispersed pituitary cells [26]. These findings suggest that there is likely a second ghrelin receptor exhibiting higher specificity for ti-ghrelin-C10 with a different tissue distribution pattern than the one currently identified (GRLN-R). In the rat, ghrelin and des-octanoyl ghrelin but not a GHS-R1a agonists (L-163-255) induced adipogenesis independent of GH secretion, suggesting that ghrelin's adipogenic action is likely mediated by a novel receptor that is distinct from GHS-R1a [3].

In mammals, ghrelin has been shown to play a role in glucose homeostasis by exhibiting diabetogenic actions, by inducing hyperglycemia in humans [38]. We have observed similar effects in tilapia. In tilapia given a single i.p. injection of ti-ghrelin-C8 (1 ng/gm BW) plasma glucose levels were significantly elevated at 4 and 8 h ($P < .01$ and $P < .05$, resp.) postinjection; the high dose (10 ng/mL) was without effect (Figure 2). It is possible that the most effective dose of ti-ghrelin-C8 on plasma glucose levels is in the picogram range. It would be of interest to determine the dose response range of ti-ghrelin-C8 on plasma glucose levels. In cultured tilapia hepatocytes, ti-ghrelin-C8 (0.1 nM) significantly ($P < .05$) stimulated glucose release, whereas at 100 nM ti-ghrelin-C8 significantly ($P < .05$) reduced the release of glucose after a 6 h incubation (Figure 3(a)). Interestingly, ti-ghrelin-C10 (0.1–10 nM) significantly stimulated the release of glucose from cultured tilapia hepatocytes (Figure 3(b)), but without altering plasma glucose levels (Figure 2). This is the only report in any teleost describing the effect of ghrelin on glucose metabolism. Future work is needed to elucidate ghrelin's role in glucose metabolism in fish, especially since fish are considered to be glucose intolerant [39], and therefore fish could be an ideal alternative model for diabetes research. We have been able to detect both GHS-R1a and GHS-R1b transcripts in tilapia liver [26]. In both porcine and rat hepatocytes, ghrelin stimulated the release of glucose

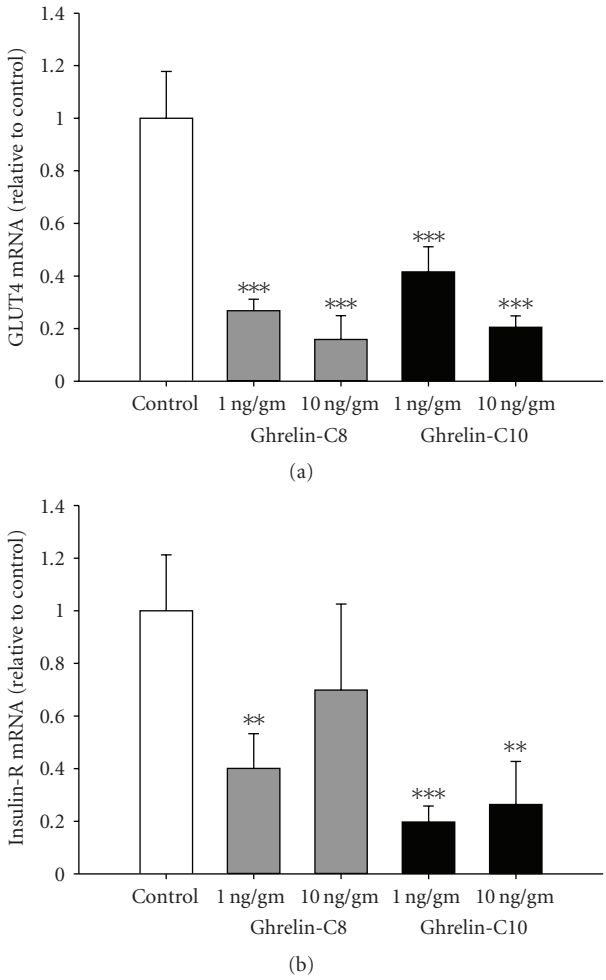

Figure 4: Effect of ghrelin-C8 and ghrelin-C10 on muscle GLUT4 (a) and insulin-R (b) mRNA levels 2 h post-injection. mRNA levels were normalized to the house keeping gene ARP. Vertical bars represent mean ± SEM (n = 5-6). ∗,∗∗, ∗ ∗ ∗ significantly different from control at $P < .05$,.01, and .001, respectively (1-way ANOVA).

[4]. However, a potent GHS, hexarelin, failed to alter plasma glucose levels. Gnanapavan and colleagues failed to identify the GHS-R1a in human liver, whereas the GHS-R1b was highly expressed; suggesting that the hyperglycemic action of ghrelin in mammals may be mediated through GHS-R1b or an unknown orphan GHSR [40]. Therefore, it is likely that the observed differential response of tilapia hepatocytes to the tilapia ghrelins is that an unknown orphan GHSR is present in tilapia that exhibits higher affinity to one ghrelin over the other. In spite of the differential effects of the tilapia ghrelin's on plasma glucose levels, we have observed that both ti-ghrelin-C8 and ti-ghrelin-C10 significantly reduced muscle mRNA levels of a putative tilapia glucose transport protein (GLUT4) and the insulin receptor (Figure 4). To our knowledge this is the first report in any vertebrate. Whether protein levels of GLUT4 and insulin receptor are changed following ghrelin treatment needs to be investigated.

The existence of ghrelin, GHS-R1a and GHS-R1b in fish suggests that the actions of ghrelin and GHSRs are conserved across vertebrate species and likely exhibit fundamental biological functions within vertebrates [41, 42]. Our data show that the two forms of tilapia ghrelin (octanoylated and decanoylated) exhibit different biological actions but that they may function together to maintain overall energy homeostasis in tilapia. It is of interest to investigate if the decanoylated form of ghrelin found in other vertebrates exhibits different biological activity than the octanoylated form. Furthermore, how different physiological states within the animal alter the circulating levels of these two different tilapia ghrelin's needs to be investigated. Currently, however, we are unable to differentiate between circulating ti-ghrelin-C8 and -C10 levels in our radioimmunoassay.

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Review Article

The Avian Proghrelin System

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To understand how the proghrelin system functions in regulating growth hormone release and food intake as well as defining its pleiotropic roles in such diverse physiological processes as energy homeostasis, gastrointestinal tract function and reproduction requires detailed knowledge of the structure and function of the components that comprise this system. These include the preproghrelin gene that encodes the proghrelin precursor protein from which two peptide hormones, ghrelin and obestatin, are derived and the cognate receptors that bind proghrelin-derived peptides to mediate their physiological actions in different tissues. Also key to the functioning of this system is the post-translational processing of the proghrelin precursor protein and the individual peptides derived from it. While this system has been intensively studied in a variety of animal species and humans over the last decade, there has been considerably less investigation of the avian proghrelin system which exhibits some unique differences compared to mammals. This review summarizes what is currently known about the proghrelin system in birds and offers new insights into the nature and function of this important endocrine system. Such information facilitates cross-species comparisons and contributes to our understanding of the evolution of the proghrelin system.

1. Introduction

Mammalian ghrelin is a 28 amino acid peptide hormone with a unique fatty acylation (n-octanoyl) modification at the serine 3 (Ser^3) position that exhibits growth hormone (GH) releasing and orexigenic activities. It was originally isolated from rat stomach and shown to be the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) in rats and humans [1]. The first report of the existence of avian ghrelin came in 2002 with the purification of the peptide, cloning of a cDNA, and preliminary testing of biological activity of the ghrelin peptide isolated from the proventriculus (the glandular portion of the avian stomach) of chickens [2]. Since that initial report, preproghrelin genes and predicted amino acid sequences for six different avian species have now been identified [3–5]. There have also been a number of reports concerning the structure and expression of the GHS-R gene in chickens, quail, and ducks. While some functions such as GH release are conserved in birds, other actions such as the effect of ghrelin on food intake are opposite to what has been found in mammals and other vertebrate species [3–5]. Moreover, the majority of studies in birds have focused on the ghrelin/GHS-R axis almost exclusively. Therefore, it is of interest to study the avian proghrelin system to determine the reasons for such differences and how they might relate to the evolution of this important hormonal system. This review focuses on the major components of the avian proghrelin system and discusses what is currently known about their structure, expression, and physiological action, while highlighting potential areas of future investigation needed to better define the operation of this system in birds.

2. Structure and Expression of the Preproghrelin Gene

To date, preproghrelin genes have been sequenced and characterized for the chicken, turkey, and duck (GenBank accession nos. AY303688, AY497549, and EF613552, resp.). In addition, cDNA sequence is now available for goose, quail, and emu preproghrelin mRNA (GenBank accession

nos. AY338465, AB244056, and AY338467, resp.). Based on this evidence it has been possible to project some of the features and genomic organization of a prototypical avian preproghrelin gene, as well as, to compare and contrast sequence variations that may be associated with functional traits in different avian species.

2.1. Gene Structure and Genomic Organization. The avian preproghrelin gene consists of five exons and four introns (Figure 1). This structure is similar to the human gene with the exception of the first exon which is larger in birds [6]. The transcriptional unit for the chicken preproghrelin gene locus, located on chromosome 12, spans 2.71 kb [7, 8]. The preproghrelin protein precursor is encoded within exons 2–5, with the mature ghrelin peptide split between exons 2 and 3 and obestatin encoded entirely within the fourth exon (Figure 1).

A recent study, that reexamined the structure and genomic organization of human and mouse preproghrelin genes, reported the existence of novel exons (including a sixth exon) containing 5′-untranslated region (5′-UTR) sequence and potential new open reading frames upstream of the originally identified first exon [9]. The existence of novel exons was confirmed by examining gene transcripts in cDNA libraries from different tissues using rapid amplification of cDNA ends (5′ RACE). No additional exons or alternatively spliced transcript variants have been reported for any avian preproghrelin gene to date. In light of this, it is interesting to note that evidence could not be found for conserved regions containing the novel upstream exons in chicken genome sequence which suggests that these novel exons may have evolved subsequent to the divergence of birds and mammals [9]. However, these findings do not rule out the possibility that a more complex structure and organization for the preproghrelin gene locus may exist in other species including birds. This is an area that clearly merits further investigation.

2.2. Sequence Variations. Analysis of different avian preproghrelin gene sequences revealed the existence of a number of polymorphisms, some of which could have functional significance. One particularly interesting feature is an 8 bp indel (insertion/deletion) found in the first exon (a non-coding exon) of egg-layer chickens (White Leghorn) that was absent in meat-type (broiler) chickens (Figure 1). This may be an important finding because these two strains differ markedly in their appetite and growth characteristics. The indel was also absent in red jungle fowl, an ancestral species for modern commercial chicken breeds [8]. This unique feature has been reported to occur at low frequency in a study summarizing sequence polymorphisms in four chicken breeds [7]. Moreover, sequence similar (but not identical) to the chicken indel and of the same size (8 bp) was present in the same location (exon 1) in the turkey, goose, duck, and emu [8, 10]. The presence of the 8 bp indel has subsequently been found to be positively associated with growth and carcass traits in meat-type chickens [11]. The turkey ghrelin gene also contains additional sequence (30 bp) at the junction of exons 1 and 2 due to exon extension (Figure 1). A single-base extension of exon 2 was found in duck, goose, and emu sequences [10]. Since both of these sequence features occur outside of the coding region of the mRNA transcript, their effects on preproghrelin gene function, if any, remain to be determined. It is possible that they could influence posttranscriptional processes such as mRNA stability or translation efficiency.

A single-nucleotide polymorphism (SNP, C223G) occurring in the 5′-UTR of the chicken preproghrelin gene was reported to determine the presence or absence of a specific transcription factor binding site (serum response factor) that could potentially influence expression of the ghrelin gene [7]. This group also reported a total of 19 SNPs in the chicken ghrelin gene sequence, the majority of these occurring within the 4 introns. There were no significant differences in these polymorphisms between egg-layer and meat-type chickens [7]. Nie et al. [7] and Richards et al. [8] reported an SNP (A/G) found in exon 5 (within the coding region) of the chicken preproghrelin gene that results in an amino acid change (Gln113Arg). However, this change would affect the C-terminal peptide region of the prohormone precursor and not in the mature ghrelin or obestatin peptides. Thus, its functional impact is unknown. There have been reports of an amino acid sequence variant form of ghrelin (des-Gln^{14}) in rats and humans that is created by the use of an alternative splice donor site located at the intron 2/exon 3 boundary. This change could potentially affect the physiological actions of mature ghrelin. However, when the purified peptide was tested, its activity (i.e., receptor binding) was comparable to intact ghrelin [12]. Moreover, this variant form is not produced at the same levels in stomach or plasma as intact ghrelin [13]. Sequence reported for all avian preproghrelin genes does not provide any evidence for an alternative splicing event that could lead to the same type of amino acid deletion in the mature avian ghrelin peptide.

2.3. Promoter Region. Flanking upstream (5′) sequence (2 kb) was examined in an attempt to determine characteristics of the putative proximal promoter region and perhaps shed some light on factors that might regulate preproghrelin gene expression in chickens [8]. Little is known about transcriptional regulation of the human gene [6, 9, 14–16] and nothing is currently known about preproghrelin gene regulation in birds. Glucagon and its second messenger, cAMP, have been reported to enhance human preproghrelin gene transcription and this has been suggested as a possible mechanism that leads to increased plasma ghrelin levels in response to fasting [15]. Similarly, growth hormone releasing hormone (GHRH) was found to upregulate gene transcription in the pituitary via a cAMP-signaling cascade [16]. The identification of two cAMP response element binding protein (CREB) sites in the putative promoter region of the chicken preproghrelin gene is consistent with the proposed gene regulation mechanism involving cAMP [8]. While an SREBP-1 site was identified just upstream of the TATA box in the chicken gene [8], a role for this transcription factor in preproghrelin gene regulation has not been reported previously in any species. The presence of multiple SRY and SOX-5 sites was also noted in this region of the chicken gene.

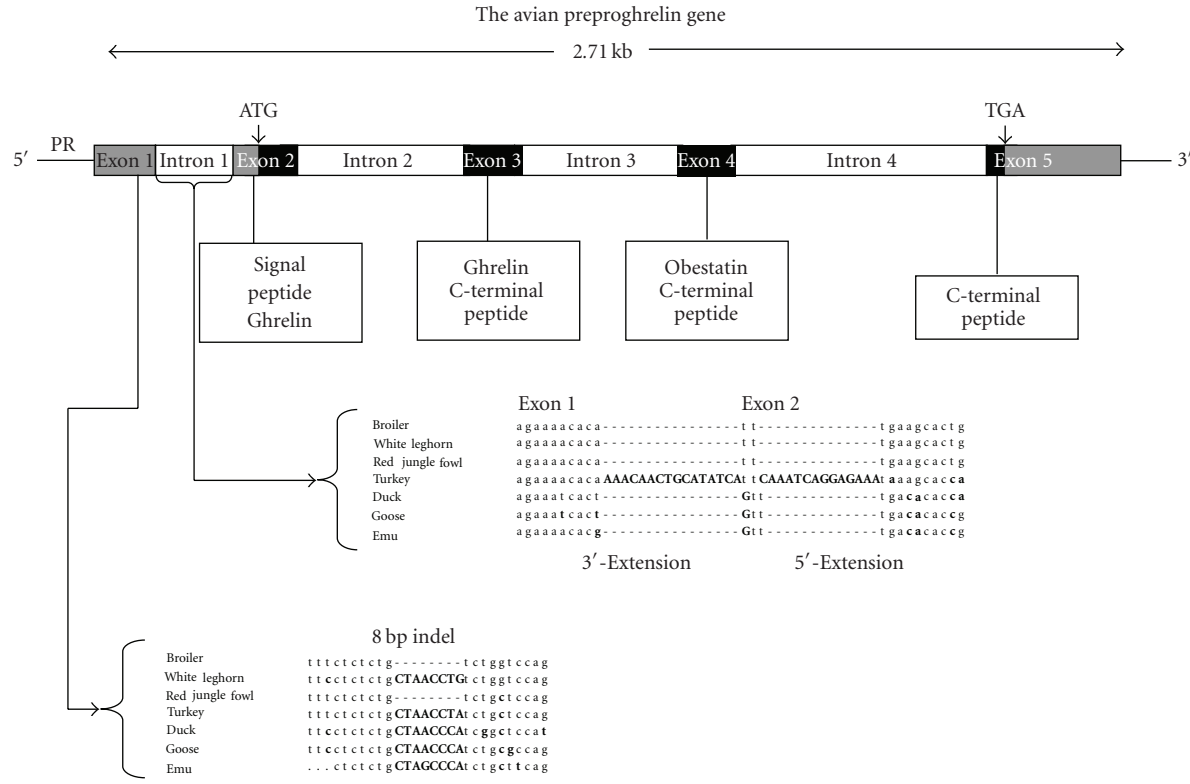

FIGURE 1: Genomic structure and organization of a prototypical avian preproghrelin gene (based on the chicken gene). The gene exhibits five exons (four coding exons shown in black) and four introns with the positions of the proximal promoter (PR) and the start (ATG) and stop (TGA) codons indicated. The portions of the preproghrelin precursor protein encoded by each exon are indicated in boxes. Also indicated are two sequence features detected in different avian genes. These include an 8 bp insertion/deletion (INDEL) located in exon 1 and exon extensions (5′ and 3′) of exons 1 and 2 detected only in the turkey.

However, the significance of these findings to preproghrelin gene regulation remains to be demonstrated.

Recent evidence suggests the involvement of more than one promoter (proximal and distal) in regulating transcription of the human preproghrelin gene to generate transcripts with differing 5′-ends and coding regions [6, 9]. There have been no reports of a corresponding alternate (distal) promoter in any avian preproghrelin gene to date. However, such findings emphasize the need for reexamination of the preproghrelin gene promoter(s) in different species to determine if such diversity exists and how that might relate to gene regulation.

2.4. mRNA Expression. Kaiya et al. [2] found preproghrelin mRNA expressed in proventriculus, brain, lung, spleen, and intestine among various chicken tissues surveyed. We reported expression of this mRNA in all tissues examined from 3-week-old male broiler chickens [8]. Proventriculus showed the highest expression followed by pancreas, brain, and intestine. In general, all studies to date indicate that the proventriculus is the predominant site for preproghrelin mRNA expression in birds reflecting an important systemic or endocrine role for the proventriculus with respect to regulating blood ghrelin levels [3–5]. However, the expression of preproghrelin mRNA in a variety of central and peripheral tissue sites also indicates the potential for local (autocrine/paracrine) effects of the proghrelin system in birds. Saito et al. [17] quantified preproghrelin mRNA levels in different regions of the brain in chickens and found that the corpus striatum expressed the highest levels followed by the cerebellum, the optic lobes, and the brainstem.

A number of studies have demonstrated the influence of feeding state on preproghrelin mRNA expression in different avian tissues [3–5]. Fasting has been reported to increase preproghrelin mRNA levels in proventriculus, but refeeding following a period of fasting did not return these levels to prefasting values [8, 18, 19]. The up-regulation of mRNA levels in the proventriculus during fasting coincides with changes in plasma acylated-ghrelin indicating an important role for the proventriculus in determining circulating hormone levels [18]. Effects of fasting on other tissues expressing preproghrelin mRNA included a reduction in pancreas, no effect on whole brain or hypothalamus, and an increase in liver [8, 18, 19]. Since the levels of mRNA expression are much lower in these tissues compared to the proventriculus, these effects most likely reflect localized (autocrine/paracrine) actions of the proghrelin system.

There have been a few studies detailing developmental changes in preproghrelin mRNA expression during embryogenesis and during the early post hatching period. Wada et al.

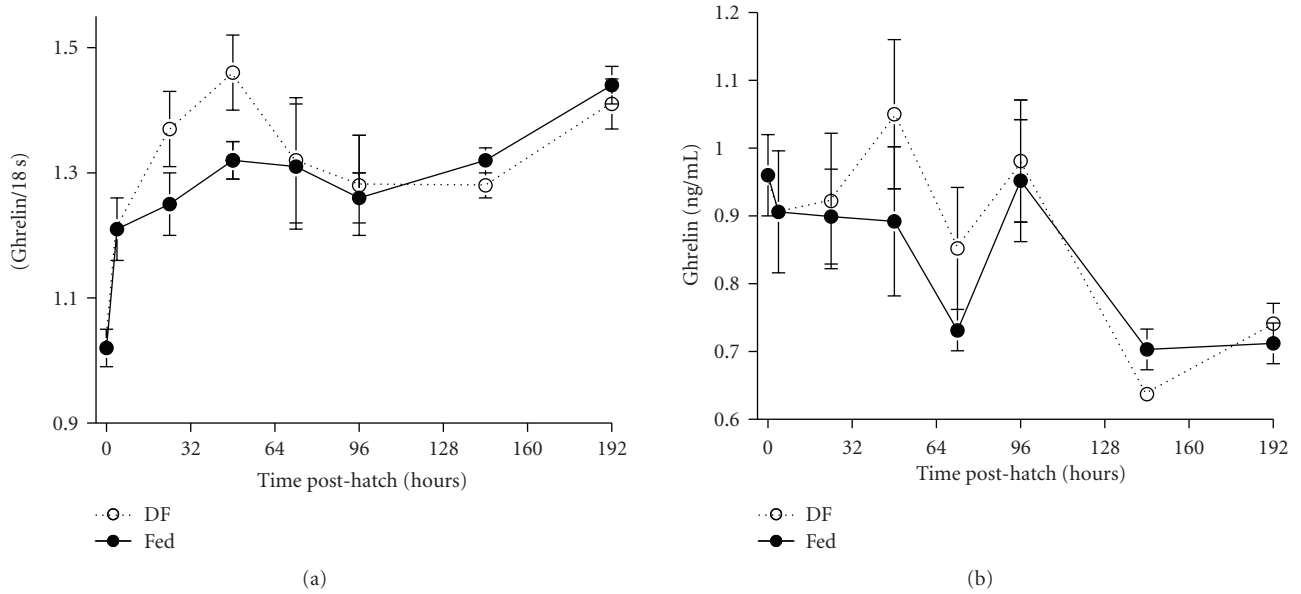

Figure 2: Expression of preproghrelin mRNA (a) in proventriculus and plasma total (acylated + des-acyl) ghrelin levels (b) in two groups of neonatal broiler chicks from hatch to 8 days posthatch. Feed was provided immediately after hatching (Fed) or was withheld for the first 48 hours after hatch (delayed feeding, DF). Values represent the mean ± SEM ($n = 6$).

[20] observed preproghrelin mRNA expression only in the proventriculus of newly hatched White Leghorn chicks, whereas in adult birds, mRNA expression was also detected in duodenum. They also found that mRNA and protein expressions were similar in proventricular mucosal X/A-like endocrine cells from adult chickens, whereas in newly hatched chicks, there was higher mRNA as compared to protein expression. Chen et al. [19] found low expression of preproghrelin mRNA in the proventriculus between days 15 to 19 of embryonic development, but expression increased dramatically by 2 days posthatch. We also found a similar ontogenic pattern from hatch (day 0) to 8 days posthatch (Figure 2(a)), with a peak at 2 days and a delay (48 hours) in feeding further enhanced the up-regulation. Furthermore, plasma levels of total ghrelin followed mRNA expression up to 2 days posthatch and then diverged with plasma ghrelin declining by 8 days posthatch (Figure 2(b)). The latter finding reflects the observations of Wada et al. [20] and Yamato et al. [21] reporting differences in mRNA expression and ghrelin protein levels in the proventriculus of newly hatched chicks and adult birds. The divergence in circulating ghrelin levels and mRNA expression in proventriculus could depend on events related to the transition from embryo to hatched chick. The perinatal period in birds is characterized by a major metabolic shift from the utilization of a high fat nutrient source (yolk) to a high carbohydrate diet (feed). During the first few days posthatch, the newly emerged chick begins feeding and ceases to rely solely on residual yolk from the yolk sac which is rapidly absorbed. Immediately after hatching (i.e., within the first 3 days), there is a dramatic up-regulation of preproghrelin mRNA expression in the proventriculus [19–21] and Figure 2(a). It has been suggested that the availability of specific nutrients (especially fatty acids such as octanoic acid) from the diet being consumed could influence the posttranslational processing (acylation) of the proghrelin protein precursor and secretion of the mature ghrelin peptide by proventricular X/A-like cells [19–21]. Thus, the divergent patterns of preproghrelin mRNA expression in the proventriculus and plasma ghrelin levels by four days posthatch may reflect differential maturation of transcriptional and posttranscriptional regulatory processes in the newly hatched chick which depend both on developmental and nutritional factors.

3. Proghrelin Precursor-Derived Peptides and Posttranslational Processing

An examination of cDNA sequences corresponding to six different avian preproghrelin mRNA transcripts predicts a consensus 116 amino acid preproghrelin precursor protein in birds (Figure 3). Cleavage of the 23 amino acid signal peptide yields a 93 amino acid proghrelin peptide. The proghrelin peptide is then further processed via a series of steps to yield a 26 or 28 amino acid ghrelin peptide and a 65 amino acid C-terminal peptide (C-ghrelin). C-ghrelin contains a putative 24 amino acid obestatin peptide which is generated after further proteolytic processing (Figure 4). The mechanism for producing mature ghrelin peptide (and possibly obestatin) from the mammalian proghrelin precursor was shown to involve limited proteolytic cleavage at a single arginine residue by the prohormone convertase PC1/3 expressed in mouse stomach [22]. A recent report found that PC1/3, PC2, and furin could process proghrelin to yield the mature ghrelin peptide in cultured mammalian cells [23]. We have previously shown that the chicken proventriculus expresses mRNA for both PC1/3 and PC2 and suggested the involvement of these two prohormone convertase enzymes in the

processing of proglucagon, another prohormone precursor [24]. However, the precise steps involved in the posttranslational processing of the avian proghrelin precursor have yet to be determined experimentally.

3.1. Ghrelin. To date, there has been only one report of the isolation, purification, and characterization of an avian proghrelin-derived peptide, that being ghrelin [2]. The mature acylated ghrelin peptide was isolated from chicken proventriculus tissue based on its ability to bind to and signal through the rat GHS-R in a cell-based assay system [2]. Chicken ghrelin was found to be a 26 amino acid peptide that exhibited 54% of amino acid sequence identity with human ghrelin (Table 1). The first seven amino acids (GSSFLSP) of chicken ghrelin are identical to human ghrelin and it is this N-terminal segment that contains the so-called "active core" (GSSF). The active core is thought to be important in binding to and activation of the GHS-R because it is at the third position (Ser^3) that attachment of a fatty acid molecule takes place which is known to be essential for ghrelin activation and receptor binding [1–5]. In fact, all avian ghrelin peptides display total conservation of the first seven N-terminal amino acids (Figure 3). Moreover, all avian mature ghrelin peptides are predicted to be 26 amino acids in length with the exception of turkey ghrelin. The turkey mature ghrelin peptide is predicted to be 28 amino acids in length due to a proline-extension at its C-terminal end, similar to human ghrelin (Figures 3 and 4). The other five avian ghrelin peptides contain two arginine residues (RR) at this position which are thought to be cleaved by a carboxypeptidase E-like enzyme during processing to produce the mature 26 amino acid ghrelin peptide [4].

Kaiya et al. [2] determined the acylation state of purified chicken ghrelin peptides and found that the Ser^3 position was acylated with a medium-chain saturated fatty acid, either octanoic (C8:0) or decanoic (C10:0) acid. Preliminary evidence also suggests the possible acylation of chicken ghrelin with a monounsaturated fatty acid, decenoic acid (C10:1) as has been reported for human ghrelin previously [5, 25]. The mechanism for the acylation and activation of ghrelin has recently been shown to involve the membrane-bound enzyme, ghrelin-O-acyltransferase or GOAT, that attaches medium-chain fatty acids to proteins [26, 27]. Furthermore, it has also been suggested that the posttranslational acylation of the proghrelin precursor occurs independently of the proteolytic processing by PC enzymes so that octanoylation does not require prior cleavage of the mature ghrelin peptide from the proghrelin precursor [22]. The presence of PC enzymes, GOAT, and n-octanoic acid in the culture medium were all found to be required to produce n-octanoyl ghrelin by mammalian cells [23].

In mammals, GOAT has been suggested to function as a gastric lipid sensor linking nutrient ingestion (i.e., presence of medium-chain fatty acids and medium-chain triacylglycerols) with actions of the ghrelin endocrine system in maintaining energy homeostasis [28]. There is some evidence to suggest a linkage between gastric lipid sensing and ghrelin activation (GOAT-ghrelin system) in birds. First, analysis of the chicken genome sequence indicated the presence of a GOAT gene ortholog (Mboat4, still uncharacterized) which is located on chromosome 4 [26]. Second, and most intriguing, is the report of Yamato et al. [21] who demonstrated that oral feeding or intraperitoneal (ip) injection of octanoic acid increased the level of octanoylated ghrelin peptide without affecting preproghrelin mRNA levels in neonatal chick proventriculus. These findings strongly suggest that medium-chain fatty acids absorbed from food can be directly utilized to acylate the ghrelin peptide in birds.

There have been a few reports in which plasma levels of ghrelin were measured in chickens and Japanese quail [8, 18, 29]. The levels detected varied widely however. We determined the plasma levels of total ghrelin in fasted and fed 3-week-old [8] and neonatal (Figure 2) broiler chickens. These levels were substantially higher than those reported by Kaiya et al. [18] in fasted and fed layer chicks and Shousha et al. [29] in adult male Japanese quail (ng/mL versus fmol/mL). The reason for these discrepancies involves the different assays employed to measure ghrelin [5]. The commercial assay used to measure total ghrelin (Linco Research, St. Charles, MO) detects both acylated and des-acyl ghrelin, whereas, Kaiya et al. [18] and Shousha et al. [29]

Table 1: Characteristics of chicken proghrelin system components.

Component[1]	Size (aa)	Chromosome	Homology (%)[2]
(1) Preproghrelin	116	12	36
(i) Signal Peptide	23		30
(ii) Ghrelin	26/28[3]		54
(iii) C-Terminal Peptide	65		31
(iv) Obestatin	24[4]		46
(2) Growth Hormone Secretagogue Receptor (GHS-R)		9	
(i) GHS-R1a	347		76
(ii) GHS-R1aV	331		76
(iii) GHS-Rtv	220		78
(3) GPR39[5]	462	7	61

[1] Information for size and homology derived from: GenBank accession nos. NP_001001131, NP_989725, and NP_001073574 for preproghrelin, growth hormone secretagogue receptor (GHS-R), and GPR39, respectively.
[2] Based on amino acid identities compared to corresponding human sequence (NP_001128413, NP_004113, and NP_001499 for preproghrelin, growth hormone secretagogue receptor (GHS-R), and GPR39, resp.).
[3] The chicken ghrelin peptide contains two arginine residues (RR) at its C-terminal end that serve as a processing signal for proteolytic cleavage and are removed by the action of carboxypeptidase to give rise to the mature 26 amino acid peptide [2]. The human ghrelin peptide contains a proline and arginine pair (PR) at its C-terminal end and gives rise to a 28 amino acid mature peptide because these two amino acids are retained.
[4] The human obestatin peptide (23 amino acids) contains a glycine residue at its C-terminal end that is used for amidation. In contrast, chicken obestatin (24 amino acids) contains a glutamic acid residue at this site and is therefore one amino acid longer than the human peptide and is most likely not amidated.
[5] Information for GPR39 is included because it is a G protein-coupled receptor related to GHS-R. However, it is now generally accepted that this orphan receptor is not the putative obestatin receptor in mammals and this is assumed to apply to birds as well.

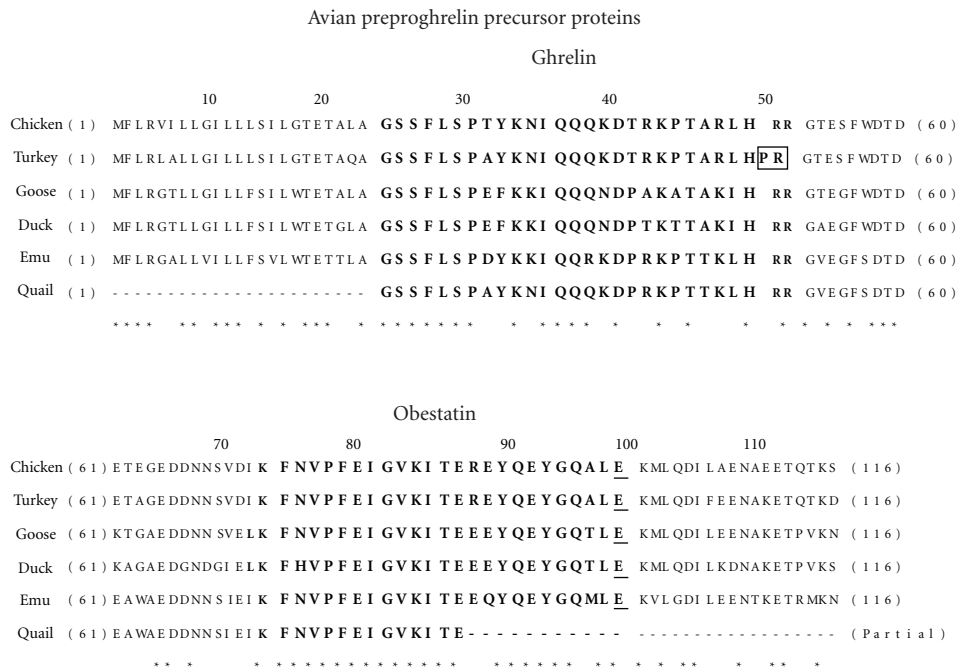

Figure 3: Amino acid comparisons of preproghrelin precursor proteins for different avian species. The locations of the mature ghrelin peptide and the putative obestatin peptide are indicated by larger bold typeface. The C-terminal glutamic acid residue (E) conserved in avian obestatin peptides is shown by bold underline typeface. Also, basic amino acids delineating proteolytic cleavage sites are shown in bold italic typeface. *indicates amino acid identity across all species. Amino acid sequences shown for chicken (broiler), turkey, goose, duck, and quail preproghrelin precursor proteins were obtained from GenBank accession nos. BAC24980, AAP93133, AAQ56122, AAQ56123, and BAE54265, respectively.

utilized a specific N-terminal directed radioimmunoassay (RIA) and sample purification (HPLC) to measure only the acylated form of ghrelin. Taken together, the combined results suggest that the level of des-acyl ghrelin is much higher than acylated ghrelin in plasma of birds. However, that has yet to be confirmed by direct measurement of both proghrelin-derived peptides in the same samples. The observations on plasma ghrelin in birds are consistent with mammalian studies which found that des-acyl ghrelin is considerably more abundant in plasma than acylated ghrelin [25, 30]. The significance of this disparity in levels is unclear; however, it is possible that both forms of ghrelin have important physiological roles in birds.

3.2. Des-acyl Ghrelin. As indicated above, indirect evidence from plasma ghrelin analyses points to the existence of des-acyl ghrelin in avian blood. The production of des-acyl ghrelin in mammals is thought to occur by deacylation of mature ghrelin occurring both in the gastric mucosa and in plasma under the action of esterase (butrylcholine esterase, paraoxonase, and lysophospholipase I) enzymes [30]. Yang et al. [26] reported that des-acyl ghrelin is not an intermediate of proghrelin processing and suggested that deacylation of mature ghrelin is the mechanism for its production as opposed to its derivation from the proghrelin precursor directly. To date, the existence of ghrelin peptide lacking an acyl-modification of Ser^3 in avian blood or tissues has yet to be confirmed experimentally [5]. Intracerebroventricular (icv) administration of synthetic des-acyl ghrelin to chickens produced no effect on feeding behavior [3]. Furthermore, in vitro administration of des-acyl ghrelin had no effect on gastrointestinal tract contractility in chickens [31]. Together these results suggest that des-acyl ghrelin does not bind to or activate the GHS-R in birds.

There have been no studies yet to determine if des-acyl ghrelin can antagonize the actions of ghrelin in birds when coadministered as has been observed in other vertebrate species such as the goldfish [4]. Despite its apparent lack of effect in chickens, growing evidence in mammals suggests that des-acyl ghrelin is active in a number of biological processes in vitro such as stimulating adipogenesis, decreasing hepatocyte glucose output, preventing cell death in cardiomyocytes, and inhibiting cellular proliferation in breast cancer cells [30]. Since it is generally recognized that des-acyl ghrelin does not interact with the GHS-R [1], these actions are thought to be mediated by a different receptor(s) which has yet to be identified [13].

3.3. Obestatin. Zhang et al. [32] originally identified obestatin as a proghrelin-derived peptide isolated from the stomach of rats. Since then, evidence for this peptide has been presented for a number of vertebrate species including birds (Figure 3). The obestatin peptide is delimited by two basic amino acid residues (lysines, K) that serve as proteolytic recognition sites in the C-terminal peptide portion of the proghrelin precursor. Avian obestatin displays one notable

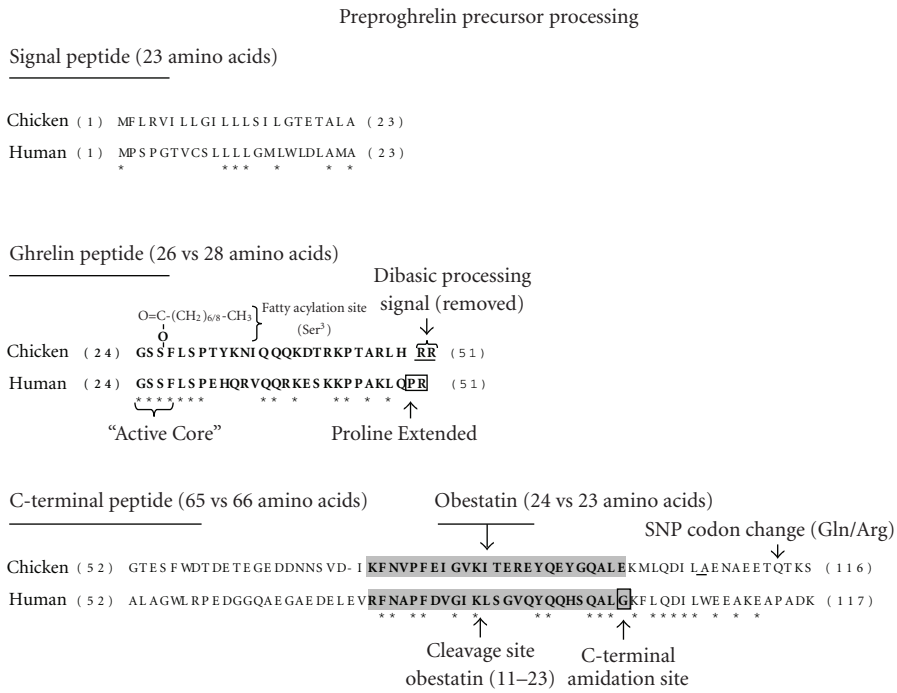

Figure 4: Comparison of amino acid sequence for chicken (GenBank accession no. NP_001001131) and human (GenBank accession no. NP_001128413) perproghrelin precursor proteins and the major peptides processed from the precursor including a signal peptide, the mature ghrelin peptide, C-terminal peptide, and obestatin (shown in gray box). *indicates amino acid identity. The C-terminal glycine (G) residue used to amidate the human obestatin peptide is indicated. Basic amino acids delineating proteolytic cleavage sites are shown in bold italic typeface. The Ser^3 acylation site within the "active core" of the mature ghrelin peptide is indicated. The location of a single nucleotide polymorphism (SNP) resulting in an amino acid change (glutamine/arginine) located in the C-terminal peptide of the chicken precursor is shown.

difference compared to its mammalian counterparts. All mammalian obestatin peptides contain a conserved C-terminal glycine residue (G) that is utilized for amidation of the peptide at this end [32]. Amidation of the C-terminus was thought to be essential for the binding of obestatin to its cognate receptor, originally proposed to be GPR39 [32]. However, all known avian obestatin peptides have a glutamic acid residue (E) in this position (Figure 3). Therefore, it must be assumed that avian obestatin (if it exists) would be 24 amino acids in length and not amidated at its C-terminal end (Figure 4). Zhang et al. [32] also identified an N-terminally truncated 13 amino acid amidated peptide (obestatin 11–23). This peptide fragment was produced by proteolytic cleavage of the full length obestatin peptide at an internal lysine residue (Lys^{10}). The same scenario is possible for avian obestatin peptides which contain a conserved lysine at the identical internal position (Figure 3). Thus, a 14 amino acid truncated obestatin peptide (obestatin 11–24) is predicted in birds which would not be C-terminally amidated (Figure 4). To date, there have been no reports of the detection or isolation of obestatin or the truncated peptide in any avian species. Given the low level of homology (46%) between avian and human obestatin peptides (Table 1), it is likely that new avian-specific antibodies will be required to detect the presence of obestatin peptides in blood and tissue samples obtained from birds. It is, however, important to isolate and purify the avian peptide and its fragment to confirm the existence of the predicted differences in structure compared to its mammalian counterpart.

3.4. Other Precursor-Derived Peptides? Another proghrelin-derived peptide that has been detected in circulation in mammals is C-ghrelin which is the C-terminal 66 amino acid peptide containing obestatin that remains after the cleavage of mature ghrelin [13]. The stomach is thought to be the major source of circulating C-ghrelin [13]. To date there is no evidence that this peptide is present in tissue or blood of any avian species, although if it does exist it is predicted to be 65 amino acids in length (Figure 4).

4. Proghrelin-Derived Peptide Receptors

Following the initial discovery of rat and human ghrelin peptides as endogenous ligands for the GHS-R, a G protein-coupled 7 transmembrane domain (TMD) receptor [1], Kaiya et al. [2] reported similar findings for chicken ghrelin which was purified based on its ability to bind to the rat GHS-R1a receptor expressed in a mammalian cell line. Subsequently, avian GHS-R genes were identified and their sequence, structure, genomic organization, and expression were studied in chickens [3–5, 8, 19, 33–36]. The findings of Zhang et al. [32] that the GHS-R related orphan receptor GPR39 was thought to be the putative obestatin receptor

led to the cloning and characterization of chicken and Japanese quail GPR39 gene orthologs [37, 38]. In light of the subsequent failure to confirm the initial findings of Zhang et al. [32], interest in avian GPR39 genes has declined. However, a brief discussion of GPR39 is included below since this receptor is structurally related to GHS-R and so that meaningful comparisons can be made in the structure and expression of the avian and mammalian genes.

4.1. Growth Hormone Secretagogue Receptor (GHS-R). Like its mammalian counterparts, the chicken GHS-R gene, located on chromosome 9, consists of two exons separated by a single intron (Figure 5). The gene encodes a 347 amino acid protein containing 7 TMDs that demonstrates high homology (>70% amino acid identity) with the human protein (Table 1). The first exon contains coding sequence for the first 5 TMDs and the sixth and seventh domains are encoded in exon 2 [33, 34]. Transcription and alternative splicing of the chicken GHS-R gene have been reported to produce three distinct transcripts, GHS-R1a, GHS-R1aV (also referred to as GHS-R1c), and GHS-Rtv (Figure 5). The GHS-R1a transcript is produced by splicing exons 1 and 2 and encodes the full length (347 amino acids) receptor protein with 7 TMDs. The GHS-R1aV transcript variant exhibits a 48 bp deletion at the 5′-end of exon 2 caused by the use of an alternative splice acceptor site during processing of the mRNA transcript [34]. This deleted sequence results in the loss of 16 amino acids from TMD 6. Therefore, the protein produced from this transcript would be predicted to lack a functional sixth domain but contain an intact seventh TMD. Because of this, the GHS-R1aV transcript is thought to encode a nonfunctional receptor similar to the GHS-R1b transcript produced by mammalian GHS-R genes [39]. However, the processed chicken GHS-1aV transcript is not identical to the mammalian GHS-R1b which is generated by termination of transcription within the intron at an alternate stop codon which produces a receptor protein that is truncated after TMD 5. The avian GHS-Rtv transcript has some sequence captured from the intervening intron including an alternate stop codon (Figure 5). This transcript would be predicted to produce a receptor protein that is truncated after TMD 5 and is thus more like the mammalian GHS-R1b [35]. Recently, Japanese quail GHS-R gene transcripts (including a GHS-R1b variant) have been identified that result from additional splicing mechanisms affecting the encoded protein (e.g., N-terminal truncation, loss of TMDs) indicating the potential for new and more complex processing of avian GHS-R mRNA transcripts (GenBank accession nos. AB469019–AB469022). Fang et al. [36] reported a number of sequence polymorphisms present in the chicken GHS-R gene. These occurred in both exons as well as the intron and were, in some cases, related to growth and carcass traits.

Expression of chicken GHS-R1a and GHS-R 1aV transcripts has been detected in all tissues examined with the highest levels of expression of GHS-R1a found in pituitary and hypothalamus [2–5, 8, 18, 33, 34]. The latter finding is consistent with the proposed role of GHS-R in mediating the effects of ghrelin on GH release and food intake. While the GHS-Rtv transcript has only been identified in ovarian cells [35], reexamination of our original findings [8] indicates that it is coexpressed along with the other two transcripts in different tissues in the broiler chicken (Figure 5). In general, the GHS-R1a transcript is the most highly expressed followed by the GHS-R1aV and GHS-Rtv. To date there have been no reports concerning the detection of GHS-R proteins so it is not yet possible to determine if each of the three mRNA transcripts results in an expressed protein in birds. However, the fact that Kaiya et al. [2] were able to utilize the rodent GHS-R1a to screen for chicken ghrelin strongly suggests that the chicken GHS-R1a protein should function in a similar way to its mammalian counterparts. While there has been no systematic attempt to determine the expression of individual GHS-R isoform mRNA, there is preliminary evidence to suggest that there may be differential tissue-specific expression of these receptor variants (Figure 5) and that could have some functional significance.

Very little is currently known about the regulation of the GHS-R gene in birds. Geelissen et al. [33] reported that chicken ghrelin, GH, and corticosterone administered to pituitary explants in vitro down-regulated the production of the GHS-R1a mRNA while thyrotropin-releasing hormone (TRH) was without effect despite its recognized ability to act as a potent GH-releasing factor in chickens [5]. These preliminary results seem to suggest the possibility for ligand-mediated negative feed-back regulation of this gene in birds [33].

4.2. GPR39 and the Search for the Obestatin Receptor. Two reports have identified and characterized GPR39 gene orthologs in the chicken and Japanese quail [37, 38]. The chicken GPR39 gene, located on chromosome 7, consists of two exons divided by a single intron and encodes a 462 amino acid 7 TMD protein that exhibits 61% amino acid identity with the human protein (Table 1). The GPR39 gene in birds exhibits very similar genomic organization to the GHS-R gene especially with respect to the positioning of the intron and the inclusion of coding sequence for the sixth and seventh TMDs in the second exon [37]. Within the deduced protein, two cytoplasmic regions located between TMDs 1&2 and TMDs 3&4, and an extracellular loop region (putative ligand-binding region) located between TMDs 6&7 are all highly conserved compared to the human protein suggestive of a similar function for this orphan receptor in birds [37]. The GPR39 gene is widely expressed in different tissues in the chicken and Japanese quail including the gastrointestinal tract (with the highest levels found in duodenum), reproductive tissues, liver, and kidney [37, 38]. Very low levels of expression were observed in the brain and pituitary unlike the GHS-R. Expression in the duodenum was found to increase dramatically during the early posthatch period in chickens, suggesting a potential role in intestinal function and/or maturation [37]. Other than cloning and sequencing GPR39 gene orthologs and determining tissue mRNA expression levels in two avian species, there has been no effort to further characterize any ligand-binding or functional aspects of this GHS-R-related

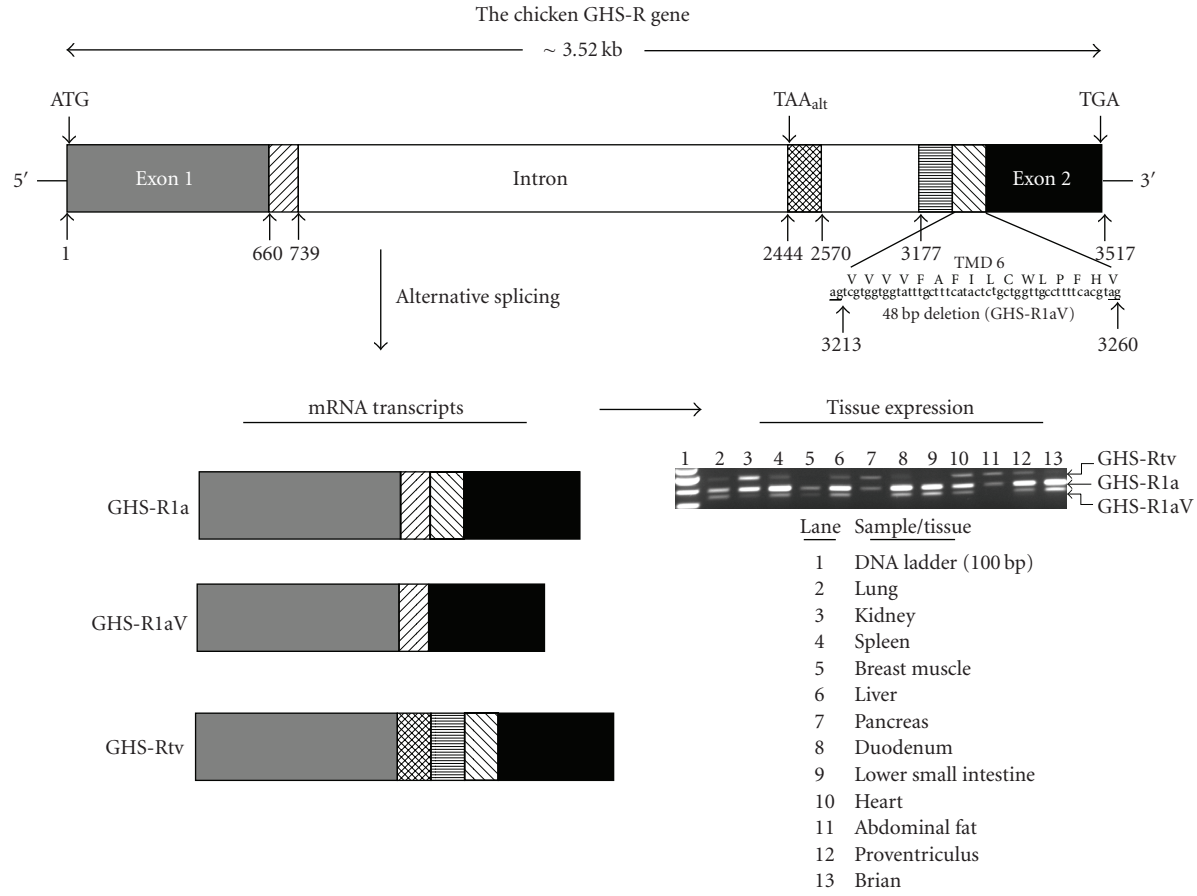

Figure 5: Genomic structure, organization, and expression of the chicken GHS-R gene consisting of two exons separated by a single intron. Different alternative splicing mechanisms are used to produce three transcript variants (GHS-R1a, GHS-R1aV, and GHS-Rtv) that encode proteins of 347, 331, and 220 amino acids, respectively. Sequence encoding a major portion of transmembrane domain 6 (TMD 6), located at the 5′-end of exon 2, is shown and is spliced out of the GHS-R1aV transcript. The GHS-Rtv variant is formed by splicing two portions of intron sequence, one of which contains an alternative stop codon (TAA$_{alt}$) which is predicted to produce a receptor protein truncated after TMD 5. Expression of each of the GHS-R mRNA transcript variants in different tissues obtained from 3-week-old broiler chickens is also shown.

receptor in birds nor has there been any attempt to determine its endogenous ligand.

5. Physiological Actions of Proghrelin-Derived Peptides

The focus of this section will be on active ghrelin (the acylated peptide), since there are very few published reports addressing the physiological actions for any other proghrelin-derived peptide in birds. With respect to ghrelin, some of its reported functions are similar to mammals such as its stimulatory effects on GH release and gastrointestinal tract contractility [3–5]. However, there are notable differences such as the effects of ghrelin on food intake and energy homeostasis. The previous sections have clearly demonstrated that both ligand and receptor are highly conserved between mammalian and avian species. This is reflected in the finding by Kaiya et al. [2] that chicken acylated-ghrelin was capable of binding to and signaling through the rat GHS-R in cultured cells. Therefore, structural variations most likely do not account for observed differences in function. In the following sections what is currently known about the pleiotropic actions of ghrelin in birds will be discussed in relation to what has been reported in mammals.

5.1. Hormone Releasing Activity. One of the principal effects of ghrelin in mammals is to stimulate GH release from the pituitary, and its ability to bind to the GHS-R formed the basis for its initial discovery [1, 40]. In birds, administration of ghrelin (human, rat, or purified chicken peptide) by intraveneous (iv) injection is known to transiently elevate circulating GH levels in both young and adult chickens in a dose- and time-dependent manner [2, 41, 42]. While it is clear that acylated-ghrelin working through the GHS-R promotes GH release in chickens, the exact mechanism(s) involved has not been completely defined in these studies. It was shown that both rat and synthetic chicken ghrelin peptides bound to and activated the GHS-R in vitro (cell culture assay system) with equal potency and stimulated GH

release into plasma in vivo with similar dose-response curves [2]. Using dispersed pituitary cells, Baudet and Harvey [42] showed that human ghrelin induced a dose-dependent GH release with potency comparable to GHRH indicating that ghrelin was capable of acting directly on pituitary somatotrophs. These findings are supported by the high level of GHS-R1a mRNA expression found in the chicken pituitary [33, 34]. However, this does not rule out the possible role of the hypothalamus in mediating the effects of ghrelin on GH release in vivo. Ahmed and Harvey [41] detected ghrelin protein expression in specific regions of the chicken hypothalamus. They suggested ghrelin, expressed locally in the central nervous system (CNS), might also participate in regulation of the hypothalamic-pituitary-GH axis. In mammals, it has also been suggested that ghrelin acts peripherally to effect GH release via stimulation of vagal afferent nerves [40]. This possibility has not yet been explored in birds.

The release of corticosterone from the adrenal gland into circulation represents a concurrent hormone releasing activity affected when ghrelin is administered to birds [2, 17]. In fact, it has been observed that the effect of ghrelin on corticosterone release is more pronounced in chickens as compared to other species including rats and humans [17]. Kaiya et al. [2] reported that iv injections of human or chicken ghrelin into young growing chicks resulted in increases in plasma corticosterone in a dose- and time-dependent manner and suggested that ghrelin could be an important regulator of adrenal function in birds. Saito et al. [17] found that icv injection of chicken ghrelin also increased plasma corticosterone in a dose- and time-dependent manner in neonatal chicks. Moreover, coadministration of the CRF receptor antagonist astressin reduced the effect of ghrelin on plasma corticosterone. Together, these findings suggest a mechanism of action for ghrelin in regulating the hypothalamic-pituitary-adrenal (HPA) axis via CRF and adrenocorticotropic hormone (ACTH) [17]. These findings are supported by expression of GHS-R1a mRNA in both the hypothalamus and the adrenal gland consistent with the potential for both local and CNS actions by ghrelin on the HPA [2, 33].

5.2. Food and Water Intake. The single most significant difference in ghrelin function between birds and mammals concerns its role in the regulation of food intake. Ghrelin is a potent orexigen in mammals when injected centrally or peripherally [1, 13, 40]. Numerous studies with birds have now clearly shown that ghrelin administered icv inhibits food intake in a dose-dependent manner under both fed and fasted conditions [2–5, 17, 29, 43, 44]. In contrast, central administration of des-acyl ghrelin had no effect on food intake in neonatal chicks [45]. However, peripheral administration of ghrelin produced some conflicting findings with respect to its effects on food intake [18, 29, 46]. These discrepancies do not appear to involve unique (to birds) differences in the structure of ghrelin and the GHS-R since icv injection of synthetic heterologous ghrelins (rat and bullfrog) produced comparable anorexigenic effects to those observed with homologous (chicken) ghrelin [44].

In mammals, the orexigenic effect of ghrelin is thought to involve the activation of hypothalamic neurons expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP) as well as downstream neurons expressing other orexigenic peptides such as orexin [40]. Thus, a logical explanation for the different effect of ghrelin on food intake in birds would involve a different site(s) and/or mechanism(s) of action for ghrelin in the CNS. Saito et al. [17] clearly showed that ghrelin administered icv to neonatal chicks failed to increase hypothalamic NPY mRNA expression. Moreover, this group also found that NPY-induced feeding could be antagonized by coadministration of ghrelin suggesting the involvement of separate neuronal/neuroendocrine pathways. They concluded that central administration of ghrelin to birds does not activate NPY-expressing neurons, but instead works via CRF and the HPA axis to bring about anorexia and increased circulating corticosterone levels. The CRF receptor antagonist astressin was able to attenuate ghrelin-induced reduction in food intake [17]. Khan et al. [46] recently reported that coadministration of the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methylester (L-NAME) to neonatal chicks attenuated the anorexigenic effect of CRF suggesting a role for NO in mediating the central effects of CRF. Interestingly, this group found no effect of coadministration of L-NAME on ghrelin-induced anorexia which implies that a CRF/NO axis may not mediate ghrelin's actions on food intake in birds.

Administration of ghrelin to mammals produces an orexigenic response whether administered centrally or peripherally [40]. In birds, the effects of ghrelin administered peripherally produced conflicting results ranging from having no effect [18] to stimulating [29] or inhibiting [47] food intake in chickens and Japanese quail. It has been suggested that very high peripheral doses of ghrelin could bypass the blood-brain barrier and act directly on the hypothalamus, whereas lower doses might work via stimulation of vagal afferent nerves in the periphery that influence central feeding regulatory circuits via neural relays in the nucleus of the solitary tract (NST) of the brain stem [5]. In support of the latter mechanism, vagotomy in mammals is known to inhibit ghrelin's ability to stimulate GH release and food intake [40]. However, this has not been studied in birds. In general, it is likely that different neurocircuits and neuroendocrine factors mediate ghrelin-induced changes in feeding behavior in birds as compared to mammals. Also important are the sites of GHS-R expression, as these will determine which neuronal circuits are impacted by ghrelin. Further study is required to more fully elucidate the nature and function of central feeding circuits and the neuroendocrine factors involved in birds.

It has been reported that centrally administered ghrelin acts as an anti-dipsogenic peptide in chickens [48]. Ghrelin-induced inhibition of water intake occurred under *ad libitum* and water-deprived conditions. In contrast, des-acyl ghrelin had no effect on water intake suggesting a role for GHS-R in mediating this effect. Recently, obestatin (synthetic chicken peptide) was reported to decrease water consumption in a line of chickens selected for low body weight [49]. In general, the mechanisms mediating the effects of

proghrelin-derived peptides on water consumption remain unknown.

5.3. Energy Homeostasis. Geelissen et al. [47] reported that peripheral (iv) administration of ghrelin to week-old male broiler chickens reduced the respiratory quotient (RQ) without affecting metabolic rate or heat production. A decrease in RQ could indicate that ghrelin induced a decline in lipogenesis and increased fatty acid oxidation in peripheral tissues and such effects would signal a switch in fuel utilization from carbohydrate to lipid. However, Geelissen et al. [47] observed no changes in plasma levels of glucose, triglycerides, fatty acids, or T_3 in response to ghrelin administration. In contrast, Shousha et al. [29] reported that both peripheral (ip) and central (icv) administration of ghrelin (rat peptide) to adult male Japanese quail invoked transient dose-dependent increases in body temperature indicative of increased thermogenesis and energy expenditure. Most of these observations in birds are in direct opposition to the reported effects of ghrelin in mammalian species in which it is reported to stimulate appetite, reduce fat oxidation (increase RQ), and induce weight gain without affecting heat production [13, 40]. Recently, Buyse et al. [50] reported that a single iv injection of chicken ghrelin into *ad libitum* fed day-old male broiler chicks reduced mRNA expression of fatty acid synthase (FAS) and two lipogenic-related transcription factors (SREBP-1 and PPAR-γ) in liver. These findings indicated a ghrelin-induced reduction in fatty acid biosynthesis at the main tissue site for lipogenesis in birds. Interestingly, the effect of peripheral ghrelin administration on expression of these same lipogenic genes in the diencephalon was opposite to that observed in liver. It was suggested that the anorexic effect of ghrelin in birds might involve regulation of FAS expression in hypothalamic neurons [50]. Again, these findings contrast sharply with what has been observed in mammals suggesting an opposite action of ghrelin on energy homeostasis which coincides with its opposite effect on food intake in birds.

5.4. Reproduction. In mammals, ghrelin and GHS-R are expressed in gonadal tissue as well as in hypothalamic neurons that function in the gonadotropic axis indicative of a regulatory role in reproduction [13]. Similar observations have been made in birds. Yoshimura et al. [51] detected ghrelin mRNA and protein expression in mucosal epithelial cells of the infundibulum and magnum portions of the oviduct in laying Japanese quail. Recently, this same group identified ghrelin in different components (i.e., yolk, albumin) of fresh fertilized chicken eggs with higher levels detected in yolk as compared to albumin [52]. While ghrelin concentration of total egg contents (i.e., yolk, albumin, and embryo) did not change during the first 5 days of incubation, it was suggested that it could play a role in embryonic cells during early phases of development of chicken embryos. A possible early developmental role for ghrelin (perhaps initially derived from maternal sources) in birds is further supported by the findings of Gahr et al. [53] who detected GHS-R expression in early developing chicken embryos and observed a 2.5-fold increase between days 4 and 5 of incubation.

There have several reports on the expression of ghrelin and GHS-R in ovarian tissue and cells suggesting the potential for autocine/paracrine effects of the proghrelin system in the avian ovary [35, 54, 55]. Administration of ghrelin or a ghrelin analog (ghrelin 1–18) to ovarian granulosa cells in culture induced markers of proliferation while decreasing markers of apoptosis, as well as stimulating the release of progesterone, estradiol, arginine-vasotocin (AVT), and insulin-like growth factor-I (IGF-I) in cultured ovarian follicular tissue fragments [35]. This suggested a role for ghrelin in regulating key ovarian functions (i.e., apoptosis, cellular proliferation, and steroid and peptide hormone secretion) in chickens mediated by GHS-R1a and postreceptor signal transduction mechanisms involving tyrosine kinase (TK), mitogen-activated protein kinase (MAPK), cyclin-dependent kinase (CDK), and protein kinase A (PKA) [54, 55].

5.5. Gastrointestinal Function. Ghrelin affects various aspects of gastrointestinal function including exocrine secretion, epithelial cell viability, and GI tract motility in mammals [13, 40]. Ghrelin immuno-reactivity has been detected predominantly in the mucosal layer of the proventriculus, duodenum, jejunum, ileum, caecum, and colon but not in the myenteric plexus of chickens and African ostrich chicks [20, 21, 56–58]. In general, the greatest number of ghrelin-immunopositive cells is found in the proventriculus and the number of these cells tends to increase with age. The high level of ghrelin mRNA and protein expression in proventriculus is consistent with a possible endocrine role for this organ in birds. A similar endocrine role has been proposed for the proventriculus in regulating circulating levels of leptin and proglucagon-derived peptide hormones [59]. In mammals, it has been reported that the stomach accounts for the majority of proghrelin-derived peptides in circulation [13, 40].

Studies utilizing isolated gut sections in culture from young growing chickens found that ghrelin stimulated contraction of the upper (crop and esophagus) and lower (colon) portions of the GI tract but had weaker effects on the intervening (proventriculus, duodenum, jejunum) regions which were more sensitive to the ghrelin-related peptide motilin [31]. Chicken ghrelin was found to have region-specific effects on contraction, whereas human and rat ghrelin produced only weak responses. Also, des-acyl ghrelin did not affect motility suggesting that a localized effect of ghrelin on GI tract motility was mediated through GHS-R which is differentially expressed in the crop (smooth muscle layer) versus the proventiculus (smooth muscle layer and enteric neurons) in chickens [31]. In a related study, Khan et al. [60] reported that icv administration of growth hormone releasing peptide-6 (GHRP-6, a ghrelin mimetic) inhibited food intake transiently but did not affect retention of food in the crop, proventriculus, or gizzard in neonatal chicks. Together, these findings suggest localized as opposed to central effects of ghrelin on gut motility in birds. However, it is not known if these effects on gut contractility have any influence on the regulation of food intake and nutrient utilization [4].

5.6. Behavioral Effects. As part of the studies of the various physiological actions of ghrelin in birds, it has been noted that this peptide also has important impacts on behavior. For example, ghrelin has been reported to induce sleep-like behavior in a dose-dependent manner when administered centrally to fed or fasted neonatal chicks suggesting that this behavior may be related to its inhibitory effect on food intake [45, 61]. However, the sleep-like behavior was preceded by a short period (less than 30 minutes) of hyperactivity and increased vocalization [61]. Since CRF also induces hyperactivity when administered icv, this behavior could have resulted by the actions of ghrelin on CRF expression in the CNS [5]. Widespread expression of ghrelin and GHS-R in different regions of the brain beyond the feeding centers located within the hypothalamus is consistent with direct involvement of the ghrelin system in controlling behavior [17, 33]. Recently, Carvajal et al. [62] reported that central administration of ghrelin induced fearful and/or anxious behavior and impaired memory retention in a dose-dependent manner in addition to inducing anorexia in neonatal chicks. The interrelationship of behavior and metabolism is an important new area to explore since it is now recognized that these processes are regulated by shared neuronal circuits in the hypothalamus and that sleep and arousal may actually represent mechanisms involved in energy conservation and expenditure, respectively [63].

6. Conclusions and Future Directions

This review discussed what is currently known about the proghrelin system in birds. While the genes encoding preproghrelin and GHS-R and their protein products are reasonably conserved in avian species, there are some significant differences in the function of the proghrelin system in birds as compared to mammals. The opposite effect of ghrelin on food intake undoubtedly involves the actions of different neural circuits and neuroendocrine pathways downstream of the ligand-receptor binding. Perhaps, by studying ghrelin's effects on food intake, it will be possible to learn more about the specific mechanisms governing feeding behavior in birds and how that regulation differs from what is known for other vertebrate species.

Despite a growing body of evidence for a proghrelin system in birds, a number of unanswered questions remain. Is the obestatin peptide hormone actually produced? If so, what are its structural characteristics and functions? Is it amidated at its C-terminal end? What, if any, are the physiological actions of des-acyl ghrelin? Are there other peptides or fragments produced from the proghrelin precursor? What proghrelin-derived peptides circulate in the blood and in what proportions? Are there additional GHS-R isoforms or related-receptors expressed and how do they mediate the actions of proghrelin-derived peptides? Why are some actions of ghrelin conserved (e.g., growth hormone release) while others are markedly different (e.g., food intake)? Working to answer these and other relevant questions about the avian proghrelin system presents a unique opportunity to better understand the structure and function of this important endocrine system and how it has evolved in different animal species including humans.

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Review Article

Ghrelin in Female and Male Reproduction

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Ghrelin and one of its functional receptors, GHS-R1a (Growth Hormone Secretagogue Receptor 1a), were firstly studied about 15 years. Ghrelin is a multifunctional peptide hormone that affects several biological functions including food intake, glucose release, cell proliferation... Ghrelin and GHS-R1a are expressed in key cells of both male and female reproductive organs in several species including fishes, birds, and mammals suggesting a well-conserved signal through the evolution and a role in the control of fertility. Ghrelin could be a component of the complex series of nutrient sensors such as adipokines, and nuclear receptors, which regulate reproduction in function of the energy stores. The objective of this paper was to report the available information about the ghrelin system and its role at the level of the hypothalamic-pituitary-gonadal axis in both sexes.

1. Introduction

Ghrelin was initially discovered as a ligand for the growth hormone secretagogue receptor (GHS-R1a) [1], and the story of its discovery has been well described in some reviews [2–4]. The peptide named "ghrelin" is a term derived from the Proto-Indo-European word "ghre" meaning "grow" and the name can also indicate the abbreviation for GH, followed by "relin" a suffix meaning releasing substance. Ghrelin is a peptide hormone secreted mainly by the stomach, although its expression has been detected in many other organs exerting both endocrine and paracrine effects [5–7]. Ghrelin has initially been reported to induce GH secretion [1]. In addition to mediating GH release through the growth hormone secretagogue receptor (GHS-R), ghrelin is involved in a series of biological functions including regulation of food intake [8], sleep [9], body weight [10], gastrointestinal motility [11], cardiovascular functions [12], cell proliferation [13], production of proinflammatory cytokines [14], and reproduction in many species. The objective of this paper is to review the available information on the role of ghrelin in reproductive processes including female and male reproduction.

2. Structure and Distribution of Ghrelin

2.1. Structure of Ghrelin. Ghrelin is a 28-amino acid peptide derived from preproghrelin [1]. It has two major endogenous forms: a des-acylated form (des-acyl ghrelin) and a form acylated at serine 3 (ghrelin). This posttranslational acylation is essential for the hormone biological activity [1, 4, 15]. The ghrelin structure, particularly that of the acyl-modification regions, is highly conserved throughout vertebrate species [1].

2.2. Distribution of Ghrelin. Ghrelin is found in mammalian species as well as nonmammalian species. The majority of ghrelin is synthesized by an endocrine cell population, the X/A-like cells, in the stomach mucosa [16]. Ghrelin is then released to the general circulation. The des-octanoylated ghrelin and n-octanoylated ghrelin are both found in rat stomach [17]. The small intestine also synthesizes ghrelin to a lesser extent with the amount of ghrelin produced diminishing with increasing distance from the pylorus [16, 18]. The expression of ghrelin has also been reported in pancreas, lymphocytes, placenta, kidney, lung, heart,

pituitary, brain, ovary, and testis [5–7]. Thus, ghrelin is a ubiquitous protein.

2.3. Regulation of Ghrelin Expression and Secretion. Circulating ghrelin levels increase with fasting and return to basal levels after refeeding in rodents and humans [19–25]. The plasma ghrelin concentration in cows decreases significantly one hour after feeding, and then recovers to prefeeding levels [26]. Starvation also increases plasma ghrelin level in prepubertal gilts [27]. Nutrient contents and also hormones are important factors for the regulation of ghrelin expression and release. For example, in the female rat stomach, estrogen decreases ghrelin mRNA expression [28]. In contrast, the ghrelin mRNA level in the rat stomach increases after the administration of insulin and leptin [17]. In cultured whole porcine follicles, GH stimulates both ghrelin synthesis and secretion, whereas IGF-I shows less influence [29]. In rodents, growth hormone-releasing hormone upregulates ghrelin mRNA in the pituitary [30].

3. Ghrelin Receptor

3.1. Structure. The ghrelin receptor is a G-protein-coupled receptor (GPCR) firstly identified in pigs and humans in 1996 [31]. It belongs to the rhodopsin-like seven-transmembrane domain (7TM) receptor family that includes the orphan GPR39 as well as receptors for the peptides motilin, neurotensin, and neuromedin [32]. It has a high degree of homology ranging from 93% to 99% identified by using molecular analysis of human, pig, dog, rat, and mouse species [31–35]. Cloned before the discovery of the peptide, the ghrelin receptor was initially described as the receptor for a number of synthetic GH secretagogues and is therefore also called the growth hormone secretagogue receptor (GHS-R1a) [31, 36]. However, some evidence indicates that several ghrelin effects are mediated not by GHS-R1a but by other types of receptors not yet identified [37]. An inactive alternative splice variant of the GHS-R subtype, termed GHS-R1b, has also been found [31]. Unlike GHS-R1a, GHS-R1b is not activated by the synthetic GHSs or ghrelin and it is unclear whether it is a functional receptor [38].

3.2. Distribution. The GHS-R1a receptor mRNA is mainly expressed in the pituitary [31] and in several structures of the brain of mammalian and nonmammalian species [39–42]. However, it is also present in the thyroid, pancreas, spleen, myocardium, adrenal gland, ovary, and testis [43]. These data suggest direct actions of ghrelin in these tissues.

3.3. Signaling Pathways. Ghrelin endocrine activities depend entirely upon the acylation and are mediated by GHSR-1a. The des-acyl ghrelin does not bind to GHSR-1a. Upon ghrelin binding to its receptor, different negative feedbacks of GHSR-1a have been described. After acute treatment of porcine pituitary cell cultures with ghrelin, Luque et al. found that ghrelin downregulated GHS-R expression [44]. Ghrelin binding to GHS-R1a also results in a rapid attenuation of the receptor responsiveness. This desensitization is the result of the uncoupling of the receptor from heterotrimeric G proteins and of the internalization of the cell surface receptors to intracellular compartments [45, 46]. Ghrelin is able to activate various signaling pathways. In GHS-R1a-expressing mammalian cells [1, 31] or in rat and human pituitary cells [47, 48], biphasic Ca^{2+} increases, due to a transient Ca^{2+} release from the intracellular store and a Ca^{2+} influx through voltage-dependent L-type calcium channel, which are observed as the signal transduction. Ghrelin has also been shown to increase AMPK (Adenosine Monophosphate-activated kinase) activity in the hypothalamus [49] and reduce it in the liver [50, 51]. It has also been reported that ghrelin could activate the MAPK 44- and 42-kDa extracellular signal-regulated protein kinases (ERK1/2) and the Akt [43–50] in different cell lines [52–55]. Ghrelin is also able to regulate the expression of several transcription factors including the nuclear factor κB, (NFκB), PPARγ, SREBP-1, and cEBP [56, 57].

4. Physiological Functions of Ghrelin in Reproductive Tissues

About ten years ago, several experiments suggested that ghrelin could act as a modulator of the male and female reproductive functions. Indeed, many in vivo and in vitro studies showed that ghrelin was able to exert its action at different levels of the hypothalamic-pituitary-gonadal axis.

4.1. Ghrelin and Gonadotropin-Releasing Hormone Secretion (GnRH). The hypothalamus has been identified as the main source of ghrelin in the central nervous system. Furthermore, as previously described, the GHS-R1a receptor mRNA has been found in many areas of the brain. In rats, systemic administration of ghrelin reduces in vivo the GnRH pulse frequency. The involvement of NPY in the mediation of the effects of ghrelin on pulsatile GnRH secretion is indicated by the complete abolition of the effects of ghrelin by the NPY-Y5 receptor antagonist [58]. GnRH secretion by hypothalamic fragments from ovariectomized females is also significantly inhibited by ghrelin [59]. In mammalian and nonmammalian species, ghrelin affects gonadotropin release acting at the level of the hypothalamus as well as directly on the pituitary gland [60].

4.2. Ghrelin and Gonadotropin Secretion. In pituitary, ghrelin suppresses LH pulse frequency in rats, sheep, monkeys [61–63], and humans [64]. Furthermore, ghrelin delays pubertal onset in male rats [59]. In rats, ghrelin is able to downregulate *Kiss1* expression in the hypothalamic medial preoptic area and this could be a contributing factor in ghrelin-related suppression of pulsatile LH secretion [65]. In contrast, in women during the menstrual cycle, administration of ghrelin does not affect basal and GnRH-induced LH and FSH secretion [66]. Opposite effects of ghrelin on LH secretion mammals and several fish species have been described. Indeed, ghrelin has been shown to stimulate LH release in goldfishes [67–69] and recently in carps [70]. Recent studies

indicate that synthetic goldfish ghrelin stimulates LH release [71]. However, the specific mechanism and the role of this activation are still unknown.

There is evidence in rats and humans that ghrelin can suppress not only LH but also FSH secretion in males and females [63]. A significant decrease of FSH was observed after seven days of continuous ghrelin infusion in male rats [64] and in the metestrus of female rats after one injection. However, in rats, ghrelin did not affect FSH secretion in the proestrous and estrous periods of the estrous cycle in females, and in gonadectomized male and female rats after single injection [64] and after chronic intermittent administration [64]. In women during the menstrual cycle, administration of ghrelin did not affect basal and GnRH-induced LH and FSH secretion [66].

The reported effects of ghrelin on LH and FSH secretion suggest that this peptide plays a key role in the reproductive functions. Beside central actions on the reproductive functions, some evidence indicates that ghrelin could exert direct effects on the female and male gonads.

4.3. Ghrelin in Female Reproduction. Emerging evidence strongly indicates that the ghrelin and ghrelin receptor (GHS-R1a and GHS-R1b) are present in the mammalian and nonmammalian ovary. For example, ghrelin is found in human, rat, pig, sheep, and chicken ovary [72–76]. In sheep ovary, ghrelin is expressed throughout the estrous cycle and pregnancy and the relative mRNA levels depend on the stage of the cycle, with the highest expression during the development of the corpora lutea (CL) and minimal expression in the regressing CL. A similar pattern is seen during pregnancy [77]. More precisely, in rodent ovary, expression of ghrelin has been demonstrated in steroidogenically active luteal and interstitial hilus cells. Expression of the functional ghrelin receptor has been reported in oocytes as well as follicular, luteal, and surface epithelium and interstitial hilus cells in rat ovary [72, 75, 78]. These observations indicate that ovarian follicular and luteal cells are potential targets for systemic or locally produced ghrelin, because they express the functional type 1a of GHS-R. They also highlight the plausibility for a role of ghrelin in the direct control of ovarian cell functions. In vivo administration of ghrelin in rats affects folliculogenesis as attested by alterations of some morphometrical and intracellular indexes in ovarian state. Indeed, it decreases the mean diameter of follicles, the number of corpora lutea, luteal cells, and oocyte and the diameter of the theca layer and the zona pellucida as well as the whole ovarian volume in the treated animals [79].

4.3.1. Effect of Ghrelin on Ovarian Steroidogenesis (Figure 1). In cultured human granulosa luteal cells, ghrelin exerts an inhibitory effect on steroidogenesis (progesterone and estradiol production) in the absence or in the presence of hCG by acting through its functional GHS-R1a [80, 81]. Moreover, the granulosa cells from ghrelin-treated rabbits secrete not only less progesterone and estradiol but also less IGF-1 and prostaglandin F than granulosa cells from untreated animals [82]. In contrast to previous reports, in cocultured granulosa and theca cells from porcine follicles, ghrelin induced estradiol secretion by modifying aromatase activity [29, 37]. Also, Ghrelin(1–18) administration in chicken causes not only an increase in progesterone and oestradiol but also secretion of arginine vasotocin and IGF-1 [76, 83].

4.3.2. Effect of Ghrelin on Ovarian Cell Proliferation and Apoptosis (Figure 1). In chicken ovarian cells, in vitro ghrelin treatments induce markers of proliferation [MAP kinase; PCNA (proliferating cell nuclear antigen), a marker of the S/phase of the cell cycle, and cyclin B1, a marker of the G2/phase] and decrease the expression of markers of apoptosis (caspase-3, bax, and bcl-2) [76, 84]. Moreover, granulosa cells from ghrelin-treated rabbits have higher expression of PCNA and lower expression of TdT (terminal deoxynucleotidyl transferase), than those from control animals.

4.3.3. Effect of Ghrelin on Oocyte Maturation and Embryo Development (Figure 1). It has also been reported that ghrelin inhibits early embryo development in mice [85]. In porcine oocytes cultured in vitro, ghrelin does not improve meiotic maturation. In contrast, it may have some inhibitory effects on the organization of microtubules and microfilaments of porcine oocytes [86]. On the contrary, some data suggest that ghrelin could enhance blastocyst viable from porcine oocytes fertilized in vitro and parthenogenetic embryos while exerting a negative effect on the structural integrity of the blastocysts [87]. Thus, the effects of ghrelin on the development of the embryo are not clear.

In addition, in rats, high levels of ghrelin receptor (GHS-R) mRNA are detected in various peripheral fetal tissues beginning on embryonic day 14 and lasting until birth. Maternal ghrelin regulates fetal development during the late stages of pregnancy [88].

4.4. Ghrelin in Male Reproduction. The testis is a complex endocrine organ where different cell types interplay to produce germ cells, under the control of several extragonadal and intragonodal hormones and growth factors. Some evidence suggests that ghrelin participates in such a regulatory network [59, 89–91] (Figure 1).

Expression of ghrelin has been demonstrated in rodents and sheep by immunostaining mainly in Leydig cells [91]. Ghrelin is also present in the human testis and particularly in Leydig and Sertoli cells but not in germ cells [92]. In human testis, the expression of ghrelin by Leydig cells is apparently linked to the degree of cell differentiation [92]. Furthermore, it is inversely correlated with the serum testosterone levels in patients with normozoospermia, obstructive azoospermia, or varicocele suggesting that ghrelin has an indirect effect on spermatogenesis [93]. In contrast to human and rodent data, in adult sheep testis, strong ghrelin immunostaining is evident not only in Leydig and Sertoli cells but also in germ cells, with an indication of increased ghrelin immunoreactivity in germ cells during the mitotic phases and the meiotic prophases of the spermatogenic cycle [74].

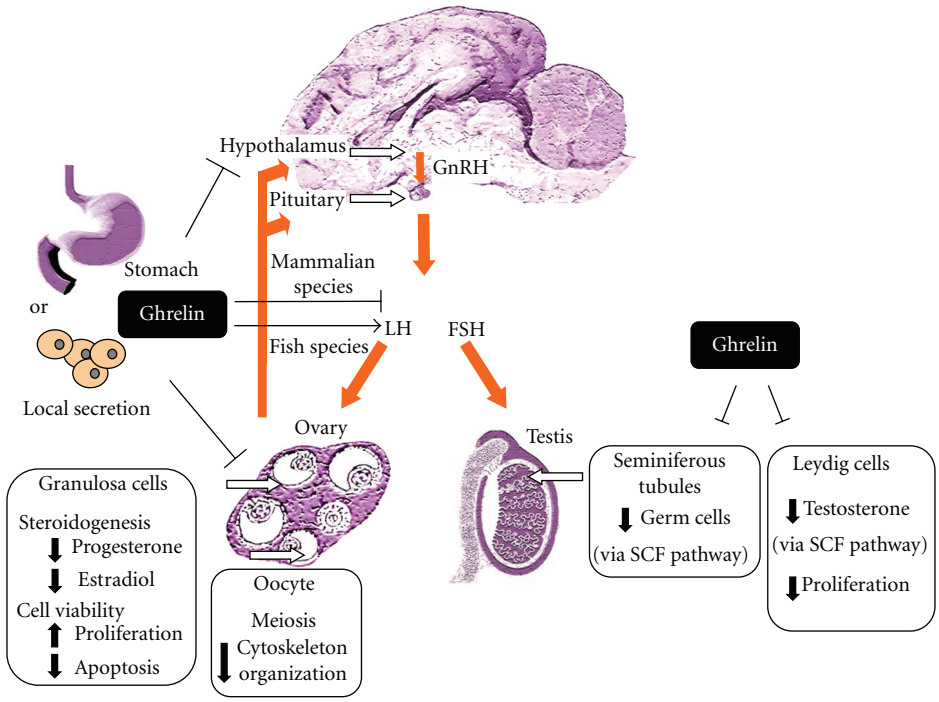

FIGURE 1: Schematic representation of the ghrelin effects at the level of the hypothalamic-pituitary-gonadal axis. Ghrelin, mainly produced by the stomach, can act through its functional receptor GHS-R1a in endocrine or/and local manner in all male and female reproductive tissues including hypothalamus, pituitary, ovary, and testis. It is well known that ovarian steroid production (oestradiol and progesterone) can modulate pituitary and hypothalamus secretions. Furthermore, GnRH produced by the hypothalamus controls LH, and FSH secretion that is known to regulate gonad functions. In mammalian species, ghrelin treatment inhibits GnRH, LH and FSH secretion at the hypothalamic and pituitary levels (red arrows). Opposite effects have been described in several species of fish. In the gonads, ghrelin exerts also inhibitory effects by altering steroidogenesis and germ cells production or viability in ovary and testis. In contrast, ghrelin treatment reduces proliferation of Leydig cells whereas it increases those of granulosa cells. SCF pathway: Stem Cell Factor pathway. ↓: decrease, ↑: increase, and ⊥: inhibition.

Thus, there are some differences between species in the localization of ghrelin protein in the testis.

Expression of the functional ghrelin receptor, GHS-R1a, has been shown in Sertoli and Leydig cells as well as seminiferous tubules in rats [79]. Some changes in the balance between 1a and 1b isoforms of GHS-R gene have been described in rat testis. Indeed, changes in the alternative splicing of the gene are observed throughout postnatal development [94]. Specifically, during pubertal development, a shift in the pattern of splicing of GHS-R gene takes place in rat testis, favouring the expression of the biological active type 1a form of the receptor and indicating that the balance between receptor subtypes may represent a novel mechanism for the regulation of ghrelin sensitivity in gonads. In humans, GHS-R1a has been located in germ cells, mainly in pachytene spermatocytes, as well as in Leydig and Sertoli cells [92]. In adult sheep, GHSR-1a protein was detected in Leydig cells as well as in Sertoli and germ cells within the tubules, and the pattern of GHSR-1a mRNA expression across the testis indicated that the mRNA was present in the interstitial area and around the periphery of the tubules.

4.4.1. Effect of Ghrelin on the Seminiferous Tubule Functions (Figure 1). These latter data suggest that ghrelin could regulate spermatogenesis by an autocrine or/and a paracrine manner. In this sense, intratesticular injection of ghrelin (15 μg for 2 days) in adult rats inhibited mRNA expression of the gene encoding stem cell factor (SCF), a key signal for germ cells production and a putative regulator of Leydig cell development. Such an inhibitory action of ghrelin on SCF has also been detected in vitro using cultures of staged seminiferous tubules [5]. The testicular SCF is a Sertoli cell product that has been involved in Leydig cell development and survival and is acting as a survival factor for the different cell types in the seminiferous epithelium such as spermatogonia in adult rats [95]. Thus, the actions of ghrelin on tubular SCF mRNA could have an impact on the regulation of spermatogenesis and also on Leydig cell proliferation.

4.4.2. Effect of Ghrelin on Testicular Steroidogenesis (Figure 1). In vitro, ghrelin significantly also inhibits in a dose-dependent manner both hCG- and cAMP-stimulated testosterone release by Leydig cells [91]. This inhibitory effect of ghrelin on testosterone secretion has been associated with decreases in the hCG-stimulated expression levels of the mRNAs for several key factors in the steroidogenic pathway (StAR, P450scc, 3ß-HSD, and testis-specific 17β-HSD type III) [91]. In vivo, the effects of ghrelin on plasma levels of

testosterone in rats depend on the nutritional state. Indeed, in fed rats, ghrelin administration induces a slight decrease in testis mass without detectable changes in final plasma levels of testosterone, whereas in food-restricted animals, where endogenous ghrelin levels are known to be increased, a chronic administration of ghrelin induces overt decrease in plasma testosterone [96]. Thus, high levels of ghrelin could contribute to male reproductive axis alterations in situations of energy deficit.

4.4.3. Effect of Ghrelin on Cell Proliferation (Figure 1). It has also been demonstrated by in vivo intratesticular injection of ghrelin that ghrelin is able to inhibit the proliferative rate of immature Leydig cells both during puberty development and after selective ablation of pre-existing mature Leydig cells by administration of EDS (ethylene dimethane sulfonate) [5]. Ghrelin and its type 1a GHS-R are expressed in testicular tumors. The expression of ghrelin but not that of GHS-R1a in Leydig tumor cells is apparently linked to the degree of cell differentiation [92].

Ghrelin is able to modulate key testicular functions such as seminiferous tubule gene expression, testosterone secretion, and Leydig cell proliferation. Thus, this peptide could operate as a novel regulator of testicular development.

5. Conclusions

In conclusion, ghrelin is a peptide hormone mainly secreted from the stomach into the circulation, but it can be synthesized by other tissues such as reproductive tissues suggesting local actions (autocrine and/or paracrine). Its functional receptor, GHS-R1a, is also expressed at different levels of the hypothalamic-pituitary-gonadal axis. As described in this paper, ghrelin may participate in the regulation of different aspects of the female and male reproductive functions from germ cell production to embryo development. These actions appear to be species-specific. Ghrelin through its various biological functions including energy metabolism by promoting fat deposition and food intake could be a key signal between energy status and control of fertility (nutrient-gene expression). However, further studies are required to gain insights into the understanding of the fine mechanisms of ghrelin action.

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Review Article

Ghrelin: Central Nervous System Sites of Action in Regulation of Energy Balance

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Ghrelin, a peptide hormone secreted by the stomach, has been shown to regulate energy homeostasis by modulating electrical activity of neurons in the central nervous system (CNS). Like many circulating satiety signals, ghrelin is a peptide hormone and is unable to cross the blood-brain barrier without a transport mechanism. In this review, we address the notion that the arcuate nucleus of the hypothalamus is the only site in the CNS that detects circulating ghrelin to trigger orexigenic responses. We consider the roles of a specialized group of CNS structures called the sensory circumventricular organs (CVOs), which are not protected by the blood-brain barrier. These areas include the subfornical organ and the area postrema and are already well known to be key areas for detection of other circulating hormones such as angiotensin II, cholecystokinin, and amylin. A growing body of evidence indicates a key role for the sensory CVOs in the regulation of energy homeostasis.

1. Introduction

Prior to 1999, the growth hormone secretagogue receptor (GHS-R) was an orphan receptor, known to evoke the release of growth hormone via a pathway independent of GHRH. The work of Kojima et al. [1] identified ghrelin, a 28-amino acid peptide secreted by the stomach, as the endogenous ligand for the GHS-R. Several facts immediately suggested that ghrelin might be involved in regulation of energy balance. First, ghrelin was secreted by the X/A-like cells in the fundus of the stomach and secretion was elevated with fasting [2], thus providing a clear signal from the gut regarding energy status. Second, it had previously been established that small molecule or peptide growth hormone secretagogues (GHSs) which activate the GHS-R also stimulated feeding [3]. Lastly, the observation that the GHS-R was strongly expressed in the NPY neurons of arcuate nucleus of the hypothalamus (ARC) [4, 5] suggested this as a potential central nervous system site of action for this peptide. In 2000, Tschop et al. [2] clearly demonstrated that ghrelin stimulated feeding and adiposity in mice and rats. Later, Cowley et al. [6] showed that ghrelin mediated activation of the GHS-R in ARC and effects which in turn modulated the electrical activity of the NPY and POMC neurons that had already been established as critical regulators of feeding behavior. These and similar results stimulated a great deal of interest because understanding the ghrelin signaling pathway was identified as a potential route in the development of successful strategies for the prevention and treatment of obesity. In spite of the overwhelming evidence that ghrelin stimulates feeding and adiposity, there is still controversy as to ghrelin's ultimate site of orexigenic activity within the CNS. This review aims to highlight some of the contentious points and to suggest some alternative explanations for the available data to this point in time.

2. Hypothalamic Actions of Ghrelin in the Regulation of Food Intake

The GHS-R is widely expressed in the body, being found in the brain, stomach, intestine, pancreas, heart, and gonads [7]. The density of the receptor is especially high in the pituitary, ARC, and dorsal vagal complex (DVC), including the area postrema (AP), the nucleus of the solitary tract

(NTS), and the dorsal motor nucleus of the vagus (DMV) [8–12]. The high levels of expression of the GHS-R within the ARC and the fact that ghrelin-producing neurons have been discovered in the hypothalamus [6] strongly suggest that ghrelin plays a role in feeding. Indeed, a role for ghrelin in regulating food intake and energy stores draws strong support from a wide variety of different studies. Microinjections of ghrelin into the ARC as well as intracerebroventricular (ICV) injections of ghrelin strongly stimulated weight gain and feeding in rats and mice [2, 13–17]. Similar injections activated NPY and AGRP neurons within the ARC, as determined by c-Fos immunolabeling [16–18]. Ghrelin receptors are highly localized on NPY neurons [4, 5, 19], and direct application of the hormone in patch-clamp experiments increased their electrical activity [6, 20, 21]. Similar results were observed using real time quantitative Ca^{++} imaging [22] and single unit recordings [23]. Perhaps some of the strongest evidence that ghrelin regulates food intake via its action in the hypothalamus is that lesioning the ARC resulted in loss of appetite-stimulating effects of both peripheral and ICV ghrelin [24, 25]. Moreover, genetic manipulations in mice clearly show that deleting NPY reduces the effect of administered ghrelin, whereas deleting both NPY and AGRP completely abolishes the orexigenic response to ghrelin [26].

But does circulating ghrelin regulate energy balance as a direct consequence of primary effects on the electrical activity of ARC neurons? Although the prevailing view in the literature is a very definitive "yes", we believe that this supposition may deserve some careful examination in view of some recent studies we will describe below. The fact that peripherally injected ghrelin not only causes weight gain and feeding [2, 15, 27, 28] but also increases c-Fos immunolabeling in the ARC, paraventricular nucleus and other areas [29–32] represent the primary data resulting in the conclusion that ghrelin directly modulates the electrical activity of GHS-R expressing neurons within the ARC by somehow crossing the blood-brain barrier (BBB), which despite alternative suggestions does exist in this region of the brain [33]. The critical issue to a true understanding of the mechanisms underlying central actions of ghrelin, we would suggest, is a clear description of how circulating ghrelin gets to and thus influences the activity of ARC and other CNS neurons involved in the regulation of energy balance. A number of different mechanisms have been suggested.

3. Transendothelial Cell Signaling

The neurons of the CNS are privileged in that they are protected by the BBB. It is well recognized that lipophobic molecules such as peptide hormones cannot cross the BBB by simple diffusion. Instead, such molecules can only communicate with neurons on the other side through limited routes. One route is indirect, involving binding of a circulating peptide signal to the luminal side of endothelial cells, which then produce diffusible messengers on the abluminal side [34, 35]. Gaskin et al. [36] have indeed shown that ICV injections of ghrelin stimulate synthesis of the diffusible messenger nitric oxide (NO), which in turn stimulates feeding. However in delivery by ICV injection, many experiments bypassed the function of the BBB. Thus the questions of whether and how ghrelin crosses the BBB still remain.

4. Specialized Transporters

Another route of communication across the BBB which has been suggested to play a role in blood-brain communication for other peptides involves the existence of specialized saturable transport mechanisms. Such transporters are able to move water soluble molecule across the BBB such that when released into the CNS side of the barrier these molecules directly contact with the neurons expressing the cognate receptors. Such transport mechanisms exist for glucose [37] and leptin although the transporter protein for leptin remains elusive (for review see [38]). Transport of circulating ghrelin across the BBB has been investigated [39–41]. Banks et al. [39] demonstrated in mice that a saturable transport system exists for transporting ghrelin from the brain to the circulation; however, no such system was identified for blood to brain transport. Interestingly, human ghrelin, which differs from mouse ghrelin by two amino acids, was a substrate for both directions of transport. Therefore while the data are suggestive, the fact that only ghrelin from another species could be transported across mouse BBB suggests that some caution should be accorded to the conclusion that a specific ghrelin transporter is responsible for transport of this peptide from the circulation to the direct milieu surrounding ARC neurons.

A further consideration with regard to the role of such a specific transporter, if it does exist, relates to the rate of transport. The plasma/CSF ratio is usually quite low, in the 4%–7% range for leptin, for example [38], and with typical concentration for ghrelin in the serum of approximately 1–3 nM [2, 15], which would translate into a transporter-mediated CSF level of about 50–150 pM. While the IC_{50} for the cloned GHS-R is reported to be 0.19–32 nM [42, 43] the observed IC_{50} values for studies examining the electrophysiological effects of ghrelin applied to neurons have been in the 1 nM to 14 nM range [44–46]. This suggests that the concentration of ghrelin found in the CSF due to a putative transport mechanism might not actually be high enough to elicit significant changes in neuronal electrical activity. Indeed, a recent investigation of plasma/CSF ratios in sheep indicated that a ten-fold increase in circulating ghrelin levels (achieved by peripheral injection) required some 40–50 minutes to increase CSF ghrelin concentrations two-fold. Moreover, the normal concentration of ghrelin in the CSF was 1000-fold lower than in the circulation [47]. Again, the CSF concentration appears for the most part to be much lower than the EC_{50} for the GHS-R.

5. The Blood-Brain Barrier in the Arcuate Nucleus

In the absence of a specialized mechanism to transport ghrelin, some have suggested that circulating ghrelin directly

accesses ARC neurons as the result of this region being endowed with a weak or modified BBB [30, 48, 49]. Others assert that the ARC has no blood-brain barrier at all [14] or that blood borne molecules can leak from the median eminence to the ARC [32]. However, anatomical evidence indicates that the ARC demonstrates BBB integrity consistent with that found in other secure locations [33, 50–52]. In fact a classic study using IV injection of horseradish peroxidase to delineate areas with no BBB clearly indicated that the BBB of ARC is similar to that of other adjacent areas [53]. Only after eight hours of incubation is HRP staining product observable this being due to retrograde transport from axonal projections to median eminence. Further work by Broadwell et al. [54] demonstrated that IV injection of horseradish peroxidase resulted in reaction product almost entirely limited to median eminence with little evidence of entry to ARC via this route. Lastly, leakage of ghrelin from the median eminence through the parenchyma and into the ARC too is highly unlikely. The median eminence is segregated from the neuropil by a layer of tanacytes which are connected by tight junctions [55] which provide a formidable barrier against movement of solute. Also, diffusion through brain tissue is limited [38, 56], as the rate is inversely proportional to the square of the distance.

Elevated levels of c-Fos immunostaining in the ARC after peripheral injection have been suggested to support the notion that ghrelin can cross the BBB (of the ARC) and modulate activity of neurons in ARC, which may in turn modulate activity in other brain areas [31, 32]. However, unless specifically designed to do so, neither c-Fos immunostaining studies can resolve order of activation of the areas showing increased neuronal activity nor can such studies show when ghrelin inhibits neuronal activity (see [44, 57]). Indeed, Date et al. [58] provide evidence suggesting that activation of ARC neurons and the observed increased c-Fos immunostaining in ARC induced by peripheral injection may be *secondary* to activation of vagal afferents and/or neurons within the NTS.

While there is no doubt that neurons of the ARC and elsewhere do respond to peripheral ghrelin administration, a mechanism by which endogenous circulating ghrelin actually binds to and activates the GHS-Rs found on neurons in the ARC and elsewhere in the hypothalamus is presently unclear.

6. Are There Other Areas Where Ghrelin Can Directly Bind to Neurons Expressing GHS-R?

Sensory circumventricular organs (CVOs) are specialized areas of the CNS that lack the normal BBB and allow ghrelin and other peptides to bind peptidergic receptors on neurons. Unlike the capillaries of most areas of the CNS, the capillaries of the sensory CVOs have fenestrations similar to those found in the periphery, and the capillaries are not enclosed by a layer of glia cell end feet. The circulation within the sensory CVOs is also specialized to facilitate detection of circulating solutes: the capillary beds are dense and exhibit tortuosities that slow blood flow and "Virchow-Robin spaces" that allow interstitial fluid to pool around the capillaries [59] (for a complete review see [60]). Sensory CVOs are particularly well endowed with a high density and wide variety of peptidergic receptors. Lastly, the sensory CVOs have strong connections to a variety of autonomic control centers. Thus the sensory CVOs act as transducers to detect signals within the circulation and communicate this information to other centers of the CNS for integration and processing. There are three sensory CVOs: the subfornical organ (SFO), area postrema (AP), and the organum vasculosum of the lamina terminalis (OVLT). Both the SFO and the AP are known to express the GHS-R and to contain ghrelin-sensitive neurons; there have not been data suggesting that the OVLT plays a role in the detection of ghrelin.

The SFO projects from the rostral wall into the third ventricle and is located dorsally to the lamina terminalis. It sends direct and indirect projections to vasopressin and oxytocin neurons of the paraventricular nucleus and the supraoptic nucleus of the hypothalamus [61]. The SFO also sends projections to the parvocellular neurons of the PVN [62], the ARC [63, 64], and other areas of the hypothalamus. Afferent projections to the SFO include the NTS, the lateral hypothalamus, the midbrain raphe, and other areas [64–66]. Thus, the SFO is in direct contact with ghrelin in the circulation and communicates with key autonomic control centers.

The AP is located in the fourth ventricle, on the dorsal surface of the medulla positioned adjacent to the NTS. The AP together with the NTS and DMV makes up the dorsal vagal complex, a major site for integration of afferent information from the gut, viscera, and circulation. The AP has reciprocal connections with a variety of targets; most notably are the NTS, the lateral parabrachial nucleus, the nucleus ambiguus, and the tegmental nuclei [67, 68]. There are also descending connections from the PVN to AP [69]. Of particular interest, however, both the NTS and lateral parabrachial nucleus send ascending connections to the PVN and ARC [70–75]. Like the SFO, the neurons of the AP are in direct contact with ghrelin and other hormones in the circulation and communicate with autonomic control centers.

There is already an established body of evidence describing the roles of two of the sensory CVOs: the subfornical organ (SFO) and the area postrema (AP) in homeostatic regulation. For example, the SFO is well known to regulate fluid balance via angiotensin II signaling, and AP is well known to play critical roles in cessation of feeding via the anorectic hormones: CCK and amylin (for reviews see [60, 76–78]). The remainder of this review will focus on the roles of SFO and AP in the processing of peripheral ghrelin signals.

7. Subfornical Organ Actions of Ghrelin

Some of the first evidence that ghrelin may play a role in regulation of energy balance by action at the SFO came in 2006 when Pulman et al. [45] demonstrated that the GHS-R was expressed in SFO and that a subpopulation of SFO neurons was dose dependently excited by the application of exogenous ghrelin. The same study revealed that amylin,

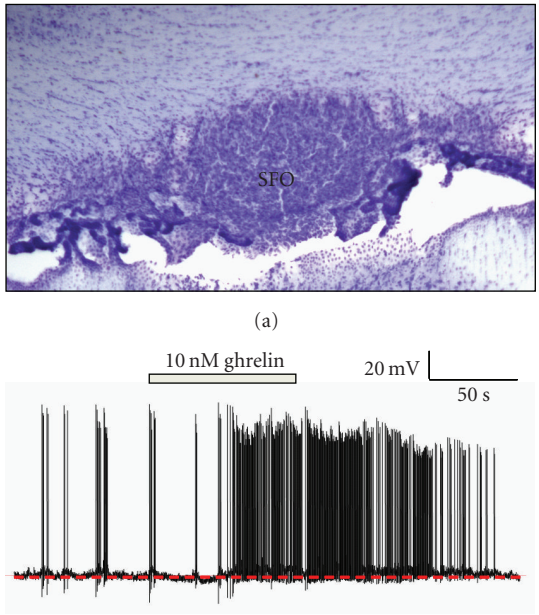

Figure 1: Ghrelin modulates the electrical activity of SFO neurons. (a) Nissl-stained coronal section showing the SFO. (b) Representative current-clamp recordings from dissociated SFO neurons that were depolarized by application of 10 nM ghrelin. This effect is mediated by activation of a cation conductance. Interestingly, separate populations of SFO neurons depolarize in response to either ghrelin or amylin (not shown). No SFO neurons are sensitive to both ghrelin and amylin; see [45].

an anorexigenic peptide, also excited a subpopulation of SFO neurons and intriguingly the subpopulations of ghrelin-sensitive and amylin-sensitive neurons were mutually exclusive (Figure 1). This suggested that orexigens and anorexigens both acted at the SFO, but via different neuronal pathways. These data are supported by the observation of Takayama et al. [32] who observed increased c-Fos staining in the SFO after peripheral ghrelin administration.

The SFO is classically thought of as a key CNS site controlling thirst and drinking (for review see [77]), and studies now also suggest that this CVO is a key site of action for regulation of not only food but also water intake by ghrelin. Two groups gave ICV injections of ghrelin into rats and observed that while ghrelin increased food intake, it also inhibited water intake [79, 80], effects which were also observed following peripherally injected ghrelin [81]. While ghrelin is primarily known for its actions on food intake, these recent observations underscore the notion that food and water intakes are both ingestive behaviors and are inextricably linked (for review see [82]).

8. Area Postrema Actions of Ghrelin

While a potential role for ghrelin in regulating energy balance at the AP has long been recognized (for review see [82, 83]), it has not received the same attention as the signaling pathways including NPY/AGRP and POMC neurons within the ARC (for a review of the notion of the ARC-centric versus distributed network hypotheses see [84]). The GHS-R is expressed in the AP [9], strongly suggesting a role for ghrelin in the regulation of AP neuronal function, a conclusion supported by studies showing that peripheral administration of ghrelin caused increases in c-Fos immunoreactivity in AP, DMV, and NTS [32, 81, 85]. This result was not surprising given the unfettered access of circulating ghrelin to AP neurons. Importantly, ablation of AP resulted in the loss of c-Fos immunostaining in DMV and NTS, suggesting that ghrelin acts directly at the AP, while acting indirectly at the DMN and NTS [85]. The effect on c-Fos staining in ARC after AP lesion and peripheral injection of ghrelin is currently unknown.

Peripheral injection of ghrelin can stimulate feeding almost instantly [47]; therefore, the speed of the detection of ghrelin signals is a point of interest. One may hypothesize that the brainstem connection to ARC, in spite of being polysynaptic, is probably faster and more consistent with latency to feeding than to the inefficient putative transport of ghrelin across the BBB [47]. Unfortunately, there are not sufficient c-Fos immunostaining data to compare the relative time course for activation of AP and NTS to the activation of ARC.

Recent observations by Gilg and Lutz [86] provide additional strong support for a central role for the AP in ghrelin-mediated feeding. They observed that lesion of AP eliminated ghrelin-induced feeding in two separate administration paradigms although an increase in body weight was still observed, likely because of the ability of ghrelin to reduce fat utilization [2]. This observation conclusively demonstrates that ghrelin-induced feeding critically depends on intact signaling at the AP. In further support of the notion that the caudal brainstem plays a pivotal role in ghrelin-induced feeding, Date et al. [58] carried out experiments lesioning the midbrain of rats to destroy NTS-ARC projections. They observed that the lesion abolishing communication between the NTS and the hypothalamus eliminated both feeding and c-Fos immunostaining in the ARC that are normally induced after peripheral injection of ghrelin. Importantly, in these studies feeding in response to ICV-administered ghrelin remained intact after the midbrain lesion, showing that hypothalamic and brainstem responses to ghrelin are separable, yet linked. Date et al. [58] argue in favor of a model where the vagus nerve detects ghrelin and transmits this information to the ARC via noradrenergic projections from the NTS. The role of vagal afferent signaling has been questioned by Arnold et al. [87] and the noradrenergic nature of the NTS efferents by Faulconbridge et al. [88]. Still, the Date model is not inconsistent with the possibility that information regarding endogenous circulating ghrelin gathered by AP and vagus is integrated within the NTS then communicated to the ARC for further integration. Clearly, the role of the AP and caudal brainstem cannot be overlooked.

Another work provides strong support for the role of the caudal brainstem in ghrelin-mediated feeding. Faulconbridge et al. observed that direct injection of ghrelin into

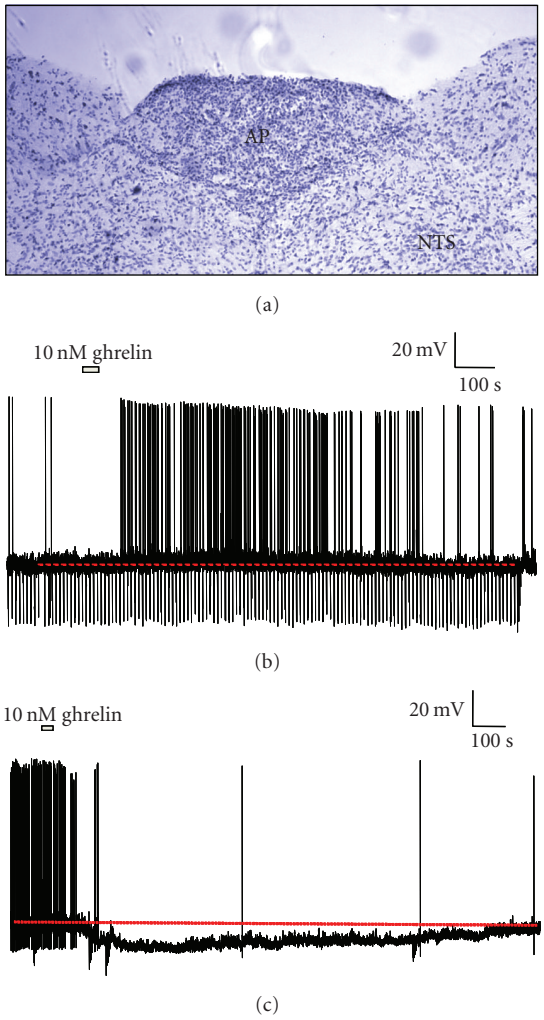

Figure 2: Ghrelin modulates the electrical activity of AP neurons. (a) Nissl-stained coronal section showing the AP, along with surrounding areas including NTS. ((b) and (c)) Representative current-clamp recordings from dissociated AP neurons that were depolarized or hyperpolarized by focally applied 10 nM ghrelin. The electrophysiological effects were caused by activation of a cation conductance (depolarization) or activation of K^+ current (hyperpolarization); see [44].

the caudal brainstem elicits feeding at similar or lower concentrations than required for ARC microinjections [89] and that the food-stimulating doses in caudal brainstem do not activate c-Fos in ARC [88]. The same group proposes a strong contribution of caudal brainstem neurons expressing NPY1 receptors [90]. While their data are not in complete agreement with Date et al. [58] regarding the role of an aminergic NTS connection to ARC, they do strongly support the notion that caudal brainstem and ARC are ghrelin-responsive areas with each contributing to ghrelin-mediated feeding.

Towards understanding the role of AP in ghrelin signaling, Fry and Ferguson [44] examined the sensitivity of AP neurons to ghrelin using patch-clamp analysis. These experiments demonstrated that about 40% of neurons within the AP were sensitive to ghrelin, exhibiting changes in electrical properties, and firing rates when exposed to the hormone. Intriguingly, half of these showed increased excitation, and the other half showed reduced excitation in response to application of physiologically relevant concentrations of ghrelin (Figure 2). The observation that cells can be inhibited by ghrelin may suggest why some studies have not observed an increase in c-Fos staining in the AP after administration of ghrelin [31]. Further experiments to investigate the projections and network properties of the ghrelin-sensitive AP neurons are ongoing.

9. Conclusions

To reiterate, there is no doubt that ARC plays a role in the regulation of energy balance by the hormone ghrelin. However, there are sufficient reasons to think that the activity of ghrelin on energy balance may not be localized entirely to the ARC but is distributed across different brain regions. In particular, the sensory CVOs are well positioned detecting circulating ghrelin and passing this information to other integrative centers for additional processing. Based on recent observations, the sensory CVOs appear to play a crucial role in the signaling pathways of ghrelin. Understanding the interaction between the sensory CVOs, the ARC, and many other CNS nuclei implicitly linked to the regulation of energy balance will hopefully lead us towards developing successful new strategies for the prevention and treatment of obesity.

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Review Article

Interactions of Gastrointestinal Peptides: Ghrelin and Its Anorexigenic Antagonists

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Food intake behaviour and energy homeostasis are strongly regulated by a complex system of humoral factors and nerval structures constituting the *brain-gut-axis*. To date the only known peripherally produced and centrally acting peptide that stimulates food intake is ghrelin, which is mainly synthesized in the stomach. Recent data indicate that the orexigenic effect of ghrelin might be influenced by other gastrointestinal peptides such as cholecystokinin (CCK), bombesin, desacyl ghrelin, peptide YY (PYY), as well as glucagon-like peptide (GLP). Therefore, we will review on the interactions of ghrelin with several gastrointestinal factors known to be involved in appetite regulation in order to elucidate the interdependency of peripheral orexigenic and anorexigenic peptides in the control of appetite.

1. Introduction

According to the current state of knowledge, control of food intake behaviour and energy homeostasis particularly relies on the complex interactions between various humoral components indicating the actual metabolic state of the organism. As a well-established hypothesis in the context of appetite regulation, the glucostatic theory suggests an important role of metabolic substrates (e.g., blood glucose levels) for the regulation of food intake [1]. Also, the assumed modulation of food intake by signals reflecting upon energy storage [2] has been validated by the discovery of the adipose tissue hormone leptin [3].

During the past decades these theories were complemented by the discovery of several additional mechanisms involved in the control of energy homeostasis. Numerous studies revealed that diverse gastrointestinal peptides are particularly responsible for the control of hunger and satiety [4]. Serving as the most important gateway connecting the endocrine with the central nervous system (CNS), the hypothalamus has been found to comprise and integrate the humorally mediated information, which reflect the metabolic state of the organism [4]. This interaction between the central nervous system and the intestinal tract by humoral factors and neuronal pathways has been named *brain-gut-axis* [4]. As a part of the *brain-gut-axis* gastrointestinal neuropeptides as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and many other humoral components are mainly involved in short-term regulation of energy homeostasis. Figure 1 provides an overview of the sites of synthesis as well as of the effects exhibited by these peripheral and central peptidergic factors responsible for the regulation of hunger and satiety.

2. Ghrelin

So far the only known peripherally produced peptide exerting a stimulating effect on food intake behaviour

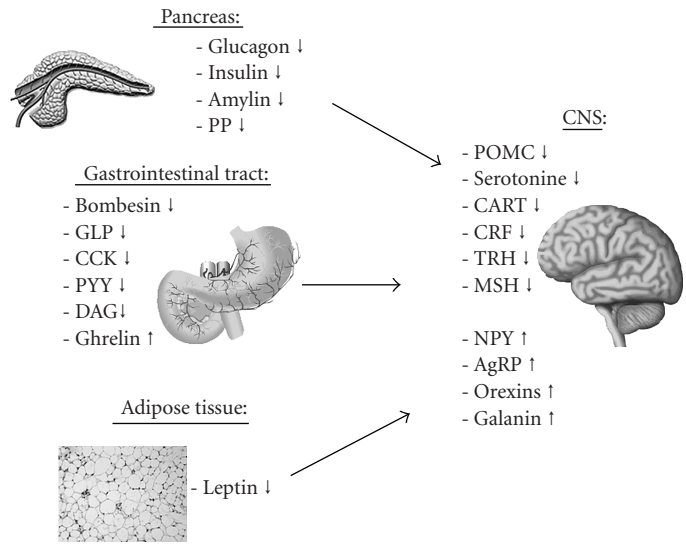

Figure 1: Peripheral and central peptides reducing (↓) or stimulating (↑) food intake (modified after Arora et al. [5]). AgRP: agouti-related peptide; CART: cocaine and amphetamine regulated transcript; CCK: cholecystokinin; CRF: corticotropin releasing factor; DAG: desacyl ghrelin; GLP: glucagon-like peptide; MSH: melanocyte stimulating hormone; NPY: neuropeptide Y; POMC: proopiomelanocortin; PP: pancreatic polypetide; PYY: peptide YY; TRH: thyreotropin releasing hormone.

is ghrelin [6]. In 1999 this peptide was discovered by Kojima et al. as the first endogenous ligand of the Growth Hormone Secretagogue Receptor (GHS-R) [6]. Ghrelin is a 28 amino acid peptide, which exhibits an esterification with an octanoyl chain at the serine residue on position three as an unique modification [6]. The acylation is catalyzed by the ghrelin-O-acyltransferase (GOAT) and converts the peptide to the biologically active form [7]. Moreover, the fatty acid residue has been found to be essential for the directed transfer *via* the blood-brain-barrier [8].

Ghrelin is mainly produced by mucosal X/A-cells of the stomach and in much smaller shares also in the pancreas, duodenum, small intestine, and coecum as well as in the heart and aorta [6, 9, 10]. Additionally, studies indicate that also regions of the brain are involved in the ghrelin synthesis as ghrelin-containing neurons were identified in the pituitary gland as well as in the arcuate nucleus of the hypothalamus [11, 12]. Moreover, ghrelin-immunopositive neurons have been described in a hypothalamic region located nearby the third ventricle [13].

Blood ghrelin levels rise preprandially, after weight loss and in the fasted state [14, 15]. Moreover, plasma ghrelin levels have been found elevated in mammals after H. pylori infection [16] as well as in patients suffering from peptic ulcers [17]. In addition, Masaoka et al. found an increase in plasma ghrelin levels and gastric preproghrelin mRNA expression in diabetic rats, whereas gastric ghrelin levels were decreased compared to nondiabetic animals [18]. In this context, zinc supplementation significantly reduced the density of ghrelin-producing cells in the fundic mucosa in diabetic animals in comparison to untreated nondiabetic controls [19].

In addition to a significant elevation of GH-secretion [6], exogenous ghrelin strongly stimulates food intake behaviour in rodents [14, 20–24] as well as in humans [25]. Likewise, elevated endogenous plasma levels of ghrelin in patients suffering from Prader-Willi-syndrome result in distinct hyperphagia [26]. In addition to its impact in the context of energy homoestasis ghrelin is also involved in the regulation of several intestinal functions, such as gastric acid secretion [27, 28] or extraintestinal actions, which are summerized in Table 1.

Studies suggest that the orexigenic effect of ghrelin is mediated *via* central mechanisms located in the arcuate nucleus (ARC) of the hypothalamus. It has been shown that intracerebroventricular (icv.) injection of ghrelin leads to a significant increase of neuronal activity within ARC as well as in the paraventricular nucleus (PVN), dorsomedial nucleus of the hypothalamus (DMH), in lateral hypothalamic areas (LHA), in the nucleus of the solitary tract (NTS), and in the area postrema (AP) [29, 30]. Interestingly, intraperitoneal (ip.) injection of ghrelin has been found to induce neuronal activity in the ARC and PVN also, but yet failed to do so in the NTS and AP [31, 32]. However, after intravenous (iv.) ghrelin injection an increase in neuronal activity in the ARC, PVN, as well as in the NTS and AP [33] or activity within ARC, NTS, and AP but not in the PVN and DMH [34] has been reported.

Although the complete central mechanism of action remains to be elucidated, it is well established that the orexigenic effect of ghrelin is mediated *via* central pathways involving neuropeptide Y (NPY) and agouti-related peptide (AgRP) in the ARC [21, 35–39]. Accordingly, ghrelin does not effect food intake behaviour in NPY-/AgRP-deficient mice [38]. These findings and the colocalization of NPY and ghrelin receptor GHS-R1a in neurons of the ARC suggest that NPY- and AgRP-positive neurons are a basic prerequisite for the ghrelin-induced orexigenic effect [12, 36]. However,

taking into account that the GHS-R1a is widely distributed in the brain [40], many other brain regions have been also found activated after ghrelin injection [32–34, 41, 42]. Therefore, it can be assumed, that there are further—yet unknown—mechanisms mediating the various effects of ghrelin. It is furthermore noteworthy that the effects of exogenous as well as endogenous ghrelin seem to be influenced by other factors of the brain-gut-axis. Therefore, some recent studies focused on the interaction between ghrelin and other humoral factors known to regulate hunger and satiety. These findings and their impact on the role of ghrelin in the hypothalamic system of food intake behaviour and energy homeostasis will be discussed in the following.

3. Interaction between Ghrelin and Peripheral Anorexigenic Peptides

3.1. Cholecystokinin. Cholecystokinin (CCK) was the first gut hormone found to reduce food intake [43]. CCK is secreted by I-cells located in the proximal small intestine as a mixture of peptides with varying numbers of amino acids, each of which possessing the required epitope for bioactivity [44]. It is widely accepted that CCK-induced satiation is mainly mediated by binding to CCK-1 receptors located on the vagus nerve [45, 46].

As the orexigenic effect of ghrelin is also partly mediated by vagal afferents, Date et al. found that peripheral injection of CCK curbs the decreased activity of gastric vagal afferents induced by ghrelin [23]. Besides, exogenous ghrelin significantly inhibits CCK-stimulated pancreatic protein secretion—even after acute subdiaphragmatic vagotomy [47]. Furthermore, it has been shown that elevated food intake after peripheral ghrelin administration is antagonized by pre- or simultaneous injection of CCK [48, 49]. Accordingly, the markedly increased neuronal activation of the hypothalamic ARC in response to peripheral ghrelin application is diminished by pre- or coapplication of CCK [48, 50]. However, peripheral ghrelin had no effect on CCK-induced neuronal activity in the PVN and the NTS [50]. Thus, it has been hypothesized that CCK inhibits the effect of ghrelin *via* vagal projections to hypothalamic pathways involving the ARC [50].

Interestingly, CCK-1 and -2 receptor deficient mice display a lower response to exogenous ghrelin and lower plasma ghrelin levels after fasting as compared to their wild-type littermates [51]. Moreover, intraduodenal infusion of ghrelin has been found to increase CCK secretion [52]. However, there are conflicting data concerning the influence of CCK on ghrelin release. Two studies indicated that exogenous CCK suppresses ghrelin release in healthy subjects, whereas after ingestion of lipids CCK seems to act on CCK-1 receptors to decrease ghrelin secretion [53, 54]. In contrast, it has been shown that CCK perfusion of isolated stomachs increases ghrelin secretion by ~ 200% [55].

In summary, there is good evidence for the functional antagonism of ghrelin and CCK on food intake whilst the exact interplay concerning the secretion of both peptides remains to be elucidated.

Table 1: Physiological effects of ghrelin.

Reference	Physiological effect
Masuda 2000, Dornoville 2004 [56, 57]	Increased gastrointestinal motility
Masuda 2000, Date 2001 [56, 58]	Influence on gastric acid secretion
Broglio 2001, Dezaki 2004, Yada 2008 [59–61]	Reduction of insulin secretion
Nagaya 2001 [62]	Decreased blood pressure
Baldanzi 2002 [63]	Inhibition of apoptosis in cardiomyocytes
Cassoni 2001 [64]	Inhibition of proliferation in breast cancer
Weikel 2003 [65]	Extension of slow-wave sleep
Asakawa 2001, Carlini 2002 [66, 67]	Anxiogenesis and memory consolidation

3.2. Bombesin. Bombesin is an anorexigenic tetradecapeptide initially isolated from the amphibian skin of Bombina bombina [68]. Since initial discovery, several mammalian bombesin-like peptides with structural homology to bombesin, such as gastrin-releasing peptide, neuromedin B, and neuromedin C, have been described [69]. Peripheral as well as central injection of bombesin reduces food intake mediated by bombesin receptors (BB1 and BB2) which are widely spread in the gastrointestinal tract as well as in the central nervous system [69–71]. Within the CNS, in particular the nucleus of the solitary tract of the brainstem has been shown to play a crucial role in the mediation of the anorexigenic effect of bombesin [72].

Concerning a possible interaction with ghrelin, evidence has been provided that coinjection of bombesin inhibits the orexigenic effect of intraperitoneal ghrelin [73]. In addition, simultaneous injection of bombesin and ghrelin significantly increased neuronal activity of CRF-immunoreactive neurons in the PVN compared to vehicle and to single ghrelin application while it did not alter ghrelin-induced neuronal activity in the ARC [73]. Therefore, it can be assumed that peripheral bombesin inhibits ghrelin-induced food intake and increases activation of CRF neurons in the PVN [73].

In addition, in goldfish (*Carassius auratus*) peripheral injection of bombesin diminished ghrelin expression levels in the gut [74]. Furthermore, while exhibiting opposing effects on food intake, application of exogenous bombesin and ghrelin both stimulated growth hormone release. However, the two peptides exerted different effects on somatostatin production, whereas peripheral ghrelin blocks the effects of bombesin on synthesis of the somatostatin mRNA [74]. Thus, the interactions between bombesin and ghrelin might account for postprandial variations found in serum GH levels and the forebrain expression of somatostatin mRNA [74].

In summary, bombesin directly interferes with sundry effects of ghrelin, most likely *via* central mechanisms.

3.3. Desacyl Ghrelin. The gastrointestinal peptide desacyl ghrelin (DAG) displays the identical amino acid sequence as ghrelin, however lacking the fatty acid residue [6]. Therefore, DAG—in contrast to ghrelin—does not interact with the GHS-R1a and thus was initially considered to be a degradation product of ghrelin without any biological effect [6]. However, recent literature indicates numerous actions of DAG (e.g., concerning cell proliferation and adipogenesis) [63, 64, 75–77]. In this context, it was found that transgenic mice over-expressing DAG showed a reduced food intake and a lower body weight compared to wild-type mice suggesting a role in the regulation of energy homeostasis [78, 79]. Also, exogenous DAG led to a significantly reduced cumulative body weight gain in adult male rats after one week of chronic infusion [80].

In addition, there is inconsistent data concerning a potentially anorexigenic effect of exogenous DAG [41, 79, 81, 82] that might be mediated by central pathways involving Urocortin and *Cocaine and Amphetamine Regulated Transcript* (CART) in the hypothalamic ARC and PVN [8, 41, 79]. However, data remain inconclusive.

Concerning a possible interaction between DAG and ghrelin, DAG was found to abrogate the metabolic effects of ghrelin after coadministration of both peptides [83]. More precisely, in rodents as well as in goldfish intraperitoneally administered ghrelin significantly increased food intake whereas simultaneously injected DAG abolished the stimulatory effect of ghrelin on feeding behaviour [83, 84]. Accordingly, the effect on neuronal activity in the ARC induced by ghrelin was significantly reduced when injected simultaneously with DAG [83]. As nesfatin-1 immunoreactive neurons in the ventromedial part of the ARC were activated by simultaneous injection of ghrelin and DAG, one might speculate that DAG suppresses ghrelin-induced food intake by curbing ghrelin-induced increased neuronal activity in the ARC and recruiting nesfatin-1 immunoreactive neurons [83].

Moreover, there is evidence indicating that DAG may counteract the role of ghrelin in the control of glucose metabolism. In humans exogenous ghrelin induced rapid changes in blood glucose and insulin levels, whereas DAG prevented the acylated ghrelin-induced effect when coadministered with acylated ghrelin [85, 86]. Furthermore, Gauna et al. found that glucose output by primary hepatocytes is time- and dose-dependently increased by incubation with ghrelin whilst this effect is counteracted by DAG coincubation [87]. Additionally, ghrelin-decreased insulin sensitivity has been reported to be prevented by intravenous coinjection of DAG [86, 88]. Besides interference with insulin secretion, in vitro DAG also abolished the effect of ghrelin on glucagon, pancreatic polypeptide, and somatostatin release [89].

Therefore, it can be summarized that DAG counteracts the effect of ghrelin on food intake, hypothalamic neuronal activation, glucagon, as well as on pancreatic polypeptide and somatostatin release. Furthermore, also opposing effects of DAG have been found on the effects of ghrelin covering insulin levels, sensitivity to insulin, as well as on blood glucose concentration.

3.4. Peptide YY. As a member of the pancreatic polypeptide family, peptide YY (PYY) is postprandially released from L-cells located in the distal gastrointestinal tract and has been reported to inhibit food intake *via* NPY-2 receptors expressed by neurons of the ARC [90, 91]. In addition to neurons of the ARC also vagal afferents projecting to the NTS have been found to be involved in the anorexigenic effect of PYY [92]. Based on the evidence that peripherally injected ghrelin acts *via* the N. vagus inducing neuronal activity in the ARC [24] a possible interaction of both peptides may be assumed theoretically.

However, recent data are conflicting as one study showed PYY infusion to significantly reduces plasma ghrelin levels in humans [93] while other reports failed to find an influence on ghrelin concentrations in mice [94] and pigs [95]. Furthermore, in mice the anorexigenic effect of intraperitoneal PYY injection has not been found to be regulated by prevailing endogenous plasma ghrelin concentrations or coinjection of ghrelin [94]. However, in contrast Chelikani et al. reported peripheral ghrelin injections in rats to attenuate PYY-induced inhibition of food intake and gastric emptying [96]. In support of these results, Riediger et al. observed in rats that subcutaneous PYY directly inhibited ghrelin-activated neurons of the ARC [97].

Taken together, available data remain inconclusive concerning the interactions of ghrelin and PYY with a need for further investigation.

3.5. Glucagon-Like Peptide. The 31 amino acid hormone glucagon-like peptide (GLP) belongs to the incretins and is postprandially secreted by L-cells in the ileum [98, 99]. The peptide has been found to significantly reduce energy intake, gastric emptying rate, and energy consumption in humans [100].

In the context of interaction, it has been shown that icv. injection of GLP-1 significantly inhibited ghrelin-induced stimulation of food intake [101]. Vice versa, also intravenous coinfusion of ghrelin has been found to significantly attenuate the GLP-1-induced reduction of food intake and its inhibitory effect on gastric emptying [96].

Moreover, it is noteworthy that GLP-1 administration has been found to prevent the initial postprandial decline in ghrelin levels, possibly due to delayed gastric emptying [102]. Furthermore, exogenous GLP-1 significantly decreased ghrelin secretion after meal ingestion in healthy man [102] as well as during vagal prestimulation in isolated rat stomachs [103]. Also, application of "the closely related peptide" GLP-2 has been reported to reduce ghrelin concentrations in humans [104]. However, Brennan et al. observed that intravenous GLP-1 injection did not exhibit any effect on ghrelin concentrations in healthy humans [53].

In conclusion, there is some evidence that GLP might diminish ghrelin-triggered effects on food intake and gastric emptying and lead to a reduction of ghrelin release.

3.6. Amylin. Amylin is an anorexigenic peptide hormone composed of 37 amino acids, which is cosecreted with insulin from pancreatic islet β-cells in response to nutrient ingestion,

incretin hormones, and neural input [105, 106]. Acute as well as chronic administration of amylin has been found to reduce food intake and body weight, which is predominantly mediated by neurons located in the area postrema [107, 108].

Initially, it has been shown that coadministration of amylin did not alter ghrelin-induced hyperphagia in rats [73]. In accordance, Osto et al. observed that the anorexigenic effect of amylin injection remained unchanged by simultanous ghrelin application in rats [109]. Thus it may be hypothesised that the metabolic state—*ad libitum* fed [73] or fasted [109] —of the animals might determine whether effects of ghrelin or amylin are predominant.

However, in conclusion interaction between ghrelin and amylin seems to be unlikely.

3.7. Pancreatic Polypeptide. The 36 amino acid peptide pancreatic polypeptide (PP) is mainly produced by cells located in the periphery of endocrine pancreatic islets. Secretion of PP is stimulated postprandially and peripheral injection of PP in rodents as well as in humans has been shown to reduce food intake and body weight, most likely mediated *via* indirect effects on the hypothalamic ARC involving the area postrema [110, 111].

Arosio et al. reported that peripheral injection of ghrelin in humans leads to a significant increase of PP levels in healthy subjects but to have a variable effect on PP release in acromegalic patients [112, 113]. In contrast, Qader and colleagues observed a dose-dependent inhibitory effect of ghrelin perfusion on PP secretion of rodents' isolated islet cells [89].

Due to this conflicting data and the lack of studies investigating coinjection of both peptides the interplay between ghrelin and PP remains to be further elucidated.

3.8. Insulin. The 51 amino acid peptide insulin is produced by pancreatic beta islet cells and is commonly recognized as the most important hormone regulating glucose homeostasis. Central injection of insulin has been shown to reduce food intake as well as body weight [114], most likely mediated *via* insulin receptors expressed on ARC neurones [115]. High blood glucose levels increase insulin release and likewise ghrelin treatment in rats has been shown to stimulate insulin secretion from isolated pancreas tissue [116, 117] as well as in vivo [118]. In contrast, in experiments conducted by other investigators ghrelin perfusion of isolated rodents pancreas suppressed insulin release in response to glucose and other secretagogues [89, 119–121] and portal vein infusion of ghrelin inhibited the glucose-induced insulin secretion [122]. In line with these results, ghrelin administration decreased insulin serum levels in rats in vivo [59, 60, 123]. Accordingly, ghrelin infusion likewise significantly suppressed C-peptide levels in gastrectomized humans [124].

However, in growth hormone-deficient humans, peripheral ghrelin induced a rapid increase in plasma insulin levels, a stimulation of lipolysis, and a reduced peripheral insulin sensitivity [86, 125]. Interestingly, in ghrelin knockout mice the usually displayed high-fat diet-induced glucose intolerance was largely prevented [126] and also ghrelin receptor knockout mice were found to have an increased insulin sensitivity [127]. Also in ob/ob mice an improvement of the diabetic phenotype has been observed after the ablation of ghrelin [128].

Vice versa, most studies revealed an inhibitory effect of exogenous insulin on ghrelin levels in humans [129–132], rats [133, 134] as well as in isolated rat stomachs [55, 103, 135]. Moreover, Murdolo et al. observed that insulin seems to be essential for the prandial suppression of ghrelin levels in humans [136]. However, challenging these results Caixas et al. found that parenteral insulin does not influence blood levels of ghrelin in humans [137], while Toshinai and colleagues even observed increased ghrelin mRNA levels in the stomach after insulin administration [138].

Furthermore, during ghrelin infusion, insulin-dependent suppression of endogenous glucose production in mice has been reported to be less effective [88]. However, coadministration of ghrelin stimulated the insulin-induced glucose uptake in adipocytes [139]. Additionally, in hepatoma cells ghrelin has been identified to regulate downstream molecules of insulin signalling [140]. As antighrelin antibodies abolished the insulin-induced neuronal activation within the nucleus tractus solitarii of the brainstem, Solomon et al. concluded that this brain area might participate in peripheral ghrelin hunger signalling mediated by insulin [141].

Taken together, ghrelin and insulin obviously interfere in the reciprocal secretion regulation in a very complex manner.

4. Summary

Discovered in 1999, investigation of ghrelin as well as ghrelin-dependent effects and interactions is a quite novel field of research. However, during the last decade effects of ghrelin have been subject to intensive investigation. As obesity is a challenging problem worldwide, especially the orexigenic effect of ghrelin has been extensively explored. In this context, various possibilities to curb the stimulating effect on food intake behaviour have been investigated with more or less promising results [142, 143]. However, so far no substance has been identified to reliably inhibit food intake during long-term treatment. Nevertheless, it has been shown that the stimulatory effect of ghrelin on food intake is diminished by several anorexigenic peptides such as CCK, bombesin, desacyl ghrelin, PYY, insulin, and GLP but not by amylin. Some of these peptides inhibit ghrelin secretion and exert opposite effects on hypothalamic neuronal activity or gastric emptying. Thus, interaction between ghrelin and these anorexigenic gastrointestinal hormones might be an auspicious approach in the context of pharmacological obesity treatment.

Moreover, in addition to the previously introduced peptides originating from the gastrointestinal tract, also the satiety factor leptin, which is primarily synthesized in the adipose tissue, interacts with ghrelin. In this context, it has been described that leptin and ghrelin diminish each others'

Table 2: Interference between ghrelin and leptin.

Reference	Interaction
Barazzoni 2003 [144]	Leptin injection reduces starvation-induced ghrelin secretion.
Toshinai 2001 [138]	Leptin administration increases ghrelin mRNA level in the stomach
Dixit 2004 [145]	Ghrelin inhibits leptin-induced cytokine expression
Nakazato 2001, Kim 2004 [21, 146]	Ghrelin reverses leptin-induced feeding reduction
Shintani 2001, Kohno 2003, Kohno 2007 [37, 39, 147]	Leptin suppresses Ghrelin-induced activation of NPY neurons within the ARC
Rosicka 2003, Park 2005 [148, 149]	Ghrelin and leptin levels are reversely correlated and depend on the BMI
Bagnasco 2002, Beretta 2002, Dube 2002, Bagnasco 2003 [150–153]	Central transgenic leptin expression elevates serum ghrelin levels

effects on food intake *via* oppositional influence on NPY-positive neurons within the ARC [37, 39]. Furthermore, as summarized in Table 2, both peptides interfere in various other ways [154, 155].

Taken together, during the last decade many aspects of appetite regulation associated with ghrelin have been elucidated. However, the brain-gut-axis—including ghrelin as the only peripheral orexigenic peptide—is a very complex system, for which our understanding to date remains limited. Thus, we can be curious for the next decades of ghrelin and its role in appetite regulation.

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Review Article

The Effect of Ingested Macronutrients on Postprandial Ghrelin Response: A Critical Review of Existing Literature Data

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Ghrelin is a powerful orexigenic gut hormone with growth hormone releasing activity. It plays a pivotal role for long-term energy balance and short-term food intake. It is also recognized as a potent signal for meal initiation. Ghrelin levels rise sharply before feeding onset, and are strongly suppressed by food ingestion. Postprandial ghrelin response is totally macronutrient specific in normal weight subjects, but is rather independent of macronutrient composition in obese. In rodents and lean individuals, isoenergetic meals of different macronutrient content suppress ghrelin to a variable extent. Carbohydrate appears to be the most effective macronutrient for ghrelin suppression, because of its rapid absorption and insulin-secreting effect. Protein induces prolonged ghrelin suppression and is considered to be the most satiating macronutrient. Fat, on the other hand, exhibits rather weak and insufficient ghrelin-suppressing capacity. The principal mediators involved in meal-induced ghrelin regulation are glucose, insulin, gastrointestinal hormones released in the postabsorptive phase, vagal activity, gastric emptying rate, and postprandial alterations in intestinal osmolarity.

1. Introduction

Ghrelin is a 28-amino-acid gastrointestinal peptide with appetite-stimulating, growth hormone-releasing and adipogenic properties [1–3]. It was originally characterized as the endogenous ligand for the hypothalamic-pituitary growth hormone secretagogue receptor type 1a (GHSR1a), stimulating the anterior gland of pituitary to produce GH [1–3]. In fact, ghrelin is the third physiological regulator of endogenous GH secretion, along with hypothalamic GH releasing hormone and somatostatin. Ghrelin is predominantly produced in the so-called X/A-like endocrine cells of gastric mucosa, and is subsequently released into bloodstream [4, 5]. Ghrelin-producing cells are mostly abundant in the oxyntic glands of gastric fundus [4, 5]. Given the widespread distribution of GHSR1a in the human body, ghrelin exerts pluripotent biological activities, affecting cardiovascular system, pancreatic endocrine function, gastrointestinal tract motility, gastric acid secretion, cell proliferation and metabolism [3].

One of the most important actions of ghrelin is its regulatory role for long-term energy homeostasis and short-term food intake [6]. There is a competitive interaction between ghrelin and leptin in hypothalamus for feeding regulation. Ghrelin activates neuropeptide Y (NPY) and Agouti-related protein (AGRP) neurons in the hypothalamic arcuate nucleus, providing a central stimulus for increased food intake and reduced energy expenditure [7]. Intracerebroventricular administration of ghrelin in rodents and peripheral administration in humans has shown to promote weight gain, by reducing fat utilization and increasing food consumption [8, 9]. Ghrelin is actually the only known appetite-stimulating gastrointestinal hormone. It acts as a circulating orexigenic signal, and has been also implicated in preprandial hunger and meal initiation. Cummings et al. were the first to show that plasma ghrelin levels increase nearly twofold immediately before feeding onset, and are strongly suppressed by food ingestion, falling to trough (nadir) levels within an hour after meal initiation [10]. This pattern of secretion is interestingly reciprocal to that of insulin, which is preprandially low and increases gradually in

the postabsorptive period [10]. Another interesting finding is that plasma ghrelin levels reflect human nutritional state [11]. Ghrelin secretion is typically up-regulated under conditions of chronic negative energy balance (anorexia nervosa, heart failure cachexia), and down-regulated in the setting of sustained positive energy balance (obesity). Furthermore, obese subjects fail to exhibit the normal postprandial decline of plasma ghrelin concentrations, observed in normal weight individuals [12].

The postmeal inhibition of gastric ghrelin production is proportional to energy load and is profoundly influenced by the meal's macronutrient content [13, 14]. In rodents and normal weight humans, the postprandial drop in ghrelin levels is more pronounced after carbohydrate (CHO) meals than after protein- or fat-enriched diet manipulations [15, 16]. The type of ingested macronutrient seems to affect differentially the magnitude and pattern of postprandial ghrelin suppression. Whether it is the direct intraluminal contact of nutrients with gastric mucosa or the insulin-mediated metabolic response to nutrient ingestion more important for postprandial ghrelin suppression remains still controversial. There is currently growing evidence that ghrelin suppression does not require the presence of nutrients in either the stomach or the duodenum, but requires effective postgastric and postabsorptive feedback mechanisms, possibly mediated by insulin and gastrointestinal hormones with anorexigenic potential [16]. Vagal activity, gastric emptying rate and postprandial increases of intestinal osmolarity are also active players in meal-induced ghrelin regulation [17, 18].

Despite the well-established stimulatory effect of ghrelin on appetite and eating behavior, little information is available regarding its relationship with fasting and postprandial energy expenditure in normal weight and obese humans. In rodents, ghrelin infusion promotes weight gain, both by increasing food intake and by decreasing energy expenditure and fat catabolism [8]. This effect is primarily due to an increase in caloric intake and respiratory quotient (RQ), suggestive of a switch from fatty acid oxidation to glycolysis leading ultimately to fat deposition. St-Pierre et al. examined the relationship between serum ghrelin and resting metabolic rate, thermic effect of food, fasting and postprandial RQ, physical activity level and peak aerobic capacity in 65 lean young women. Significant inverse correlations were reported between ghrelin, resting metabolic rate and thermic effect of food, persisting after adjustment for fat-free mass, fat mass and insulin levels [19]. These results suggest that higher levels of ghrelin are associated with low levels of resting and postprandial thermogenesis, indicating that the metabolic effects of ghrelin may extend far beyond the regulation of satiety and substrate oxidation, serving as a biomarker for decreased energy expenditure in humans. On the other hand, the relationship between ghrelin and energy expenditure in obesity constitutes a matter of debate. In a study by Marzullo et al., the obese subjects with low resting energy expenditure (impaired energy balance) exhibited lower active ghrelin levels, compared with obese subjects with high energy expenditure, indicating that ghrelin secretion and activity might be decreased in cases of obesity with impaired energy expenditure, as part of an obesity-related compensatory mechanism [20].

The present review aims to shed light on the underlying mechanisms of postprandial ghrelin regulation. In addition, a significant body of clinical and experimental data will be discussed, elucidating the macronutrient-specific effect of several isocaloric test meals on postprandial ghrelin levels. We searched PubMed and other electronic databases for high quality articles written in English, using the following search terms: ghrelin, macronutrients, carbohydrate, protein, fat and postprandial response.

2. Underlying Pathophysiology of Postprandial Ghrelin Regulation

A recent study in humans demonstrated that ghrelin levels can be suppressed by sham feeding (when nutrients are only smelled, chewed or tasted without being swallowed), as well as by actual feeding, indicating the importance of cephalic response to nutrient intake and supporting the role of vagal activity for the control of postprandial ghrelin secretion [17]. The vagally mediated cephalic phase appears to have a major role in initiating the postprandial fall in ghrelin levels, which are thereafter maintained suppressed, by other—as yet not entirely elucidated—gastrointestinal or postabsorptive mechanisms, mediating the nutrient-related ghrelin response [17]. The gastric phase alone appears to play no role in the regulation of ghrelin secretion, because neither gastric distension alone, nor activation of chemical nutrient-sensing mechanisms of gastric mucosa (gastric chemosensitization) can modulate ghrelin levels [18]. In an interesting experiment in rats, intragastric infusion of glucose reduced ghrelin levels by approximately 50%, while water infusion had no effect [21]. However, when gastric emptying was prevented through the inflation of a pyloric cuff (gastric distension), glucose and water infusions were similarly ineffective to suppress ghrelin. These experimental findings indicate that gastric distension and chemosensation are both insufficient to induce a ghrelin response [21]. Prandial ghrelin regulation is probably mediated by intestinal signals generated downstream of Treitz ligament, meaning that postgastric feedback is definitely required for an adequate inhibitory ghrelin response [16]. In this intestinal phase of food ingestion, there seems to be a prominent macronutrient effect, determining the depth and duration of postprandial ghrelin suppression [17]. The macronutrient-related patterns of ghrelin response imply that either the direct exposure of gastrointestinal mucosa to ingested nutrients, or the increased circulating levels of nutrients or other related hormones, can influence postprandial ghrelin levels in a macronutrient-specific manner [17, 22]. Candidate mediators involved in the regulation of postprandial ghrelin secretion are glucose, insulin, cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), peptide YY (PYY), pancreatic polypeptide (PP), oxyntomodulin and somatostatin (SS). Most of these molecules are gastrointestinal hormones, which delay gastric emptying and display insulinotropic and anorexigenic activities [18, 21].

Insulin and glucose are thought to be dynamic modulators of plasma ghrelin concentrations in rodents and humans [23–25]. Hyperglycemia and hyperinsulinemia tend to decrease, while hypoglycemia and insulin deficiency tend to increase circulating ghrelin levels. Both intravenous and oral administration of glucose leads to a significant decline in circulating ghrelin, indicating that ghrelin secretion may be suppressed, at least in part, by an increased plasma glucose level in healthy humans [23, 26]. For intravenous glucose administration in particular, Möhlig et al. showed that glucose elicits a significant decrease of plasma ghrelin concentrations, whereas intravenous free fatty acid or arginine load does not affect circulating ghrelin levels [27]. Insulin-mediated glucose uptake and metabolism may also control postprandial ghrelin levels, while insulin sensitivity is considered to be an important determinant of postprandial ghrelin suppression [28]. A great increase in plasma free fatty acids, as a result of constant intravenous lipid infusion, failed to suppress plasma ghrelin, while ghrelin decreased by almost 50% under hyperinsulinemic euglycemic clamp conditions [27].

The effect of acute hyperinsulinemia on plasma ghrelin concentrations is still a matter of debate. Clinical data regarding the existing interrelation between ghrelin and insulin are rather conflicting. According to the study of Flanagan et al., using a stepped hyperinsulinemic eu-, hypo- and hyperglycemic glucose clamp, insulin may suppress circulating ghrelin independently of glucose, although glucose might have an additional synergistic effect [29]. In the same direction, Murdolo et al. tested the hypothesis that insulin is the driving force for postprandial ghrelin suppression by comparing the effects of meal ingestion on plasma ghrelin levels between insulin-deficient patients with type 1 diabetes and healthy controls [30]. The investigators concluded that insulin is essential for meal-induced plasma ghrelin suppression, commenting that severe insulin deficiency in uncontrolled type 1 diabetic subjects may partly explain the episodes of hyperphagia observed in these patients, through compromising postprandial ghrelin response [30].

The exact mechanism of insulin affecting plasma ghrelin concentrations remains to be established. Insulin might inhibit ghrelin synthesis or secretion, either directly or indirectly. Studies in rats have shown that insulin-induced hypoglycemia increases, instead of decreasing, ghrelin mRNA levels in the gastric fundus, providing no evidence for a direct inhibitory action of insulin on ghrelin synthesis [11]. However, in healthy humans, plasma ghrelin concentrations are decreased during insulin-induced hypoglycemia, suggesting species-specific differences between rodents and humans [31]. Since ghrelin-producing cells are closely associated with the capillary network of the lamina propria of gastric mucosa, their function might be under endocrine control [5]. However, there is still no evidence for insulin receptors on the surface of ghrelin-producing cells. Indirect pathways for insulin suppressive effects on ghrelin synthesis and secretion include activation of hypothalamic insulin receptors and modulation of the cellular flux of glucose or free fatty acids [32]. There seems to be an interaction between nutrients and insulin in reducing circulating ghrelin levels. A greater insulin-induced glucose uptake by X/A-like cells might inhibit ghrelin synthesis and/or secretion [30].

Contrary to the findings mentioned above, Caixás et al. reported that, unlike food intake, the subcutaneous administration of a short-acting insulin analog was not able to suppress ghrelin levels [33]. In concordance with this study, Schaller et al. concluded that meal-related ghrelin suppression is not directly regulated by glucose or insulin, since a reduction in ghrelin was observed only at supraphysiological insulin concentrations, while hyperglycemia did not decrease ghrelin at all [34]. Contrary to Murdolo, Spranger et al. observed a substantial postprandial decrease of plasma ghrelin in an insulin-deficient patient with type 1 diabetes following a carbohydrate challenge, while a subsequent bolus of subcutaneous short-acting insulin induced no further changes in circulating ghrelin [35].

Taking everything into consideration, glucose and insulin are unlikely to explain the entire postprandial ghrelin response, since ghrelin remains suppressed long after the normalization of glucose and insulin levels, and furthermore, because lipids tend to suppress ghrelin in the absence of substantial increases in glucose or insulin [16]. Other hormonal mediators (such as CCK, GLP-1 and GIP) released in the postabsorptive phase in response to nutrient stimuli, appear to orchestrate the whole postmeal ghrelin response, enhancing the inhibitory effects of glucose and insulin, which are still remaining the principal contributors. The inverse temporal relationship between circulating concentrations of ghrelin and insulin, reported in a large number of clinical studies, substantiates the key regulatory role of insulin for ghrelin regulation [36–38].

It would be also interesting to examine the role of leptin in postprandial ghrelin regulation, since ghrelin and leptin are part of a dynamic peripheral feedback system that regulates body weight and energy homeostasis by modulating satiety. Data concerning the relationship between ghrelin and leptin at fasting and postprandial state are rather contradictory. In vitro studies have previously shown that leptin inhibits ghrelin production from the gastric mucosa, and leptin levels in humans appear to be inversely related to ghrelin concentrations [39]. On the other hand, the interesting observation by Cummings et al. that leptin and intermeal ghrelin levels display diurnal rhythms that are in phase with one another suggests that leptin and ghrelin might be coordinately regulated [10]. The same study reported a subtle postprandial drop in leptin levels that may reflect meal-related regulation of gastric leptin, according to the investigators [10]. The relationship between insulin and leptin is more clearly defined and may partially explain the ghrelin-leptin interrelationship. Previous studies indicate that leptin secretion is regulated by insulin-mediated glucose metabolism, suggesting that insulin is a positive modulator of leptin concentrations [40]. This is why consumption of high-fat meals and high-fructose beverages that produce smaller postprandial glucose and insulin responses compared with isocaloric high-carbohydrate meals, have shown to reduce 24-hour circulating leptin concentrations in humans [40]. Accepting that leptin and insulin are positively associated, it is conceivable that postprandial

surges of insulin are related to increased leptin levels, and given the reciprocal relationship between insulin and ghrelin, a similar inverse relationship might be supported for leptin and ghrelin as well. Because insulin and leptin function as key signals conveying information on energy intake and body fat stores to the central nervous system for the long-term regulation of food intake and energy homeostasis, it is possible that reduced insulin and leptin production, as well as increased ghrelin levels, contribute to increased energy intake, weight gain, and obesity in humans [40].

3. Carbohydrate Ingestion and Postprandial Ghrelin Response

In an interesting study by Foster-Schubert et al., three different macronutrient preloads of equal caloric content, volume and energy density (protein-, fat-, and CHO-based beverages) were compared for their relative efficacy to suppress total and acylated (bioactive) ghrelin levels [41]. Total ghrelin levels decreased significantly more after CHO or protein ingestion than after lipids, while ghrelin's nadir (lowest) levels were reached most rapidly after the CHO-enriched preload (within 99 minutes). For both acylated and total ghrelin concentrations, investigators observed marked and macronutrient-dependent differences during the first 3 hours versus the subsequent 3 hours of the postprandial study period. More specifically, they observed only after CHO ingestion a marked rebound of acylated ghrelin to 37% above baseline levels, during the second 3 hours of the 6 hour post-ingestive period. This study reveals a previously unidentified pattern in the response of acylated and total ghrelin after CHO ingestion. Ghrelin levels decreased in the initial 3 hours, followed by a marked overshoot to above the pre-ingestion baseline during the second 3 hours. No such overshoot was observed after protein or lipid ingestion, both of which suppressed acylated and total ghrelin levels until study completion. These observations suggest very different effects of high CHO meals in the early versus the later postabsorptive phase, indicating that ingested CHO might prompt an early hunger rebound. Such findings have important clinical implications for design of dietary regimens [41].

The faster gastric emptying after CHO ingestion, compared with lipids or proteins, can partly explain the strong and rapid postprandial ghrelin suppression in the first phase [41]. The rapid removal of CHO from stomach should cause a rapid, strong suppression of ghrelin levels, an effect that might be short lived, because these nutrients are quickly absorbed and metabolized. If insulin-mediated glucose disposal is more important for ghrelin suppression than mere insulin levels, the late post-CHO ghrelin overshoot may result from reduced intracellular glucose metabolism, when glucose levels decreased below baseline [41].

Not all types of dietary CHO are likely to have the same effect on postprandial ghrelin levels. In a study comparing high-glucose with high-fructose meals, the mean postprandial suppression of ghrelin was markedly attenuated and significantly less pronounced after consuming the high-fructose meals [40]. Fructose, unlike glucose, does not stimulate insulin secretion from pancreatic beta cells, presumably because of the low number of fructose transporters (GLUT5) on beta cell membrane [40]. Intravenous fructose infusion increases only marginally circulating insulin concentrations, while ingested fructose is ineffective in eliciting postprandial insulin secretion. What is more, fructose does not increase insulin-mediated glucose metabolism or circulating leptin levels [40]. Given the key role of insulin for postprandial ghrelin suppression, ingested fructose suppresses ghrelin poorly. The failure of fructose to effectively suppress ghrelin (impaired satiety), along with the reduced insulin and leptin concentrations, could lead to an increased caloric intake and ultimately contribute to obesity, during chronic consumption of diets high in fructose [40].

CHO-enriched test meals, containing both simple and complex carbohydrates, have been used in various clinical studies investigating the differential response of postprandial ghrelin to meals of different macronutrient composition. In all of them, and particularly in normal weight subjects, CHO ingestion provoked a significant postprandial ghrelin decline by approximately 30% from baseline values within 2 hours after meal onset [37, 38, 40–42]. A common finding in all these studies is the inverse correlation between postprandial ghrelin and insulin concentrations throughout the whole study period. While the suppressant effect of CHO on ghrelin levels is well established and taken for granted, the biphasic pattern of ghrelin suppression after CHO intake [41] and the clinically meaningful distinction between glucose and fructose [40], are novel thought-provoking findings that warrant further investigation.

4. Protein Ingestion and Postprandial Ghrelin Response

Dietary protein is considered to be the most satiating macronutrient [43]. The higher satiety associated with protein consumption may be at least partially mediated by a protein-induced prolonged postprandial ghrelin suppression [43]. Such a reduction in the orexigenic signal might delay the initiation of a subsequent feeding episode or lower hunger and energy intake. The prolonged suppression of ghrelin after protein intake might relate to the protracted emptying of proteins from stomach, causing a more sustained activation of post-gastric ghrelin-suppressing mechanisms [41]. Additional mechanisms that account for the significant satiating effect of dietary protein include the following: proteins have a larger thermic effect than CHO or fat, since they cannot be stored in the body, but need to be metabolized immediately [44]. Moreover, increased circulating concentrations of amino acids after protein intake stimulate hepatic gluconeogenesis preventing hypoglycemia, and thus promoting satiety [44]. In rats fed on protein-enriched diets, intestinal gluconeogenesis is also induced in the postabsorptive phase [45]. Last but not least, proteins stimulate the secretion of specific gastrointestinal peptides (CCK, GLP-1, GIP) that delay gastric emptying and increase satiety [44].

In a study of three isoenergetic meals (balanced, high-fat and high-protein) consumed by healthy young women, acylated ghrelin fell significantly after ingestion of both balanced and high-protein meals, while ghrelin persisted at significantly lower levels than baseline for a longer duration, following the high-protein meal [36]. Apart from prolonging postprandial ghrelin suppression, liquid protein preloads have also shown to prolong the elevation of anorexigenic gastrointestinal hormones, such as CCK and GLP-1 [43]. These responses are observed irrespective of the type of protein consumed (soy, whey, or gluten) [43]. In support of this, Lang et al. has demonstrated no effect of protein type (egg albumin, casein, gelatin, soy protein, pea protein and wheat gluten) on satiety, 24 hour energy intake and postprandial glucose and insulin concentrations [46]. In a further randomized crossover study in healthy adult males, the high-protein meal maintained significantly lower ghrelin levels at 180 minutes compared with the high-CHO and high-fat meals, indicating that dietary protein exhibits longer-term postprandial ghrelin suppression and enhanced satiety [37]. According to Blom et al., the high-protein breakfast decreased postprandial ghrelin secretion more than did the high-CHO breakfast [44]. It also increased glucagon and CCK, tended to increase GIP and GLP-1, and decreased gastric emptying rate, without affecting however ad libitum energy intake.

Despite the accumulating evidence supporting the satiating and ghrelin-suppressing capacity of dietary protein, there have been a few studies suggesting that protein ingestion stimulates, instead of suppressing, ghrelin levels [38, 47], while an additional study indicated that the satiating effect of protein is practically unrelated to postprandial ghrelin secretion [48].

5. Fat Ingestion and Postprandial Ghrelin Response

Ingested lipids appear to suppress the orexigenic hormone ghrelin less effectively than do CHO or protein [41]. The relatively weak ability of this macronutrient to suppress ghrelin can be attributed to the poor stimulation of insulin secretion by lipids as well as to the lower osmolarity of lipid meals and beverages. Postprandial increases of intestinal osmolarity are believed to promote ghrelin suppression. However, lipids contribute fewer osmolar units compared with an isocaloric consumption of CHO or proteins [41].

In a study by Pavlatos et al., total ghrelin levels did not decline significantly after a fat-rich meal in normal weight women, as opposed to an isoenergetic protein-rich meal [49]. In a similarly designed study by Tentolouris et al., fat consumption has also displayed a diminished capacity to induce satiety [50]. In this study, the effect of two isocaloric test meals (one rich in CHO and one rich in fat) on postprandial active ghrelin concentrations was comparatively evaluated in lean and obese women. After the fat-rich meal, active ghrelin levels were not significantly suppressed, even in the lean participants. The investigators conclude that increased fat intake might promote obesity not only through its high caloric content and adverse metabolic effects, but also through its failure to suppress postprandial hunger. Erdmann et al. reported a different (more delayed) time pattern of ghrelin suppression after fat ingestion, compared with CHO [47]. More specifically, the fat-rich meal decreased plasma ghrelin levels, but the nadir was reached towards the end of the study period, namely at 180 minutes.

The potential impact of varying fatty acid composition (saturated, monounsaturated and polyunsaturated fat) on postprandial ghrelin response has been only scarcely investigated. In a relevant double-blind crossover study, researchers assessed two high-fat test meals, one with a high saturated to unsaturated fat ratio (70/30) and the other with a low ratio (55/45), and concluded that increasing saturated fat consumption had no deleterious effects on fasting and postprandial plasma ghrelin concentrations [51].

6. Effect of BMI on Nutrient-Related Ghrelin Regulation

BMI, body fat and indices of central fat distribution are inversely associated with fasting plasma ghrelin concentrations. A large number of clinical studies have shown that obese subjects tend to display lower total and acylated ghrelin levels in the fasting state compared with normal weight individuals [50]. This finding appears to be an appropriate compensatory response, so that obese individuals will not get any fatter and lean individuals will not get any thinner (adaptive mechanism for prevention of obesity and cachexia resp.). It has been proposed that the sustained positive energy balance observed in obesity suppresses maximally circulating ghrelin levels, and thus limits flexibility for further short-term feeding regulation. The impaired cholinergic (vagal) regulation of postprandial drop in ghrelin concentrations might be also responsible for the dysregulated ghrelin control in obese subjects [52]. Furthermore, obese subjects are often insulin-resistant and thus hyperinsulinemic, and insulin is a well established inhibitory signal for ghrelin secretion. To the best of our knowledge, the differential rate and magnitude of preprandial rise in ghrelin levels has not been comparatively evaluated in lean and obese individuals. As already mentioned, it is widely accepted that obese subjects exhibit significantly lower fasting ghrelin concentrations than lean, but whether the rate of preprandial ghrelin increase is actually differentiated between lean and obese subjects has been scarcely addressed. In fact, most of the studies that used frequent blood sampling protocols in order to assess the diurnal plasma ghrelin profile in subjects of varying BMI (preprandial and postprandial hormonal alterations), reported no specific BMI-related differences between lean and obese participants in terms of preprandial rate of ghrelin increase [10].

Another important aspect of ghrelin regulation in obese subjects is the blunted postprandial ghrelin response. This means that obese subjects have low ghrelin levels preprandially, but postprandial ghrelin secretion is not sufficiently suppressed, suggesting a severe defect in ghrelin-induced

satiety mechanisms, which makes them feel still hungry, even though they have just completed their meal. Pavlatos et al. have shown that neither a protein- nor a fat-rich meal was able to elicit a significant acute ghrelin response in obese women [49]. In the same direction, Tentolouris et al. reported that a high-CHO meal (with a well established ghrelin-suppressing potential in lean individuals) was also insufficient to suppress postprandial active ghrelin levels in obese women, indicating a considerable secretory and possibly satiety impairment in these subjects [50]. Another interesting conclusion of the same study was that, the leaner a person is, the higher his fasting ghrelin is, and the steepest its postprandial decline. This means that a lean subject feels quite hungry before meals, but afterwards feels easily satiated. Apart from ghrelin, additional hormonal factors that contribute to this auto-regulation of body weight homeostasis in normal weight subjects include GLP-1, GIP, and PYY, which delay gastric emptying, induce satiety and prevent hyperphagia, and are significantly more functional in lean subjects compared with the obese [53]. However, as a person gains weight, this autoregulatory effect appears to become severely compromised. An obese subject cannot experience postprandial fullness, independently of the macronutrient composition of his meal [50].

The fact that lean subjects display higher fasting ghrelin levels than obese does not necessarily mean that they also consume greater amounts of food. On the contrary, food intake is most likely to be increased in obese subjects, because of the blunted postprandial ghrelin response, as described above. Besides, the effect of ghrelin on hunger and satiety sensations is not necessarily translated into alterations in *ad libitum* energy intake, as shown by the study of Erdmann et al. [38]. An additional study by Druce et al. showed that low-dose infusion of ghrelin increased *ad libitum* energy intake at a buffet meal only in the obese group, and not in the lean, indicating that obese people are highly sensitive to the appetite-stimulating effects of ghrelin, even when the circulating ghrelin is low [54]. As a result, the absolute difference of fasting ghrelin levels between lean and obese subjects is not a major determinant of subsequent food intake, since other factors such as endogenous sensitivity to circulating ghrelin, ghrelin activity and postprandial ghrelin changes are thought to play an important role, as well.

Additional factors that can in part explain the suppressed basal ghrelin levels in obese subjects include hyperleptinaemia and increased circulating levels of IL-1b (interleukin 1b), since both leptin and IL-1b are thought to inhibit ghrelin secretion [7]. Hyperleptinaemia is observed frequently in obesity due to leptin resistance, and high levels of IL-1b and other inflammatory mediators are also a common finding in patients with obesity and metabolic syndrome. Concerning the blunted postprandial ghrelin response in obese subjects, the impaired post-meal elevation of gastrointestinal hormones with anorexigenic and insulinomimetic properties, such as GLP-1, GIP and PYY, has been implicated as an additional significant contributor [55]. As far as insulin resistance is concerned, its role for ghrelin regulation is different in fasting and postprandial state. In fasting, insulin resistance and thus hyperinsulinemia lead to decreased fasting ghrelin levels. Fasting plasma ghrelin concentrations are lower in insulin-resistant obese adults, compared with equally obese individuals with relatively higher insulin sensitivity [28]. On the other hand, postabsorptive insulin resistance and impaired intracellular insulin signaling lead to inadequately suppressed and thus increased levels of ghrelin, since insulin sensitivity is regarded as prerequisite for sufficient postprandial ghrelin suppression [28].

The macronutrient-specific effect of meals on postprandial ghrelin levels has interesting implications only in normal weight individuals. In the obese population, the macronutrient effect appears to become blunted and is therefore of minor importance. This hypothesis is further corroborated by a recent study by Heinonen et al., where obese individuals with metabolic syndrome elicited no differences in plasma ghrelin or feelings of hunger and satiety, after consuming two high-CHO meals producing different insulin responses (whole-grain rye bread and wheat bread) [56]. Despite the different insulin response, ghrelin levels did not change in obese patients in response to either type of bread meals. In addition, ghrelin levels did not correlate with insulin or glucose, indicating that regulation of ghrelin might be altered in obese patients with metabolic syndrome independently of insulin [56]. An additional study by Moran et al. revealed a dysregulation of ghrelin homeostasis in overweight women with polycystic ovary syndrome (PCOS), suggesting that women with PCOS exhibit similar ghrelin abnormalities with obese women (down-regulated fasting ghrelin, blunted postprandial ghrelin suppression), and this disorder was not differentially affected by diet macronutrient composition [53].

Diet-induced weight loss, contrary to gastric bypass surgery where ghrelin levels remain dramatically decreased, has shown to elevate fasting ghrelin levels and normalize postprandial ghrelin response [57]. This means that when a person loses a significant amount of weight by diet he might feel a greater preprandial desire to eat, but his postprandial satiety is significantly improved. The greater sensitivity to vagal stimulation after weight loss may result in a more pronounced drop in postprandial ghrelin levels, in addition to the improvement in insulin sensitivity, which is a major determinant of postprandial ghrelin suppression [57]. Romon et al. reported that diet-induced weight reduction preferentially improves ghrelin response to a high-CHO meal, compared with a high-fat meal, indicating that weight loss might selectively improve the response of ghrelin to carbohydrate [57].

7. Acute Effect of Ethanol and Smoking on Plasma Ghrelin Levels

Zimmermann et al. addressed the interesting question, whether acute ethanol ingestion affects ghrelin secretion [58]. Ghrelin declined significantly within 15 minutes after alcohol drinking, fell to a minimum of 66% of baseline at 75 minutes and remained suppressed until the last sample at 2 hours. Given that alcohol seems to acutely attenuate circulating ghrelin levels and is also known for its

satiating power, one might expect from alcohol to promote weight loss. However, its considerable caloric density and its detrimental overall health effects should not be overlooked.

As far as smoking is concerned, in an interesting study by Kokkinos et al., acute cigarette smoking induced no significant suppression of post-smoking ghrelin in habitual smokers, possibly desensitized to any possible effect of smoking on ghrelin, through prolonged nicotine exposure [59]. On the other hand, there was a progressive decline of ghrelin in non-smokers, reaching its nadir 60 minutes after smoking. Fasting total ghrelin levels were not significantly different between smokers and non-smokers, indicating that smoking is unlikely to exert a long-term anorectic effect in smoking populations. The significant decrease in circulating ghrelin after smoking cessation, reported by Lee et al., provides further evidence for lack of correlation between smoking status and suppressed plasma ghrelin concentrations [60].

8. Summary, Conclusions, and Perspectives

Many clinical studies have used isoenergetic test meals (protein-, fat- and CHO-rich) in order to examine the relative efficacy of each macronutrient to suppress postprandial ghrelin. Even though the overall concept in these studies is common, the experimental design (meal composition, measured parameters, blood sampling intervals, duration of post-ingestive period) is slightly or moderately different. This discrepancy may be in part responsible for heterogeneity in findings. Trying to delineate the central message behind all these divergent data, carbohydrate appears to be the most effective macronutrient in terms of postprandial ghrelin suppression, possibly because of its glucose-elevating and insulin-secreting effect. However, recent data indicate that CHO ingestion may provoke a delayed ghrelin rebound in the later postabsorptive period, questioning the role of CHO-rich meals in weight loss dietary approaches. Besides, all types of dietary CHO are not equally effective. Fructose-enriched meals display a poor ghrelin-suppressing capacity, promoting increased caloric intake, weight gain and obesity under conditions of chronic consumption. The most satiating macronutrient appears to be dietary protein. Protein induces prolonged ghrelin suppression and elevation of gut-derived anorexigenic hormones that delay gastric emptying regardless of the type of protein consumed. However, the influence of solid forms of protein (turkey, pork) on postprandial ghrelin levels may require assessment over a longer period of time than 3-4 hours, since slow gastric emptying delays postprandial ghrelin nadir. As far as fat is concerned, it appears to be the least potent ghrelin-suppressant, even in normal weight subjects. Some studies have shown that fat decreases ghrelin concentrations, but later or more weakly than other macronutrients. At the same time, other studies report that fatty meals have absolutely no effect on postprandial ghrelin levels. In obese subjects, postprandial ghrelin response is blunted, and the macronutrient effect on ghrelin levels appears to be rather neutral. However, weight loss restores ghrelin response and leads to a significant improvement of ghrelin-mediated appetite regulation.

From now on, it would be interesting to evaluate the long-term effect of macronutrient-enriched diet manipulations on fasting and postprandial ghrelin levels. Additional parameters that could possibly influence ghrelin response and should be further investigated are food form and viscosity (liquid, solid, semi-solid products), portion size and meal duration.

Abbreviations

GH: Growth hormone
GHSR1a: Growth hormone secretagogue receptor type 1a
NPY: Neuropeptide Y
AGRP: Agouti-related protein
CCK: Cholecystokinin
GLP-1: Glucagon-like peptide 1
GIP: Glucose-dependent insulinotropic polypeptide
PYY: Peptide YY
PP: Pancreatic polypeptide
SS: Somatostatin.

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Review Article

Ghrelin's Roles in Stress, Mood, and Anxiety Regulation

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Several studies suggest that the peptide hormone ghrelin mediates some of the usual behavioral responses to acute and chronic stress. Circulating ghrelin levels have been found to rise following stress. It has been proposed that this elevated ghrelin helps animals cope with stress by generating antidepressant-like behavioral adaptations, although another study suggests that decreasing CNS ghrelin expression has antidepressant-like effects. Ghrelin also seems to have effects on anxiety, although these have been shown to be alternatively anxiogenic or anxiolytic. The current review discusses our current understanding of ghrelin's roles in stress, mood, and anxiety.

1. Introduction

Metabolic syndrome and psychiatric disorders have become leading threats to the public health worldwide, and associations between the two now have been reported in several studies. For instance, a growing body of literature indicates that obesity is an important environmental risk factor for developing affective disorders. As an example, in a large cross sectional epidemiological U.S. study, a body mass index ≥ 30 was found to be associated with a 25% higher rate of mood disorders [1, 2]. Conversely, other studies suggest that psychological stress can increase the risk of developing obesity. For instance, a longitudinal study found that major depression in late adolescent girls was associated with a 2.3-fold increased risk of obesity in adulthood [3]. Also, a chart review of U.S. veterans with posttraumatic stress disorder showed a significantly increased rate of obesity [4]. Thus, it seems likely that certain circulating hormones and critical neuroanatomical circuits exist that regulate both energy homeostasis and our psychological state. Work from a handful of laboratories now suggests that the peptide hormone ghrelin is one such mediator of both behaviors linked to food intake and body weight and behaviors associated with psychosocial stress, mood, and anxiety.

2. Changes in Ghrelin Associated with Psychosocial Stress

We and others have found that rises in ghrelin occur not only in response to states of energy insufficiency [5–8] but also following stress [9] (Figure 1). For example, elevations in either gastric ghrelin mRNA or total plasma ghrelin have been observed in response to various models of acute stress, including following a tail pinch stress protocol in ddy mice and following a water avoidance stress protocol in Wistar Kyoto and Sprague-Dawley rat [10, 11]. Also, rises in desacyl and acylated ghrelin plasma levels, preproghrelin mRNA levels, and numbers of ghrelin cells were shown in Wistar rats following 5 days continuous exposure to 2 cm of water [12]. Our own study found that acylated ghrelin levels rise in C57BL6/J mice in response to chronic social defeat stress (CSDS); in particular, ghrelin was significantly elevated on the day following the 10-day CSDS protocol and remained elevated when assessed again one month later [9]. In addition, human subjects subjected acutely to psychosocial stress also display increased plasma ghrelin [13]. Supportively, epinephrine, which increases with stress, can increase circulating ghrelin levels [14].

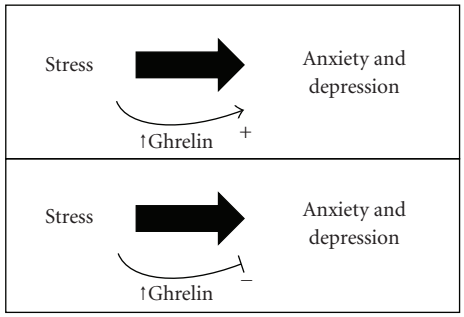

Figure 1: Two opposing models for ghrelin action in the behavioral responses to stress. Published studies all seem to agree that ghrelin levels rise upon stress of various types. Some studies suggest that rising ghrelin would contribute to the mechanisms responsible for the development of stress-induced depression and anxiety (upper panel), while others suggest that rising ghrelin helps minimize what otherwise would be more severe manifestations of depression and anxiety following stress (lower panel).

3. Ghrelin's Role in Mood

The effects of these stress-induced increases in ghrelin likely include effects on metabolism-related physiology and behavior as well as effects on mood. Our own work using mouse models has revealed that increasing circulating ghrelin levels by 10 days of calorie restriction or by acute s.c. injection produces antidepressant-like responses in the forced swim test [9]. However, caloric restriction no longer induced these responses in mice lacking ghrelin receptors (GHSR-null mice), thus suggesting that interference with ghrelin signaling negates the antidepressant-like behaviors associated with calorie restriction [9]. Also, upon challenge with the CSDS protocol, GHSR-null mice manifested greater social isolation (another marker of depressive-like behavior) than did wild-type littermates [9]. Thus, it has been suggested that activation of ghrelin signaling pathways in response to chronic stress may be a homeostatic adaptation that helps individuals cope with stress (Figure 1, lower panel).

We are aware of only one other study that examines the effects on mood of manipulations to ghrelin expression [15]. For this latter trial, behaviors were examined in rats subsequent to i.c.v. administration of antisense ghrelin oligonucleotides. Rats receiving the antisense ghrelin DNA exhibited much less immobility in the forced swim test as compared to rats receiving scrambled oligonucleotides, thus suggesting an antidepressant-like effect [15]. Associative studies that examine a ghrelin-mood relationship also exist, including one in which a GHSR polymorphism was found associated with major depressive disorder [16] and another in which total plasma ghrelin levels were compared among subjects with major depression, schizophrenia, and controls [17].

It is also important to note that ghrelin now has been shown in a handful of studies to affect reward behavior of various types. For example, ghrelin lowers the threshold dose of cocaine required to establish a conditioned place preference, is required for alcohol reward, and itself can elicit a conditioned place preference [18–20]. Ghrelin also increases neuronal activity in brain reward centers in humans shown images of appealing foods [21] and has been shown to enhance the rewarding value of high-fat diet when administered to *ad lib*-fed mice [22]. These findings are relevant to the discussion of mood as anhedonia is a major component of most forms of depression and as reward behaviors and mood-related behaviors share many of the same neural circuits (see below) [23].

4. Ghrelin's Role in Anxiety

Several groups have investigated ghrelin's effects on anxiety-like behaviors. Using models identical to those described above, we showed that increasing circulating ghrelin levels by calorie restricting mice for ten days or by acute s.c. administration of ghrelin to *ad lib*-fed C57BL6/J mice produces anxiolytic-like responses in the elevated plus maze [9]. However, when GHSR-null mice were calorie restricted, no longer were these anxiolytic-like behavioral responses observed [9]. Thus, we proposed that ghrelin has anxiolytic-like effects and that ghrelin signaling is required for the anxiolytic-like effects of caloric restriction [9]. Our observations seem to be supported by a report showing that Wistar Kyoto rats, which are thought to display more anxiety-like behaviors than Sprague-Dawley and other rat strains, have lower plasma levels of ghrelin than Sprague-Dawley rats [24]. Furthermore, although stress-induced elevations in circulating ghrelin have been noted in both Wistar Kyoto and Sprague-Dawley rat strains, the magnitude of those elevations was significantly lower in the anxiety-prone Wistar Kyoto animals than in the Sprague-Dawley animals [11].

These findings of anxiolytic-like effects of raised ghrelin levels differ from the results of several other studies. In one of these studies, i.c.v. or i.p. administration of ghrelin to ddy mice decreased duration of time in and number of entries into the open arms of an elevated plus maze (anxiogenic-like actions) when assessed ten minutes after injection [10]. Another group demonstrated that i.c.v. administration of ghrelin or its direct microinjection into the hippocampus, amygdale, or dorsal raphe nucleus induced anxiety-like behaviors in certain rat strains when assessed 5 minutes later in the elevated plus maze, open field test and step-down/inhibitory avoidance test [25, 26]. Also, i.c.v. administration of antisense ghrelin oligonucleotides induced not only antidepressant-like behaviors but also anxiolytic-like responses in rats [15]. Finally, a recent study demonstrated that i.c.v. administration of ghrelin to chicks can induce anxiogenesis [27].

The reasons for the varied anxiety-related behavioral responses to changes in ghrelin signaling are not clear, at present (Figure 1, upper versus lower panels). They could potentially be due to differences in dose, route of administration, timing of administration, timing of behavioral test after administration, strain or species, or other experimental details such as handling of animals. Strain-dependent differences in performance in various behavioral tasks, such as the elevated plus maze and forced swim test

are not uncommon (as an example, in one study, only one out of four inbred strains of mice exhibited sensitivity to fluoxetine in the forced swim test [28]). Further studies will be required to sort out how these discrepant anxiety-related animal study findings for ghrelin translate into behavioral effects in humans.

5. Potential Mechanisms by Which Ghrelin Regulates Mood

The mechanisms by which ghrelin affects mood-related behaviors have not yet been fully elucidated, but likely include interaction with its receptors in one or more brain sites critical to mood determination. We have shown that ghrelin's ability to decrease immobility in the forced swim test is dependent on the presence of orexin [9], and previous work has demonstrated that the antidepressant-like responses to calorie restriction (which also causes an increase in ghrelin) requires orexin [29]. Both direct and indirect links between ghrelin and orexin exist. The most direct link would involve binding of ghrelin to GHSRs present on orexin neurons. Such would be supported by previous studies demonstrating GHSRs within the lateral hypothalamic area of rat, where orexin-containing neuronal cell bodies exist [30], as well as those showing that ghrelin can induce action potentials in isolated orexin neurons [31]. Alternatively, ghrelin might indirectly engage the orexin system by targeting neurons at other locations which, in turn, project to the lateral hypothalamic area. For instance, several studies suggest that ghrelin directly engages its receptors on AgRP/NPY neurons of the hypothalamic arcuate nucleus (as reviewed in [32, 33]), which are also known to project to lateral hypothalamic orexin neurons [34].

Ghrelin's actions on mood also might involve direct interaction with GHSR-containing neurons that exist within the ventral tegmental area. There is growing evidence for a role of the ventral tegmental area (VTA) and its dopaminergic projections to the nucleus accumbens in mood regulation and depression [23]. These circuits, in addition to related projections from the VTA to the amygdala and limbic regions of neocortex, are particularly involved in motivation, the valuation of rewards, the establishment of reward-associated memories and the ability to experience pleasure; impairment of all of these features prominently in the manifestation of depression [35, 36]. As just one example of VTA involvement in depression, chronic social defeat stress has been shown to be associated with a significant increase in VTA dopamine neuron firing rates [37]. In fact, ghrelin also increases action potential frequency in ventral tegmental area neurons and induces dopamine release into the nucleus accumbens [38–40]. Furthermore, ventral tegmental area microinjection of ghrelin increases food intake while microinjection of a GHSR antagonist into the VTA decreases food intake in response to i.p.-injected ghrelin [38, 41].

A large body of work has identified the hippocampus as being involved in antidepressant efficacy and other aspects of depression, including that associated with stress [42]. Many studies have shown that the hippocampus together with the neocortex mediates cognitive aspects of depression such as memory impairment and feelings of worthlessness, hopelessness, guilt, and suicidality [42]. Also, antidepressant therapy stimulates hippocampal neurogenesis, in a time course that seems consistent with the delayed onset of therapeutic action of antidepressant agents [43]. Of interest, GHSRs are known to be expressed within all regions of the hippocampus [30, 44, 45]. In addition, peripherally administered ghrelin is taken up by and increases spine synapse density within the hippocampus [46]. Ghrelin-deficient mice perform poorly in tests of behavioral memory, while ghrelin administration reverses these deficits [46]. Direct microinjection of ghrelin into the hippocampus dose-dependently increases memory retention [26]. Ghrelin also recently has been shown to stimulate cellular proliferation and differentiation of adult rat hippocampal progenitor cells [47, 48], thus suggesting that ghrelin also might induce hippocampal neurogenesis.

Finally, ghrelin's action on mood may be mediated through the modulation of brain inflammation. Mounting evidence indicates that inflammation may play a role in psychiatric diseases (as reviewed in [49, 50]). For example, correlative studies have suggested the association between inflammation markers and depressive symptoms [51]. In addition, several studies have shown that administration of cytokine or cytokine inducers such as LPS or vaccine can lead to the development of depressive symptoms, while antiinflammatory therapy generates antidepressant-like effects [52–54]. GHSRs have been found to be expressed in immunocytes [55] and ghrelin or ghrelin mimetics also have been shown to have immunosuppressive actions via the inhibition of proinflammatory cytokines such as IL1-beta, IL-6, and TNF-alpha [56–59]. Together, these data suggest that the stress-induced elevations in ghrelin may help to alleviate the potential damage that could be caused by inflammation within the brain.

6. Summary

Several groups have now demonstrated that rises in ghrelin occur not only during periods of energy insufficiency but also following either acute or chronic stress. Investigations into the ramifications of these stress-associated ghrelin increases are only in their early stages. Our own work suggests that these raised ghrelin levels may help to minimize the deleterious, depression-like behaviors often associated with stress, but perhaps at the expense of a worsened metabolic profile. Future studies are needed to sort out ghrelin's effects on anxiety-like behaviors, as these have been shown by different groups to be either anxiogenic or anxiolytic. Certainly, it is crucial that these differences in the proposed action of ghrelin on anxiety-like behaviors be resolved given the impact that they might have on the side-effect profile of any GHSR antagonist in development as an antiobesity or antidiabetes agent. Also, the antidepressant-like actions and possible anxiolytic-like actions of ghrelin potentially might enhance the effectiveness of ghrelin mimetics being considered for the treatment of cachexia or anorexia nervosa.

Future studies should also be directed towards determining the mechanisms by which ghrelin acts to have its effects on mood-related and anxiety-related behaviors as well as the pathways responsible for the stress-induced elevations in ghrelin.

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Review Article

The Prokinetic Face of Ghrelin

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This review evaluated published data regarding the effects of ghrelin on GI motility using the PubMed database for English articles from 1999 to September 2009. Our strategy was to combine all available information from previous literature, in order to provide a complete structured review on the prokinetic properties of exogenous ghrelin and its potential use for treatment of various GI dysmotility ailments. We classified the literature into two major groups, depending on whether studies were done in health or in disease. We sub-classified the studies into stomach, small intestinal and colon studies, and broke them down further into studies done in vitro, in vivo (animals) and in humans. Further more, the reviewed studies were presented in a chronological order to guide the readers across the scientific advances in the field. The review shows evidences that ghrelin and its (receptor) agonists possess a strong prokinetic potential to serve in the treatment of diabetic, neurogenic or idiopathic gastroparesis and possibly, chemotherapy-associated dyspepsia, postoperative, septic or post-burn ileus, opiate-induced bowel dysfunction and chronic idiopathic constipation. Further research is necessary to close the gap in knowledge about the effect of ghrelin on the human intestines in health and disease.

1. Introduction

Ghrelin is a 28-amino acid motilin-related peptide hormone mainly secreted by the X/A-like enteroendocrine cells of the oxyntic (parietal) mucosa of the gastric fundus. It is the endogenous ligand for the growth-hormone secretagogue receptor (GHS-R) 1a, recently discovered by two independent research groups [1, 2]. The discovery of ghrelin bore great importance in the scientific media for the following reasons: (1) ghrelin is the only endogenous (natural) ligand for the GHS-R, ever discovered; (2) ghrelin shares similar structure with motilin, the so-long called "orphan peptide," based on its unique structure; (3) ghrelin is the first known case of a peptide hormone modified by a fatty acid, the *n*-octanoic acid; a step necessary for ghrelin to exert its effects [1]; (4) ghrelin is a hunger hormone that stimulates appetite, food intake and promotes weight gain, that is, ghrelin antagonist would serve as a potential treatment for obesity. In addition, ghrelin's poly-faceted properties include prokinetic and possible anti-inflammatory abilities. Although there has been several excellent reviews published recently on the prokinetic effects on ghrelin, some were brief [3, 4], or combined with motilin [5, 6], and some were focused on the effect of ghrelin on the interdigestive motility [7], gastrointestinal [GI] disorders [8, 9], or a certain subset of disease [10]. In the current review, we offer a complete detailed review solely on the prokinetic effects of exogenous ghrelin on the stomach and intestines in health and in disease.

2. Methods

This review evaluated published data regarding the prokinetic properties of exogenous ghrelin using the PubMed database for English articles from 1999 to September 2009. Our strategy was to combine and distil all currently available studies and reviews in order to provide an overview on exogenous ghrelin and its prokinetic abilities along the gut in health and disease. The search was performed by combining the terms "ghrelin" with "prokinetic" or with "gastrointestinal motility." Clinical trials and review articles were specifically identified, and their reference citation lists were searched for additional publications not identified in the database searches. We classified the literature into two major groups, depending on whether studies were done in health or in disease. We sub-classified the studies into

stomach, small intestinal and colon, and broke them down further into in vitro, in vivo (animals) and in humans. The reviewed studies were presented in a chronological order to guide the readers across the scientific advances in the field. We have combined all the available information from previous literature, in order to provide a complete review on the prokinetic properties of exogenous ghrelin and its potential applications for the treatment of various gastrointestinal (GI) dysmotility ailments.

3. Effects of Ghrelin on Gastric Motility in Health

3.1. In Vitro Gastric Tissues in Health. In vitro studies demonstrated the prokinetic potential of ghrelin or its (receptor) agonists in enhancing gastric muscle contractility via activation of the growth hormone secretagogue receptor (GHS-R) and direct neural stimulation of the enteric nervous system (ENSs); an effect involving the cholinergic and tachykininergic pathways. Earliest studies were done in 2003 by Dass et al. on rodent gastric fundic circular muscle strips. Ghrelin concentration dependently increased the amplitude of cholinergic off-contractions at concentrations from 0.1 to 10 μM. Ghrelin did not affect the neurogenic electrical field stimulation (EFSs) induced contractions [11]. They confirmed their results in 2005 [12]. Only a year later, were they able to prove that ghrelin increased the EFS-induced contractions using a concentration of 1 μM [13]. Fukuda et al. confirmed this effect on the antrum and body longitudinal muscle strips using the same concentration of 1 μM [14]. Depoortere et al. showed that ghrelin and ghrelin receptor synthetic peptide agonist, growth hormone receptor peptide 6 (GHRP-6), enhanced off-contractions on both the fundus and the antrum [15]. They went further to report such effects in the presence of N_{ω}-Nitro-$_{L}$-arginine-methyl-ester-hydrochloride (L-NAME), to verify the involvement of nitric oxide (NO) in the EFS-induced relaxation on-response. Ghrelin or GHRP-6 also enhanced L-NAME-induced contractions; an effect that was reproducible in mice [15, 16]. The same research group confirmed the involvement of GHS-R in ghrelin's excitatory effect on gastric muscle tissue either by direct identification of the GHS-R 1a transcripts in the muscle strips, or by testing the effect of treatment with the synthetic nopeptide GHS-R agonist (capromorelin) [15, 16]. In the same year, Levin et al. [17], reported that ghrelin's excitatory effect on the gastric fundus was blocked by the pretreatment with atropine, suggesting the involvement of the cholinergic pathway. Such conclusion was confirmed a year later by Bassil et al. who showed the involvement of the tachykininergic pathway, as well [13]. Recently, ghrelin's excitatory effects have also been reported in the upper gut of birds [18, 19].

3.2. In Vivo—Animal Stomach in Health. In vivo studies showed the role of ghrelin in the regulation of the migrating motor complex (MMC) in the fasting state and the involvement of the activation of GHS-R and neuropeptide Y (NPY) and possibly, vagal cholinergic neurons. In 2003, Fujino et al. reported that IV ghrelin induced MMC in the antrum of vagotomized fed rats via activation of the GHS-R and NPY neurons [20]. In fact, ghrelin appears to be the endogenous signal for the MMC in rodents [21]. In 2008, Taniguchi et al. showed that ghrelin infusion increased the motility index (MI) of antral phase III-like contractions, dose dependently, in conscious freely moving rats [7]. A year later, they confirmed the same finding of increase MI in conscious freely moving mice [22]. Zheng et al. reported that ghrelin-induced phase III-like contractions in the antrum of freely moving mice are mediated via the vagal cholinergic pathway [23]. In dogs, however, ghrelin was reported to have no effect on MMCs, suggesting a possible species-related variation [24]. Such variation may be related to the fact that rodents do not express the motilin receptor, whereas in dog, expressing ghrelin and motilin receptors, it is possible that the endogenous signal for the MMC to be modulated by motilin, with no response to ghrelin.

In vivo studies revealed opposite effects of ghrelin on the proximal and distal gastric tone in anaesthetized rats. Kobashi et al. reported that ICV ghrelin, or direct injection of ghrelin into the dorsal vagal complex (DVC), relaxed the proximal stomach, while ICV ghrelin at a higher dose contracted the distal stomach [25]. These results suggested the presence of GHS-R in the DVC, as well as the involvement of vagal preganglionic neurons in the action of ghrelin on gastric tone.

In vivo studies debated the effect of ghrelin on gastric myoelectrical activity (GMA). We have previously reported no effects of ghrelin on GMA in healthy dogs [26] and have confirmed it in rats (Sallam—unpublished). However, Tümer et al. reported an enhancement of GMA following ghrelin treatment using the same dose we used in rats [27]. We tend to disagree that the reported enhancement was substantial considering that it was within 5%–10%; moreover, the rats had an almost 90% of normal slow waves before the ghrelin treatment.

A number of in vivo studies preceded the in vitro studies and demonstrated prokinetic effects of ghrelin on gastric motility exerted via a vagally mediated mechanism. Masuda et al. were the first to report that IV ghrelin enhanced gastric contractions dose-dependently in anesthetized rats (from 0.8, 4, and 20 μg/kg) and the effect was blocked by the pretreatment with atropine or vagotomy [28]. Later, Trudel et al. however, could not reproduce the results in anaesthetized rats; instead they performed the study in conscious rats and reported that IV ghrelin dose-dependently (5 and 20 μg/kg) accelerated gastric emptying [29]. A similar effect was reported with subcutaneous ghrelin (100 nmol/Kg) in conscious mice [30]. Fukuda et al. reported that IV ghrelin (20 μg/kg) accelerated gastric emptying of both nutrient and non-nutrient meals in conscious rats. Pretreatment with capsaicin blocked the effect of ghrelin on only the nutrient meal [14]. Using ghrelin infusion of 500 pmol/Kg/min and a similar gastric emptying assessment method, Levin et al. could not reproduce Fukuda's results regarding ghrelin's acceleration of the nutrient meal [17]. However, a number of other studies confirmed the ability of intravenous (IV) or intraperitoneal (IP) ghrelin or ghrelin (receptor) agonists in

Table 1: Prokinetic effects of exogenous ghrelin on gastric motility in healthy human volunteers (D-Lys). GHRP-6: GHRP receptor antagonist; GE: gastric emptying; GHRP-6: ghrelin secretagogue receptor 6 (non-synthetic ghrelin receptor agonist); GHS-R: growth hormone secretagogue receptor; IV: intravenous; MI; motility index; MMC: migrating motor complex; SPECT: single photon emission computed tomography; VAS: visual analogue scale.

Author	Subjects	Study design	Ghrelin type	Effective dose	Methods	Results
Levin F., [39]	Healthy volunteers (5M, 3F)	Randomized, double-blind, placebo-controlled, crossover	Ghrelin	10 pmol/Kg/min for 180 min after meal	Assessment of solid GE by scintigraphy	Ghrelin accelerated the rate of GE
Tack J., [35]	Healthy fasting volunteers (4F, 5M)	Cross-sectional	Ghrelin (Clinalfa, Switzerland)	40 μg IV infusion over 30 min given 20 min after the end of phase III of MMCs	Assessment of antroduodenal motility and gastric tone by manometry and barostat	Ghrelin induced premature phase III contractions and increased the tone of the proximal stomach
Cremonini F., [37]	Healthy volunteers: obese (5M, 20F) and normal weight (13F)	Randomized, parallel-group, one dose, double-blind, placebo-controlled	Ghrelin (Clinalfa, Switzerland)	0.33 μg/Kg IV given 10 minutes after IV injection of ^{99m}Tc-pertechnetate	Assessment of gastric volume and emptying by SPECT Assessment of symptoms by VAS	Ghrelin marginally decreased fasting gastric volumes, but not GE or symptoms
Bisschops R., [36]	Healthy volunteers ($n = 9$)	No information	(i) Ghrelin (ii) GHRP-6 (Clinalfa, Switzerland)	40 μg IV infusion over 30 min given 20 min after the end of phase III of MMCs	Assessment of antroduodenal pressures by manometry and gastric tone by barostat	(i) Ghrelin induced phase III contractions of gastric origin; those were of increased amplitude and duration (ii) Ghrelin increased proximal gastric tone
Ang D., [38]	Healthy volunteers (4M, 6F)	Randomized, placebo-controlled, double-blind, cross over	Ghrelin	40 μg IV infusion over 30 min given10 min before meal	Assessment of gastric accommodation by barostat	(i) Ghrelin inhibited gastric accommodation and decreased postprandial gastric volumes (ii) Ghrelin had no effects on postprandial symptoms

the acceleration of gastric emptying in rats [15, 27, 31, 32] and mice [16, 32, 33], using a variety of methods for the assessment of gastric emptying. Such results were equally reproducible in ghrelin receptor knockout mice, suggesting a minor role of ghrelin in gastric emptying regulation [34]. In dogs, ghrelin was reported to show no effect on gastric emptying in one study, but excitatory effects on gastric contractions in another [24, 26].

3.3. In Human—Stomach of Healthy Subjects. Studies on the prokinetic effects of exogenous ghrelin on the stomach of healthy human volunteers are summarized in Table 1. Researchers have been able to reproduce some of the in vitro and in vivo results in healthy humans. The effects of ghrelin on the fasting, not fed, human stomach was similar to in vivo studies. In fasted volunteers, ghrelin induced phase III-like contractions [35]; an effect that was confirmed two years later by Bisschops [36]. However, both research groups, in addition to Cremonini et al. [37], reported that ghrelin increased the tone of the proximal stomach in the fasting state in healthy volunteers. Even postprandially, ghrelin increased the tone of the proximal stomach [38]. Such result is contradictory to an in vivo study in which ghrelin decreased proximal stomach tone in rats [25]. However, it is noteworthy that this particular in vivo study was performed in anesthetized, not conscious animals.

In 2006, researchers debated the prokinetic effect of ghrelin on gastric emptying [37, 39]. It is possible that the use of different techniques for assessment of gastric emptying might have been responsible for this discrepancy.

Ghrelin was reported to inhibit gastric accommodation in healthy volunteers [38]. This raises the question of whether ghrelin would induce side effects, similar to erythromycin, the macrolide antibiotic and motilin receptor agonist that inhibits postprandial accommodation and induces postprandial symptoms. However, ghrelin has been shown not to affect meal related symptoms in healthy obese or lean subjects [37, 38]. This is conceivable, as healthy volunteers would not be expected to have pronounced postprandial symptoms.

4. Effects of Ghrelin on Gastric Motility in Disease

4.1. In Vitro Gastric Tissues in Disease. Two recent studies by Qiu et al. reported that ghrelin or GHRP-6 increased the amplitude of carbachol-induced contractions of the gastric fundus of various diabetic rodent models [40, 41]. These results were reproducible in vivo (see below).

4.2. In Vivo Gastric Motility in Animal Model of Diseases. Studies on the prokinetic effects of exogenous ghrelin on diseased stomach in vivo are summarized in Table 2. A number of studies have reported that ghrelin or GHRP-6 accelerated or normalized gastric emptying in a variety of diseased animal models; these include: diabetic, postoperative, or morphine, or septic, or burn induced ileus, and cisplatin induced dyspepsia models. (1) *Diabetic model*: Qiu et al. published 3 papers in 2008 confirming their in vitro findings in two diabetic rodent models [40, 41, 47]. They also found that the excitatory effects of ghrelin or GHRP-6 on gastric emptying in diabetic gastroparesis were mediated via the cholinergic pathway. (2) *Postoperative ileus and/or morphine-treated model*: In 2002, Trudel et al. showed that IV ghrelin (20 μg/kg) normalized gastric emptying in a postoperative ileus rat model [29]. A year later, they confirmed the same finding in dogs [42]. In 2005, the same group reported that RC-1139, a ghrelin receptor agonist, accelerated gastric emptying, dose-dependently, in postoperative ileus or in healthy morphine-treated rodent model. However, when postoperative ileus animals were also given a dose of morphine, a higher dose of RC-1139 was needed to accelerate gastric emptying [31]. In 2007, Venkova et al. tested another ghrelin receptor agonist, TZP-101 and showed its ability to accelerate gastric emptying in a postoperative ileus rodent model whether or not it was aggravated by morphine [45]. (3) *Septic model*: in 2004, De Winter et al. reported that IP ghrelin or GHRP-6 accelerated gastric emptying in a lipopolysaccharide (LPS)-induced septic ileus rat model [33]. In 2009, Chen et al. confirmed that ghrelin accelerated gastric emptying in a LPS septic ileus mouse model, but at a much lower dose [46]. (4) *Burn model*: in 2007, we have reported the ability of ghrelin to normalize gastric emptying in a 60% total body surface area (TSBA) rat model; an effect mediated by the cholinergic pathway [44]. (5) *Cisplatin-induced dyspepsia model*: in 2006, Liu et al [43]. reported that IP ghrelin improved gastric emptying in a mouse model of dyspepsia.

4.3. In Human—Patients with Gastric Motility Disorders. Studies on the prokinetic effects of exogenous ghrelin in dyspeptic and/or gastroparetic patients are summarized in Table 3. From 2005 till present, most of the researchers seem to agree that ghrelin can accelerate gastric emptying in dyspeptic and/or gastroparetic patients. Unlike animal studies, the effects of ghrelin on gastric emptying in these patients were irrespective to vagal contribution [49, 50], giving hope to neuropathy gastroparetic patients. In 2009, Ejskjaer et al. reported that TZP-101, the ghrelin receptor agonist, also accelerated gastric emptying in diabetic patients with gastroparesis [51].

Though the effect of ghrelin on patients' symptoms remained debatable [36, 48, 49], TZP-101 was reported to show no effect on the postprandial symptoms of diabetic gastroparesis patients and its safety profile has been determined in a phase I trial [51, 52].

Despite the optimistic results of acute ghrelin administration on gastric motility in patients with gastroparesis, many researchers showed their concern regarding the side effects of the chronic use of ghrelin. Being the ligand of growth hormone secretagogue receptor, ghrelin has been shown to induce growth hormone secretion [53] and insulin resistance [54]; such effects would cause serious unfavorable side effects in particular subsets of patients. Modification of ghrelin (receptor) agonists might be necessary to avoid such problems. Long term studies will show whether the ghrelin receptor would be desensitized by chronic activation, similar to the motilin receptor. Treatment alteration between ghrelin and motilin receptor agonists has been suggested [3]. The new ghrelin receptor agonist, TZP-101, is promising as it did not induce growth hormone secretion following either peripheral or central administration [53]. TZP-101 has also been claimed to have a lower tendency to provoke ghrelin receptor desensitization [5, 52, 55].

5. Effects of Ghrelin on Intestinal Motility in Health

5.1. In Vitro—Intestinal Tissues in Health. In the small intestine, studies reported a prokinetic effect of ghrelin on jejunal contractility in rodents, involving direct activation of the GHS-R on the myenteric neurons and the cholinergic pathway. In 2004, Fukuda et al. reported that ghrelin enhanced EFS-induced contractions in longitudinal jejunal muscles [14] and Edholm et al. reported that ghrelin enhanced acetylcholine-induced contractions in circular jejunal muscles; an effect mediated via the cholinergic pathway [56]. In 2008, Bisschops proved that not only ghrelin, but GHRP-6, dose-dependently activated the myenteric neurons by eliciting a Ca^{2+} transient; this depended on direct activation of the GHS-R [36].

In the colonic tissues, studies have shown that the prokinetic effects of ghrelin were species-specific. While it induced colonic contractions in fish or birds [19, 57], ghrelin had no effect on the colon of rodents or humans [11, 12].

5.2. In Vivo—Intestinal Motility in Healthy Animals. In vivo studies showed that ghrelin induced intestinal MMCs in fed rats involving the activation of the cholinergic pathway, and the NO, NPY or 5-hydroxytryptamine 4 (5-HT_4) receptors. In 2003, Fujino et al. reported that ghrelin induced MMCs in fed and/or vagotomized rats; this effect was blocked by immunoneutralization of the NPY receptor [20]. In 2004, Edholm reported that ghrelin dose-dependently shortened the intestinal MMC cycles. Pretreatment with atropine blocked the ghrelin effect [56]. In 2007, Wang et al. reproduced the exact same results in rats and showed that

Table 2: Prokinetic effects of exogenous ghrelin on diseased stomach in vivo. GE: gastric emptying; GHRP-6: ghrelin secretagogue receptor 6 (non-synthetic ghrelin receptor agonist); h: hour; iNOS: inducible nitric oxide synthase; IP: intraperitoneal; IV: intravenous; L-NAME: N_{ω}-Nitro-$_{L}$-arginine-methyl-ester-hydrochloride; NO: nitric oxide; Postop: postoperative; SD: Sprague Dawley.

Author	Species	Ghrelin type	Effective dose	Methods	Results	Mechanism of action
Trudel L., [29]	Conscious postop ileus model (SD male rats)	Human ghrelin-28 (IGBMC, France)	20 μg/kg IV given immediately after meal	Assessment of GE by gastric retention of a phenol red-marked meal	Ghrelin reversed the postop delayed GE	
Trudel L., [42]	Conscious postop ileus model (female mongrel dogs)	Ghrelin (IGBMC, France)	(i) 100 μg/kg IV on day 2 (ii) 4 μg/kg IV on day 3 (iii) 20 μg/kg IV on day 4given after meal	Assessment of GE by acetaminophen method	Ghrelin reversed the postop delayed GE	
De Winter B., [33]	Conscious LPS septic ileus model (Swiss OFI mice)	(i) Rat ghrelin (Tocris, UK) (ii) GHRP-6 (Bachem, UK)	(i) Ghrelin: 100 μg/kg (ii) GHRP-6 : 20 and 100 μg/kg given IP 1h prior to meal	Assessment of GE by the gastric retention of an Evans blue-marked meal	Ghrelin and GHRP-6 accelerated GE in LPS septic ileus mice	
Poitras P., [31]	Conscious postop ileus ± morphine-treated rat model (male SD rats)	Ghrelin receptor agonist RC-1139 (Rejuvenon Corp., USA)	2.5–10 mg /Kg IV given immediately after meal	Assessment of GE by the retention of ^{99m}Tc-labelled meal	(i) RC-1139 accelerated GE dose-dependently in postop ileus rats (ii) RC-1139 at 10 mg/Kg accelerated GE in postop ileus + morphine-treated rats	
Liu Y., [43]	Conscious Cisplatin-treated adult male C57/6J black mice	Rat ghrelin (Bachem Ltd, UK)	1 mg/Kg, IP b.i.d	Assessment of gastric emptying by the wet weight of gastric content	Ghrelin improved GE	
Sallam H., [44]	Conscious scald-burned model (SD male rats)	Ghrelin (Tocris, USA)	2 nmol/rat given IP 20 min before meal	Assessment of GE by gastric retention of a phenol red-marked meal	Ghrelin accelerated GE; an effect blocked by pretreatment with atropine	Ghrelin's effects on gastric motility involve the cholinergic pathway
Venkova K., [45]	Conscious postop ileus ± morphine-treated rat model (male SD rats)	Ghrelin receptor agonist TZP-101 (Tranzyme Pharma Canada)	0.1–1 mg /Kg (1ml) IV given 1-2 min before meal	Assessment of GE by the retention of ^{99m}Tc-labelled meal	TZP-101 accelerated GE dose-dependently in postop ileus rats ± morphine	
Qui et al. [40]	Diabetic mouse model (IP-alloxan-treated C57 mice)	Rat ghrelin GHRP-6(Tocris, UK)	50–200 μg/Kg given IP prior to meal	Assessment of GE by gastric retention of a phenol red-marked meal	Ghrelin and GHRP-6 at all doses accelerated GE; an effect blocked by atropine or L-NAME.	The effects of Ghrelin and GHRP-6 on GE in diabetic gastroparesis is mediated via the cholinergic pathways
Qui et al. [41]	Diabetic guinea pig model (IP-STZ-treated)	Ghrelin GHRP-6	20, 50 and 100 μg/Kg given IP prior to meal	Assessment of GE by gastric retention of a phenol red-marked meal	Ghrelin and GHRP-6 at all doses accelerated GE; an effect blocked by atropine.	The effects of Ghrelin and GHRP-6 on GE in diabetic gastroparesis are mediated via the cholinergic pathways

Table 2: Continued.

Author	Species	Ghrelin type	Effective dose	Methods	Results	Mechanism of action
Chen Y., [46]	LPS endotoxemia mouse model (male ICR mice)	Rat ghrelin (Global Peptide Services, UDA)	20 μg/Kg IP given 15 min before meal	(i) Assessment of GE by gastric retention of a phenol red-marked meal (ii) Assessment of plasma NO production by fluorometry (iii) Assessment of iNos expression by immunohisto-chemistry	(i) Ghrelin had no effect on GE (ii) Ghrelin normalized endotoxemia-induced delayed GE (iii) Ghrelin 20 μg/Kg reduced plasma NO and iNOS expression in the submucosa and musculosa of the stomach	Ghrelin's effect on LPS-delayed GE are mediated via the down regulation of NO
Zheng Q., [47]	Diabetic mouse model (IP-alloxan-treated C57 mice)	GHRP-6 (Tocris, UK)	200 μg/kg given IP prior to meal	Assessment of GE by gastric retention of a phenol red-marked meal	GHRP-6 accelerated diabetic-induced delayed GE; an effect blocked by pretreatment with atropine	GHRP-6 effects on gastric motility involve the cholinergic pathway

the NO and 5-HT pathways might be involved in the action of ghrelin on intestinal interdigestive motility [58]. One year later, Taniguchi et al. pinpointed the involvement of the 5-HT_4 receptor in the ghrelin's action on intestinal MMCs [7]. Recently, using a manometric method for simultaneous assessment of gastro-duodenal motility, Tanaka et al. showed that ghrelin induced MMCs in fed conscious freely moving mice [22]. In dogs, however, ghrelin was shown to have no effect on intestinal MMCs [24].

Using a variety of techniques for the assessment of intestinal transit, researchers proved that ghrelin or its receptor agonist accelerated intestinal transit in rodents. In 2002, Trudel et al. showed that IV ghrelin 20 μg/kg accelerated intestinal transit in rats [29]. This prokinetic effect of ghrelin on intestinal transit has been confirmed by several researchers using IV or IP ghrelin using the same, or different dose in rats [14, 15] or mice [32, 46]. Ghrelin receptor agonists GHRP-6 or EX-1314 has also been shown to have similar prokinetic results. EX-1314 did not accelerate the intestinal transit in ghrelin receptor knockout mice, confirming the necessity of GHS-R activation for the prokinetic action of ghrelin, in accordance to what has been shown in the in vitro studies [15, 32, 36].

As for the colon, studies showed that to induce colon propulsion in conscious, not anesthetized, rats, central administration of ghrelin was needed. Although, ghrelin may exert an indirect effect on the colon merely by triggering upper GI MMCs, such possibility was not evidenced in the literature. The lack of direct effect of ghrelin on the colon may possibly be due to the lack of ghrelin immunoreactive cells and/or ghrelin receptors in the colon [1, 59, 60]. In 2002, Trudel et al. reported no effect of IV ghrelin on the colon of conscious rats [29]. In 2005, Tebbe et al. showed that ghrelin, injected in the paraventricular nucleus (PVN), stimulated colonic motility, dose dependently, in freely moving conscious rats. This effect involved central activation of the PVN via the corticotrophin releasing factor 1 (CRF_1) and the NPY_1 receptors [61, 62]. In 2006, Shimizu et al. reported that intrathecal ghrelin increased the colonic propulsive function in anesthetized rats. Shimizu et al. reported similar effects with intrathecal, or IV, or IV infusion of CP464709, a synthetic ghrelin receptor agonist which exerted defecation in conscious rats that was dependent on intact pelvic nerves [63]. In 2009, Charoenthongtrakul et al. reported that oral EX-1314 increased fecal output in conscious mice [32]. Shafton et al. tested a centrally acting ghrelin receptor agonist, GSK894281 that was administrated orally in conscious rats. They reported a dose-dependent increase in the fecal output both acutely and after 8 days of treatment [64].

5.3. In Human—Intestines of Healthy Subjects. Tack et al. reported premature intestinal phase III contractions following IV ghrelin in healthy volunteers. These contractions were of gastric origin [35]. Although ghrelin, like motilin, triggered intestinal MMCs in humans, but unlike motilin, plasma ghrelin has not been reported to fluctuate with phase III, suggesting that motilin remains the main hormone dominating interdigestive motility in man.

No studies were found on the effects of ghrelin on colon motility in healthy subjects.

6. Effects of Ghrelin on Intestinal Motility in Disease

6.1. In Vitro—Intestinal Tissues in Disease. No studies were found on the effects of ghrelin on small intestinal tissues obtained from diseased animal models.

However, ghrelin effects on colitis rodent model were reported. Recently, De Smet et al. showed that ghrelin decreased the colonic inhibitory responses in healthy mice and aggravated colitis in a dextran sodium sulfate (DDS)-induced colitis mice model [65]. This is contradictory to several studies in which ghrelin exerted an anti-inflammatory

Table 3: Prokinetic effects of exogenous ghrelin in dyspeptic and/or gastroparetic patients. IDDM: insulin-dependent diabetes mellitus; GE: gastric emptying; GHRP-6: ghrelin secretagogue receptor 6 (non-synthetic ghrelin receptor agonist); GHS-R: growth hormone secretagogue receptor; h: hour; IV: intravenous; VAS: visual analogue scale.

Author	Subjects	Study design	Ghrelin type	Effective dose	Methods	Results
Tack J., [48]	Six dyspeptic patients (5F, 1M)	Cross-sectional	Ghrelin (Clinalfa, Switzerland)	40 μg IV over given 30 min at the start of the meal	(i) Assessment of GE rates for solids and liquids by the ^{14}C octanoic acid and ^{13}C glycin breath tests (ii) Assessment of the intensity of 6 meal-related symptoms	Ghrelin accelerated GE for both liquids and solids, as well as meal-related symptom scores
Murray C., [49]	Ten IDDM gastroparetic patients (5M, 5F)	Randomized, double blinded, cross-over	Synthetic human ghrelin (Bachem, UK)	5 pmol/Kg/min IV over 2 h	(i) Assessment of GE rate by real time ultrasonography (ii) Assessment of the symptoms by VAS	Ghrelin accelerated GE rate, but had no effect on patients' symptoms, despite impaired cardiovagal tone
Binn M., [50]	Six gastroparetic patients (all F; 1 with truncal vagotomy)	Cross-sectional	Synthetic human ghrelin (Merck Biosciences, Switzerland)	20 μg/ml IV over 1 minute	Assessment of GE by C^{13}-octanoic acid breath test	Ghrelin accelerated gastroparetic-induced delayed GE, even despite vagotomy
Bisschops R., [36]	Dyspeptic patients with delayed GE ($n = 6$)	No information	(i) Ghrelin (ii) GHRP-6 (Clinalfa, Switzerland)	40 μg IV infusion over 30 min given 20 min at the start of the meal	(i) Assessment of GE by ^{14}C octanoic acid and ^{13}C glycin breath test (ii) Assessment of meal-related symptoms	(i) Ghrelin accelerated GE of liquids significantly and of solids marginally (ii) Ghrelin decreased the cumulative meal-related symptom scores
Ejskjaer N., [51]	Diabetic patients with gastroparesis (5M, 5F)	Randomized, double-blind, placebo-controlled, single dose, cross-over	TZP-101 (Tranzyme Pharma)	80, 160, 320 and 600 μg/Kg IV over 30 min after meal	Assessment of GE by scintigraphy	TZP-101 accelerated GE of both liquid and solid components of the meal; no significant effect on symptoms

effect in animal models of colitis [66, 67]. Further investigation is needed to explore the role of ghrelin in colitis-induced dysmotility.

6.2. In Vivo—Intestinal Motility in Animal Model of Diseases. Studies on the prokinetic effects of exogenous ghrelin on diseases intestines in vivo are summarized in Table 4. Similar to its effects on gastric emptying, ghrelin or its receptor agonists have been reported to accelerate or normalize intestinal transit in a variety of diseased animal models; these include diabetic, postoperative, or morphine, or septic, or burn induced ileus, and opiate-induced bowel disorder models. (1) *Diabetic model*: Zheng et al. showed that ghrelin or GHRP-6 increased intestinal transit; this effect was mediated via the cholinergic pathway [47]. (2) *Postoperative ileus and/or morphine-treated model*: Venkova et al. tested another ghrelin receptor agonist, TZP-101, that was also shown to accelerate gastric emptying in postoperative ileus rodent model whether or not it was aggravated by morphine [45]. (3) *Septic model*: controversial results have been reported with IP ghrelin in LPS-induced septic ileus rodent models. While De Winter et al. showed that ghrelin or GHRP-6 (100 μg/kg) had no effect in septic rats [33], Chen et al. showed that ghrelin (20 μg/kg) normalized the intestinal transit in septic mice [46]. (4) *Burn model*: in 2007, we have reported that ghrelin normalized the intestinal transit in a 60% TSBA rat model; this effect was mediated via the cholinergic pathway [44]. (5) *Opiate-induced bowel disorder model*: recently, Charoenthongtrakul et al. reported that EX-1314 normalized opiate-induced delayed intestinal transit in mice [32].

As for the colon, studies showed that IV administration of ghrelin agonists (TZP-101, ipamorelin, or GHRP-6) accelerated the colon transit in a postoperative ileus rat model [68, 69], while IP ghrelin had no effect on colon motility in a scald burn rat model [44].

Table 4: Prokinetic effects of exogenous ghrelin on intestinal motility in vivo in disease. CT: colon transit; GHRP-6: ghrelin secretagogue receptor 6 (non-synthetic ghrelin receptor agonist); h: hour; ICV: intracerebrovascular; iNOS: inducible nitric oxide synthase; IP: intraperitoneal; IT: intestinal transit; IV: intravenous; LPS: lipopolysaccharide; NO: nitric oxide; NPY: neuropeptide Y; Postop: postoperative; SC: subcutaneous; SD: Sprague Dawley.

Author	Species	Ghrelin type	Effective dose	Methods	Results	Mechanism of action
De Winter B., [33]	Conscious healthy and LPS septic ileus model (Swiss OFI mice)	(i) Rat ghrelin (Tocris, UK) (ii) GHRP-6 (Bachem, UK)	(i) Ghrelin: 100 μg/kg (ii) GHRP-6 : 20 and 100 μg/kg IP 1 h prior to meal	Assessment of IT by the transit of an Evans blue-marked meal	Ghrelin and GHRP-6, at either dose] had no prokinetic effect on IT in healthy or diseased mice	
Sallam H., [44]	Conscious scald-burned model (SD male rats)	Ghrelin (Tocris, USA)	2 nmol/rat given IP 20 min before meal	(ii) Assessment of IT and CT by the transit of a phenol red-marked meal	Ghrelin accelerated IT but had no effect on CT	Ghrelin's effects on intestinal motility are mediated via the cholinergic pathway
Venkova K., [45]	Conscious postop ileus ± morphine-treated rat model (male SD rats)	Ghrelin receptor agonist TZP-101 (Tranzyme Pharma Canada)	0.3–1 mg /Kg (1ml) IV given 1-2 min before meal	Assessment of IT by the transit of ^{99m}Tc-labelled meal	TZP-101 accelerated IT dose-dependently in postop ileus rats ± morphine	
Zheng Q., [47]	Conscious diabetic mouse model (IP-alloxan-treated C57 mice)	GHRP-6 (Tocris, UK)	200 μg/kg given IP prior to meal	Assessment of IT and CT by the transit of a phenol red-marked meal	GHRP-6 accelerated IT, but not CT; an effect blocked by pretreatment with atropine	GHRP-6 effects on intestinal motility involve the cholinergic pathway
Charoenth-ongtrakul S., [32]	Conscious opiate-induced bowel disorder mice model (male lean C57BL/6 mice)	Ghrelin receptor agonist EX-1314 (Elixir Pharma-ceuticals)	300 μg/Kg given PO 5 min prior to meal	Assessment of IT by percentage of distance of charcoal travelled/ total length of small intestine	EX-1314 reversed opiate-induced delayed IT	
Chen Y., [46]	LPS endotoxemia mouse model (male ICR mice)	Rat ghrelin (Global Peptide Services, UDA)	20 μg/Kg IP given 15 min before meal	(i) Assessment of IT by the of distance charcoal travelled/ total length of small intestine (ii) Assessment of plasma NO production by fluorometry (iii) Assessment of iNos expression by immunohisto-chemistry	(i) Ghrelin normalized endotoxemia-induced delayed IT (ii) Ghrelin reduced plasma NO and iNOS expression in the submucosa and musculosa of the duodenum	Ghrelin's effect on LPS-delayed IT transit is mediated via the down regulation of NO
Fraser G., [68]	Conscious postop ileus rat model (male SD rats)	(i) Ghrelin receptor agonist TZP-101 (Tranzyme Pharma, Canada)	0.3–1 mg /Kg (t.i.d) IV given at 15 min, 2 and 4 h after surgery	Assessment of CT by monitoring the time of appearance and weight of fecal pellet output marked with trypan blue dye	TZP-101 accelerated CT dose-dependently at 12 and 24 h after surgery	

Table 4: Continued.

Author	Species	Ghrelin type	Effective dose	Methods	Results	Mechanism of action
Venkova K., [69]	Conscious postop ileus rat model (male SD rats)	(i) Ghrelin receptor agonist and selective growth hormone secretagogue ipamorelin (Albany Molecular Research, Inc., NY) (ii) GHRP-6 (Sigma-Aldrich, MO)	(i) Ipamorelin 1 mg /Kg IV one dose or 0.1 repetitive doses (ii) GHRP-6 20 μg/Kg IV bolus given after dosing of 4 doses/day at 3 h intervals for 2 days after surgery	Assessment of CT by monitoring the time of appearance and weight of fecal pellet output marked with trypan blue dye	Ipamorelin and GHRP-6 accelerated CT 48 h after surgery	

6.3. In Human—Patients with Intestinal Motility Disorders. No studies were found regarding the effects of ghrelin in patients with intestinal motility disorders.

7. Summary and Conclusion

In conscious animals, exogenous ghrelin was reported to (1) induce gastric and intestinal MMCs in fed rodents, but not in the canine; (2) exert controversial effects on gastric myoelectrical activity in rodents; (3) induce antral contractions in dogs; (4) accelerate gastric emptying in healthy, diabetic, postoperative, or morphine, or septic, or burn-induced ileus, and cisplatin-induced dyspepsia animal models; (5) accelerate intestinal transit in healthy, diabetic, postoperative, or morphine, or septic, or burn induced ileus, and opiate-induced bowel disorder rodent models; (6) accelerate colonic transit in healthy rodents, when centrally administrated.

Clinically, exogenous ghrelin was reported to (1) induce gastric and intestinal MMCs in fasted healthy subjects; (2) increase fundic tone in both fasted and fed healthy subjects; (3) exert controversial effects on gastric emptying and have no effect on postprandial symptoms in healthy subjects; (4) accelerate gastric emptying in dyspeptic and/or gastroparetic patients and have debatable effects on postprandial symptoms in these patients. Luckily, the prokinetic effects of ghrelin in gastroparesis and/or dyspepsia patients were independent of vagal involvement.

The prokinetic effects of ghrelin on GI motility involve the exclusive activation of the GHS-R 1a receptor, not the motilin receptor, the enteric nervous system (specifically the myenteric plexus), excitatory neurons involving 5-HT4 and NO, capsaicin-sensitive afferent neurons, tachykininergic motor neurons, as well as intact vagal cholinergic neurons.

Oral ghrelin use is limited due to its instant inhibition by the gastric acidic milieu; however, other routes for its administration are possible. The emergence of IV ghrelin agonists (e.g., synthetic peptide GHRP-6; synthetic non-peptide capromorelin or ipamorelin) or ghrelin receptor agonists (e.g., GSK894281, EX-1314, EX-1315, RC-1139, TZP-101) are paving the way for possible uses in patient treatment. Oral TZP-102 soon followed and has been tested in healthy volunteers [70]. Other oral agonists include TZP-102, EX-1314 and RC-1141.

In conclusion, the prokinetic face of ghrelin enables it to serve as a strong tool in the clinical practice for the treatment of various GI dysmotility ailments. The prokinetic properties of ghrelin or its (receptor) agonists have the potential to serve in the treatment of diabetic, neurogenic or idiopathic gastroparesis and possibly, chemotherapy-associated dyspepsia, postoperative, septic or post-burn ileus, opiate-induced bowel dysfunction and chronic idiopathic constipation. Further research is necessary to close the gap in knowledge about the effect of ghrelin on the human intestines in health and disease.

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Review Article

The Roles of Motilin and Ghrelin in Gastrointestinal Motility

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In structure, ghrelin resembles motilin. The two peptides are considered to be members of the motilin-ghrelin peptide family. Motilin is considered to be an endocrine regulator of the interdigestive migrating contractions, the fasted motor pattern in the gastrointestinal (GI) tract. It has been reported that ghrelin stimulates GI motility. The gastrokinetic capacity of ghrelin has been well documented in the rodent. However, there have been few positive reports of the gastrokinetic capacity of ghrelin in dogs. Some reports with human subjects have shown that an i.v. ghrelin injection accelerated gastric emptying of a meal and improved meal-related symptoms. These results suggest that ghrelin has potential as a prokinetic. However, it seems unlikely that plasma ghrelin would play a physiological role in these digestive physiological events and stimulate gastric emptying, as these outcomes would appear to be in contradiction with the suppression of the endogenous release of ghrelin after eating. The physiological roles of ghrelin need to be clarified.

1. Introduction

Ghrelin is a 28-amino-acid peptide predominantly produced by endocrine cells in the oxyntic mucosa of the stomach as an endogenous ligand for the growth hormone (GH) secretagogue receptor [1–4]. Initially, ghrelin was identified as having properties related to the release of GH [5]. Studies have shown that the infusion of ghrelin increases circulating plasma GH in rodents and humans [6–10]. However, other actions of ghrelin have emerged, such as its effects on the glucose metabolism and insulin release [11, 12], cardiovascular actions [13, 14], and food intake and control of energy balance [15, 16].

Structurally, ghrelin resembles motilin. Motilin is a 22-amino-acid peptide synthesized from endocrine cells of the duodeno-jejunal mucosa. Motilin and ghrelin precursors share almost 50% similarity in their amino-acid sequences, and the receptors of both peptides are part of the same family of G protein-coupled receptors and share 53% overall amino-acid sequence identity [17]. Based upon their structural similarity, the two peptides are now considered to be members of the new motilin-ghrelin peptide family.

Motilin regulates the interdigestive migrating contractions (IMC), the fasted motor pattern in the gastrointestinal (GI) tract [18]. Motilin plasma levels increase cyclically every 90–120 minutes during the interdigestive fasting period, and this cyclical release of motilin disappears after ingestion of a meal. These cyclical peaks of plasma motilin are synchronized to strong peristaltic contractions initiated from the stomach and migrating to the duodenum and small intestine. This pattern of migrating waves is known as the phase III contraction of IMC.

Ghrelin has also been reported to stimulate GI motility [19–22]. Ghrelin administration induces phase III-like contractions in the rat stomach [20]. Ghrelin also induces premature phase III contractions of IMC in the human stomach [21]. Vantrappen et al. [23] reported that motilin induces phase III contractions at a lower dose than ghrelin. On the other hand, high doses of motilin [24] and low doses of ghrelin [5] stimulate GH secretion. As indicated by Peeters [25], these results suggest that both peptides may cross-react with their receptors.

Endogenous ghrelin has been reported to be involved in mediating phase III-like contractions in the stomach of rats [26] and mice [27]. However, we revealed that ghrelin

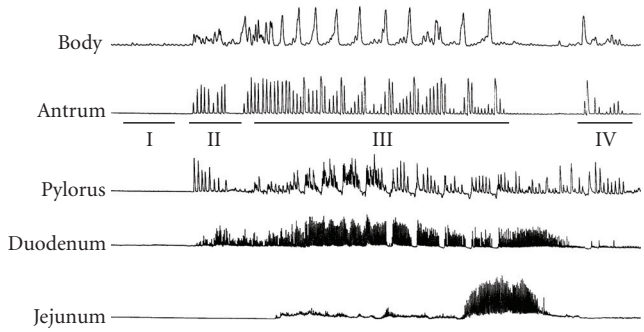

Figure 1: The typical four phases of IMC in the dog. Phase I: quiescence. Phase II: phase of irregular contractile activity. Phase III: intensive rhythmic contractions. Phase IV: rapid decline of activity before complete quiescence.

administration did not stimulate GI motility in conscious dogs [28]. Whether ghrelin activates GI motility in dogs and humans is controversial. This review focuses on the capacity of ghrelin to act on GI motility and compares the findings with those of motilin mainly in the dog.

2. Motilin and Gastrointestinal Motility

Because the motilin receptor exists as a pseudogene only in rodents [29, 30], studies on motilin regarding GI motility in animal models are scarce and have been limited to dogs. Motilin is considered to be a unique hormone playing a role in the interdigestive period, rather than, as in the case of most hormones, in the postprandial period. In the interdigestive state, GI motility is characterized by cycling IMC originating in the stomach and propagating along the small intestine. IMCs are assumed to have an important housekeeping role by forcefully pushing the content of the gut forward while cleaning the bowel of debris and bacteria that would otherwise accumulate and lead to bacterial overgrowth and compromise nutrient absorption from the small intestine, resulting in the sensation of hunger. The four phases of IMC were first described by Szurszewski [31]. Figure 1 shows the typical four phases of IMC in the dog. Phase I is quiescence. Phase II is irregular contractile activity. Phase III is characterized by intense, rhythmic contractions starting in the lower esophageal sphincter (LES) and stomach and migrating down the small bowel to the terminal ileum. In Phase IV, the activity rapidly declines until complete quiescence. Soon after motilin's discovery, it was suggested that motilin induces hunger contractions [32]. Itoh et al. [33] showed that the exogenous administration of motilin initiates premature phase III contractions in the stomach that are quite similar to the spontaneously occurring phase III contractions in dogs. This notion is supported by a study by Peeters et al. [34], who described phase III motor activities starting in the stomach or the upper duodenum that are associated with plasma motilin peaks, and another by Lee et al. [35], who reported that immunoneutralization of circulating motilin suppresses phase III contractions.

Many observations suggest the presence of motilin receptors on smooth muscle cells and on neurons of the GI tract. Itoh et al. [36] found that the effect of motilin on phase III activity in dogs was blocked by a 5-hydroxytryptamine-3 (5-HT_3) antagonist. This finding suggests that the motilin-induced signal may be mediated via 5-HT_3 receptors on the vagal afferents. The signal is then transmitted to the stomach via vagal efferents that induce the release of endogenous acetylcholine, since anticholinergic agents block the effect of motilin. On the other hand, in many in vitro studies, the induction of contractility by motilin was observed to be resistant to tetrodotoxin, which suggests that motilin receptors are present on smooth-muscle cells [37–39] and the contractile effects of motilin are mediated through a direct action on smooth muscle cells. Most evidence now points to the existence of motilin receptors on nerves as well as on muscles.

3. Ghrelin and Gastrointestinal Motility

As for the case of motilin, Tack et al. [21] demonstrated that, in humans, the administration of ghrelin induces a premature gastric phase III, which is not mediated through the release of motilin. Unlike motilin, ghrelin also induced phase III-like contractions in rats and mice [20, 26, 27]. These observations suggest that while the pharmacological effects of ghrelin were demonstrated, the involvement of ghrelin in the control of normal interdigestive motility was not.

In dogs, we revealed that an i.v. injection of synthesized canine ghrelin did not stimulate motor activity in the digestive tract (Figure 2), although it did stimulate the release of a GH [28]. Kudoh et al. [40] also reported that neither the growth hormone-releasing peptide-2 nor ghrelin evoked GI contractions in the interdigestive state. These results differ from those obtained with studies using rodents. It is reasonable to expect that the action of a peptide will change from species to species. It remains to be determined whether ghrelin could be the surrogate of motilin in rats. The role of endogenous ghrelin in the regulation of phase III-like contractions remains unclear. To date, unlike the case of motilin [34], fluctuation of plasma ghrelin levels in synchrony with phase III activity fronts has not been reported.

4. Motilin as a Prokinetic

Motilin has a therapeutic potential as a pharmacological agent in stimulating gastric motility and accelerating gastric emptying of foods. Itoh et al. [41] first showed that erythromycin, a macrolide antibiotic, interacts with the motilin receptor and mimicked the effect of motilin on GI motility during the interdigestive state in dogs. In addition, in humans, erythromycin induces phase III activity [42], and the effect is dose-related in healthy volunteers and patients with diabetic gastroparesis [43]. Erythromycin derivatives devoid of antibiotic activity but with strong affinity for motilin receptors, also called motilides [44], were clinically

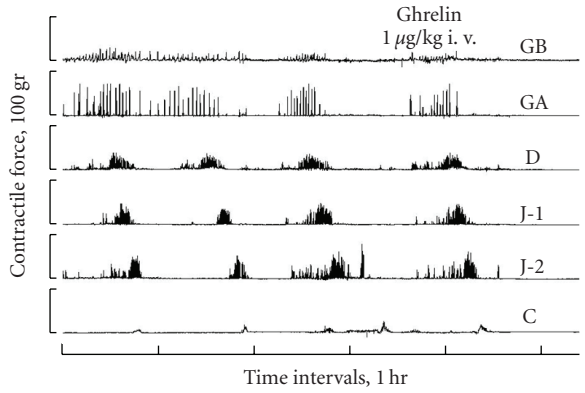

Figure 2: Examples of the effect of canine ghrelin at 1 and 10 μg kg BW^{-1} on the myoelectrical activity in a conscious dog, measured at a gastric body (GB), gastric antrum (GA), duodenum (D), jejunum (J-1 and J-2), and colon (C). Ghrelin did not alter the interval and amplitude of phase III (1 μg kg BW^{-1}) (from [28]).

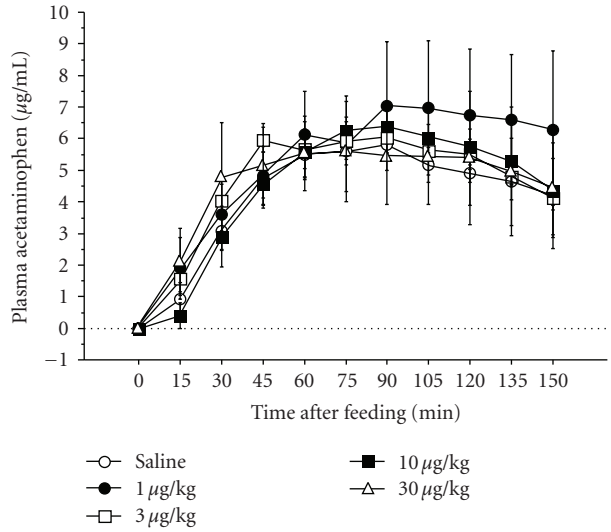

Figure 3: Effects of canine ghrelin on gastric emptying in conscious dogs. Saline and canine ghrelin were administered intravenously. Each symbol represents the mean ± S.E.M. every 15 minutes in three dogs. Differences were not significant ($P > .05$) (from [28]).

tested. Clinical studies of ABT-229, one of the motilides, demonstrated the acceleration of gastric emptying in healthy volunteers; however, ABT-229 failed to improve symptoms in patients with functional dyspepsia and diabetic gastroparesis [45, 46]. Disappointing results with ABT-229 decreased the interest in this field of research. However, it was pointed out that several factors associated with the drug (long half-life and/or tachyphylaxis [47] and possible effect on gastric accommodation [48–50]) and the study design (selection of patient population [51]) may have contributed to the negative outcome [52].

A new motilide, GM-611 or mitemcinal, led to promising new results. Takanashi et al. [53] confirmed mitemcinal as a selective and full motilin receptor agonist in in vitro pharmacological studies. Similarly to the case of motilin, intravenous administration of mitemcinal in dogs [54] stimulated interdigestive, as well as digestive, gastroduodenal motor activity, and its effect was blocked by the motilin-receptor antagonist GM-109. Colonic motility in dogs [55] could also be stimulated by mitemcinal given orally. Randomized controlled trials in 392 insulin-requiring diabetics revealed that symptoms attributable to gastroparesis could be ameliorated with 10 mg mitemcinal twice daily than with a placebo and without inducing significant adverse effects [56].

5. Ghrelin as a Prokinetic

Masuda et al. [19] suggested that ghrelin could stimulate gastric contractions in rats. Trudel et al. [57] documented that ghrelin accelerates gastric emptying and the small intestinal transit of a liquid meal and is a strong prokinetic agent capable of reversing the postoperative gastric ileus in conscious rats. Poitras et al. [58] confirmed that the ghrelin analog RC-1139 is a potent gastrokinetic in rat: it reversed gastric postoperative ileus, even in the presence of opiates.

The gastrokinetic capacity of ghrelin had been well documented in the rodent. Trudel et al. [59] demonstrated that, as found earlier in rodents, ghrelin accelerates the normal gastric emptying of a meal and was a potent prokinetic agent that improved postoperative gastric ileus in dogs. On the contrary, we [28] showed that an i.v. injection of ghrelin did not accelerate gastric emptying in dogs (Figure 3). To date, encouraging results of the gastrokinetic capacity of ghrelin in dogs are few.

In humans, Binn et al. [60] showed that an i.v. ghrelin injection accelerated gastric emptying of a meal even in the presence of deficient gastric innervation. Tack et al. [61] obtained similar results in which, in idiopathic gastroparesis, the administration of ghrelin enhanced gastric emptying and improved meal-related symptoms. These observations suggest the potential for ghrelin as a prokinetic. TZP-101, a synthetic ghrelin-receptor agonist, has been shown to be an active gastrokinetic agent in rats [62] and has already been tested in humans [63]. However, it seems difficult to believe that plasma ghrelin could play a physiological role in these digestive physiological events. Most evidence indicates that ghrelin plasma levels are high during the fasting period and decrease after meal ingestion. Most GI peptides increase after a meal. Motilin and ghrelin are the only hormones known to decrease in the postprandial period [64]. The observed biological action of the peptide, stimulation of meal gastric emptying, appears to be in contradiction with its endogenous release being suppressed after eating.

6. Conclusion

Ghrelin is of great interest, as is motilin, to the GI physiologist. The value of ghrelin as a prokinetic agent may soon be revealed. However, the physiological roles of ghrelin, especially in dogs and humans, need to be clarified.

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Review Article

Ghrelin, Des-Acyl Ghrelin, and Obestatin: Regulatory Roles on the Gastrointestinal Motility

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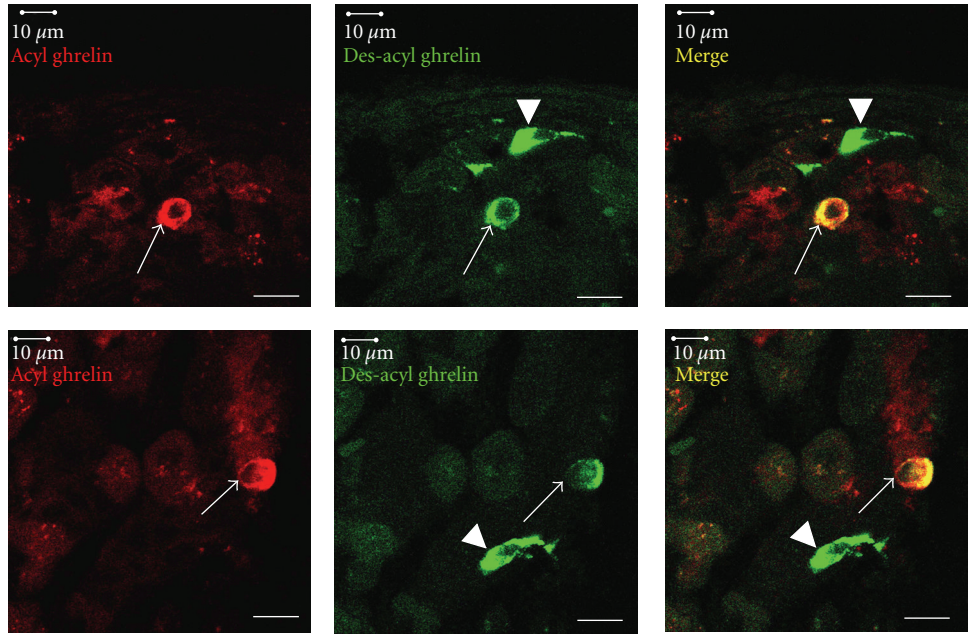
Ghrelin, des-acyl ghrelin, and obestatin are derived from a common prohormone, preproghrelin by posttranslational processing, originating from endocrine cells in the stomach. To examine the regulatory roles of these peptides, we applied the manometric measurement of gastrointestinal motility in freely moving conscious rat or mouse model. Ghrelin exerts stimulatory effects on the motility of antrum and duodenum in both fed and fasted state of animals. Des-acyl ghrelin exerts inhibitory effects on the motility of antrum but not on the motility of duodenum in the fasted state of animals. Obestatin exerts inhibitory effects on the motility of antrum and duodenum in the fed state but not in the fasted state of animals. NPY Y2 and Y4 receptors in the brain may mediate the action of ghrelin, CRF type 2 receptor in the brain may mediate the action of des-acyl ghrelin, whereas CRF type 1 and type 2 receptors in the brain may mediate the action of obestatin. Vagal afferent pathways might be involved in the action of ghrelin, but not involved in the action of des-acyl ghrelin, whereas vagal afferent pathways might be partially involved in the action of obestatin.

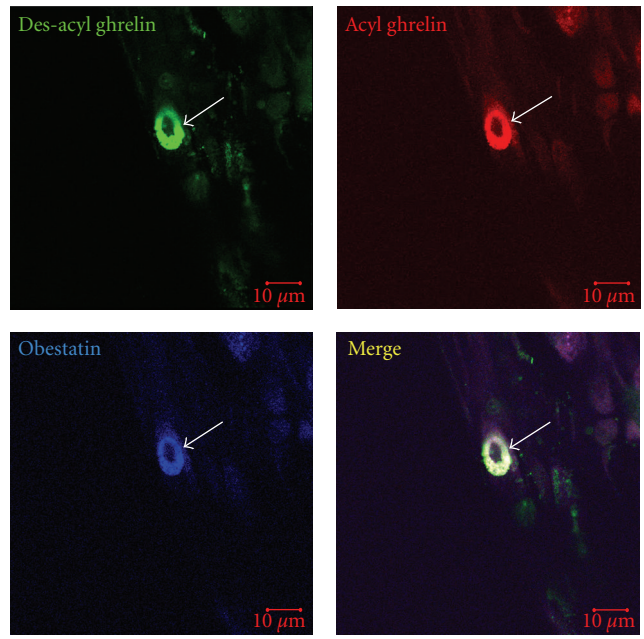
1. Introduction

Ghrelin, des-acyl ghrelin, and obestatin are derived from a prohormone, preproghrelin by posttranslational processing. Ghrelin was first identified as endogenous ligand for growth hormone secretagogue receptors (GHS-R) with O-n-octanoyl acid modification at serine 3 position [1]. Des-acyl ghrelin, on the other hand, has the same amino acid sequence with no O-n-octanoyl acid modification [1]. Obestatin was found by a bioinformatics approach to be encoded by preproghrelin [2]. Obestatin was initially reported to be endogenous ligand for orphan G protein-coupled receptor GPR39 [2]; however recent studies have found no specific binding of obestatin to various types of GPR39-expressing cells [3–5]. Ghrelin is a potent stimulator of food intake and gastrointestinal motility [6], while des-acyl ghrelin exerts opposite effects on food intake and gastrointestinal motility [7]. The effects of obestatin on food intake and gastrointestinal motility have been controversial [8–13].

Recently we developed conscious rat and mouse models to measure physiological fed and fasted motor activities in the gastrointestinal tracts [14–18]. By using these models we succeeded to examine the effects of ghrelin, des-acyl ghrelin, and obestatin on gastroduodenal motility and involvement of hypothalamic peptides mediating the action of these peptides. In this review, we overview the different effects of ghrelin, des-acyl ghrelin, and obestatin on the upper gastrointestinal motility with special attention being paid to brain-gut interactions.



(a)



(b)

Figure 1: Localization of ghrelin, des-acyl ghrelin and obestatin in the rat stomach. (a) Immunofluorescence double staining for acyl ghrelin- (red) and des-acyl ghrelin-positive (green) reaction in the antral mucosa of rat stomach. Acyl ghrelin-positive reaction and des-acyl ghrelin-positive reaction are colocalized in closed-type cells (arrows), whereas des-acyl ghrelin-positive reaction is localized in open-type cells (arrowheads). (b) Immunofluorescence triple staining for des-acyl ghrelin (green), acyl ghrelin (red) and obestatin (blue) in the antral mucosa of rat stomach. Three peptides are colocalized in the closed-type cells (arrows). Bars = 10 μm.

2. Localization of Ghrelin, Des-Acyl Ghrelin, and Obestatin in the Rat Stomach

The localization of ghrelin in the stomach has been studied in various animals by using the specific antibody for ghrelin [19, 20]; however, the localization of des-acyl ghrelin in the stomach has been scarcely examined. We developed antibodies specific for ghrelin (antirat octanoyl ghrelin (1-15) -cys-KLH serum) and for des-acyl ghrelin (antirat des-octanoyl ghrelin (1-15) -cis-KLH serum) and successfully detected the different localization of ghrelin and des-acyl ghrelin in the rat stomach [21].

Both ghrelin- and des-acyl ghrelin-immunoreactive cells were distributed in the oxyntic and antral mucosa of the rat stomach, with higher density in the antral mucosa than oxyntic mucosa. Immunofluorescence double staining showed that ghrelin- and des-acyl ghrelin-positive reactions overlapped in closed-type round cells, whereas des-acyl ghrelin-positive reaction was found in open-type cells in which ghrelin was negative (Figure 1(a)). Ghrelin/des-acyl ghrelin-positive closed-type cells contain obestatin (Figure 1(b)); on the other hand des-acyl ghrelin-positive open-type cells contain somatostatin [21].

The characteristic features of open-type cells that contain des-acyl ghrelin and closed-type cells that contain ghrelin indicate that they may respond differently to intraluminal factors. It is highly possible that open-type cells may react to luminal stimuli more than closed-type cells. Therefore we investigated the effects of different intragastric pH levels on the release of ghrelin and that of des-acyl ghrelin from the ex vivo perfused rat stomach [21]. In a preliminary study we measured the intragastric pH levels in the fasting and fed states of rats and found that intragastric pH in the fasting state was pH 4, whereas that in the fed state was pH 2 [16]. Our results showed that the release of ghrelin was not affected by intragastric pH, whereas the release of des-acyl ghrelin was increased at intragastric pH 2 compared to that at intragastric pH 4 [21]. This result suggests that des-acyl ghrelin-containing cells may sense the intragastric pH via their cytoplasmic processes and release the peptide in accordance with the lower intragastric pH. The fact that the release of des-acyl ghrelin is stimulated by lower intragastric pH seems reasonable because des-acyl ghrelin may act as a satiety signal [6, 7] in the fed state of animals.

3. Manometric Measurement of Gastrointestinal Motility in Conscious Mice and Rats

We developed freely moving conscious animal model to measure the gastrointestinal motility in rats [15] and mice [18]. This model permits the measurement of gastrointestinal motility in animals in the physiological fed and fasted states by a manometric method [15, 18]. In the fasted state, the cyclic changes of pressure waves were detected in both antrum and duodenum, including the quiescence period during which relatively low amplitude contractions occur (phase I-like contractions), followed by a grouping of strong contractions (phase III-like contractions). The frequencies of phase III-like contractions in the fasted motility in the antrum and duodenum in mice (6.0 ± 0.2/h and 6.0 ± 0.3/h, resp.) were significantly ($P < .05$) higher than those in rats (5.3 ± 0.5/h, 5.6 ± 0.8/h, resp.) [15, 18]. After food intake, such fasted motor pattern was disrupted and replaced by a fed motor pattern, which consisted of irregular contractions of high frequency.

4. Ghrelin and Gastroduodenal Motility

Intracerebroventricular (i.c.v.) and intravenous (i.v.) injection of ghrelin stimulated the % motor index (%MI) in the antrum and induced the fasted motor activity in the duodenum when given in the fed state of animals [16, 18] (Figure 2(a)). I.c.v. and i.v. injection of ghrelin increased the frequency of phase III-like contractions in both antrum and duodenum when given in the fasted state of animals [16]. The effects of i.v. injection of ghrelin on gastroduodenal motility were blocked by i.v. injection of GHS-R antagonist but not by i.c.v. injection of GHS-R antagonist [16]. Immunoneutralization of NPY in the brain blocked the stimulatory effects of ghrelin on the gastroduodenal motility [16] (Figure 2(b)). These results indicate that ghrelin released from the stomach may act on the ghrelin receptor on vagal afferent nerve terminals and NPY neurons in the brain may mediate the action of ghrelin on the gastroduodenal motility (Figures 2(c) and 2(d)). Our previous study showed that immunoneutralization of NPY in the brain completely blocked the phase III-like contractions in the duodenum of normal rats, and Y2 and Y4 receptor agonists induced the phase III-like contractions in the duodenum when given in the fed state of animals [15]. Combined together, in normal animals ghrelin may stimulate gastroduodenal motility by activating the GHS-R on vagal afferent nerve terminals and affect NPY neurons in the hypothalamus, and Y2 and/or Y4 receptors in the brain may mediate the action of ghrelin (Figure 2(d), Table 1). Once the brain mechanism is eliminated by truncal vagotomy, ghrelin might be primarily involved in the regulation of fasted molility through GHS-R on the stomach and duodenum [16].

Human ghrelin has a structural resemblance to human motilin, and human ghrelin receptors exhibit a 50% identity with human motilin receptors [22]. Therefore the role of ghrelin in the gastrointestinal motility is comparable with that of motilin [23, 24]. Motilin originates from the endocrine cells in the duodenum [23], while ghrelin originates from the endocrine cells in the stomach [20]; both of them are involved in the regulation of phase III contractions in the gastrointestinal tracts. Motilin induces fasted motility in the stomach and duodenum when it is given peripherally but not when given centrally [24, 25], while ghrelin induces fasted motility in the duodenum when it is given both peripherally and centrally [16]. Since it is known that gastric acidification modulates the action of motilin [26], we examined the relationship between the effects of ghrelin on gastroduodenal motility and intragastric pH. The results showed that within 30 minutes after feeding low intragastric pH (pH 2.5 ± 0.2) inhibited the effects i.v. injected ghrelin on gastroduodenal motility, and that this effect was reversed by an increase of intragastric pH (pH 5.4 ± 0.6) within 60 minutes after feeding, or by pretreatment of famotidine (intragastric pH 6.0–6.7) [16]. These results suggest that the sensitivity of the GHS-R in the gastrointestinal tract might be inhibited by low intragastric pH.

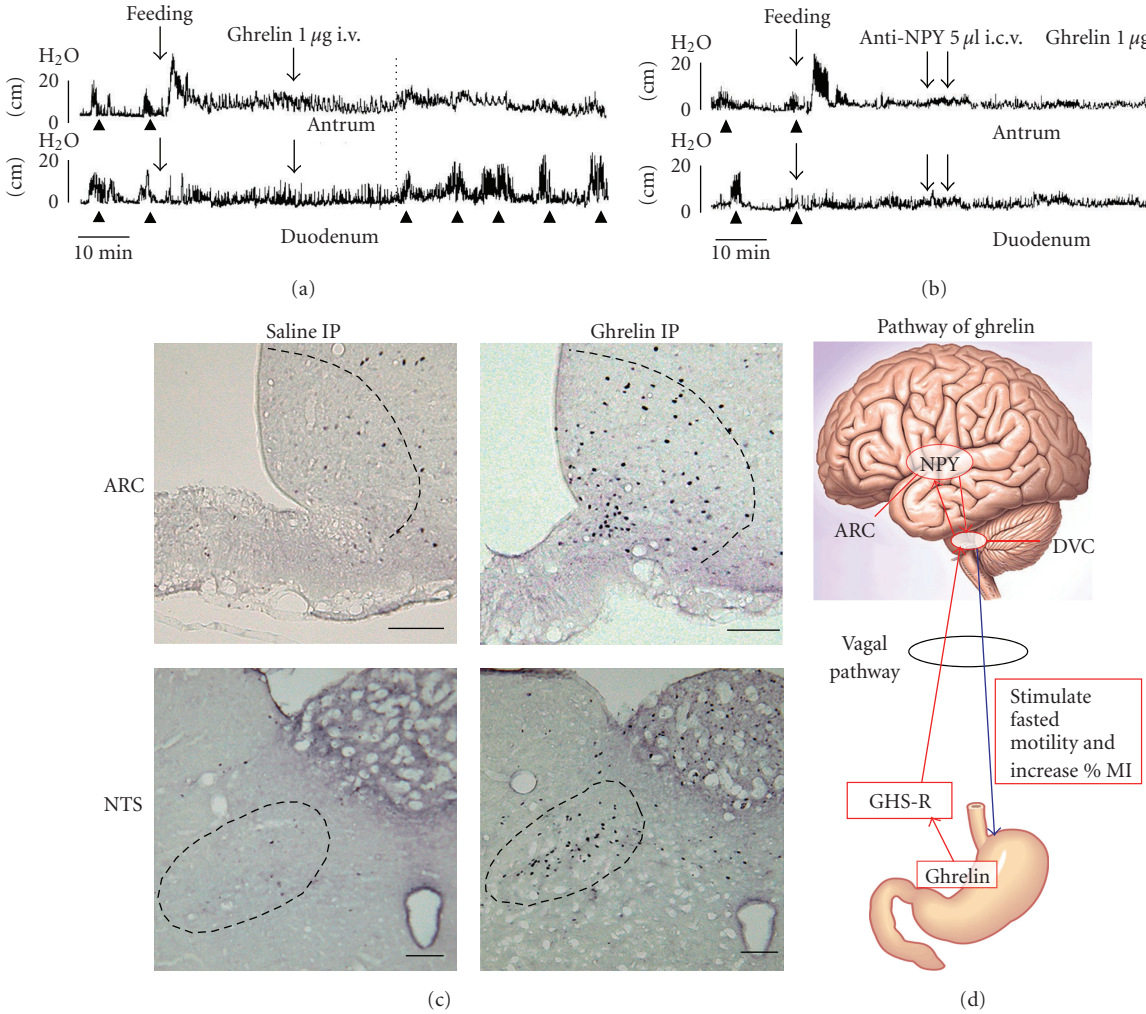


Figure 2: Effects of ghrelin on the gastroduodenal motility. (a) Effects of i.v. injection of ghrelin on the fed motor activity of the antrum and duodenum. I.v. injection of ghrelin induces the fasted pattern in the duodenum and increases the motor activity in the antrum. (b) I.c.v. injection NPY antiserum completely blocks the effect of i.v. injection of ghrelin. (c) The density of c-*Fos*-positive cells in the arcuate nucleus (ARC) and NTS is increased by i.p. injection of ghrelin compared to saline-injected control. (d) Summary diagram of the effects of ghrelin on the gastroduodenal motility and brain mechanism mediating its action.

Table 1: Summary of the regulatory roles of ghrelin, des-acyl ghrelin and obestatin on the gastroduodenal motility.

	ghrelin		des-acyl ghrelin		obestatin	
	Fasted state	Fed state	Fasted state	Fed state	Fasted state	Fed state
Stomach	↑	↑	↓	—	—	↓
Duodenum	↑	↑	—	—	—	↓
Hypothalamic neuron	NPY		urocortin 2		CRF, urocortin 2	
Brain receptor	Y2, Y4		CRF type 2		CRF type 1, type 2	
Vagal afferent pathway	+		—		+	

5. Des-Acyl Ghrelin and Gastroduodenal Motility

Central and peripheral administration of des-acyl ghrelin has been shown to significantly decrease food intake in food-deprived mice and decrease gastric emptying [6]. Transgenic mice with overexpression of the des-acyl ghrelin gene exhibited a decrease in body weight, food intake, and fat mass weight accompanied by moderately decreased linear growth compared with their nontransgenic littermates [6]. In rats, des-acyl ghrelin injected intraperitoneally (i.p.) effectively decreased food intake in food-deprived rats and decreased the dark-phase food intake in free-feeding rats but failed to decrease the light-phase food intake in free-feeding rats [7].

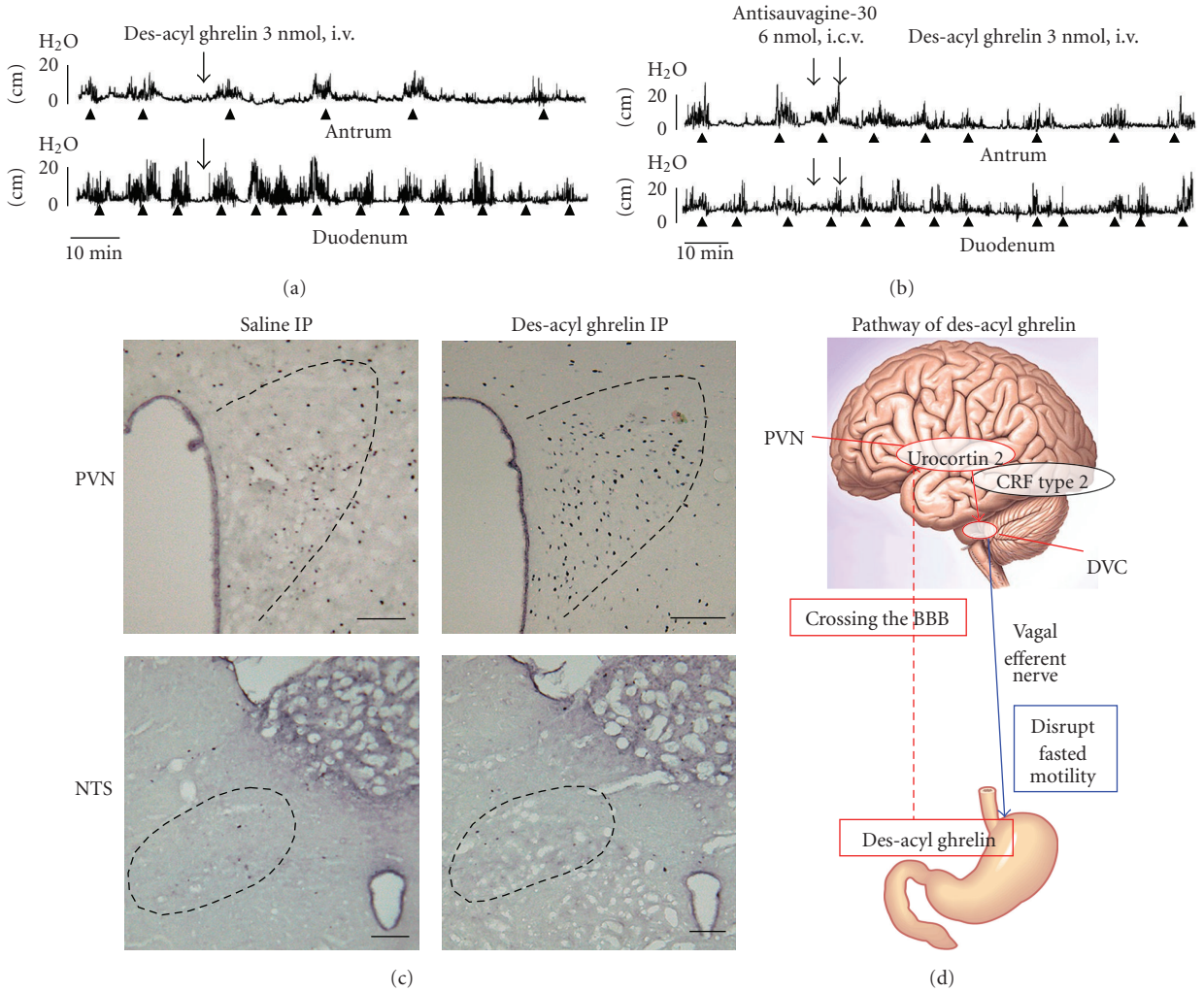


Figure 3: Effects of des-acyl ghrelin on the gastroduodenal motility. (a) Effects of i.v. injection of des-acyl ghrelin on the fasted motor activities of the antrum and duodenum. I.v. injection of des-acyl ghrelin decreases the frequency of phase III-like contractions in the antrum but not in the duodenum. (b) The decreased frequency of phase III-like contractions induced by i.v. injection of des-acyl ghrelin is restored to normal in pretreatment of i.c.v. injection of the selective CRF type 2 receptor antagonist antisauvagine-30. (c) The density of c-*Fos*-positive cells in the PVN is increased by i.p. injection of des-acyl ghrelin compared to saline-injected control, whereas that in the NTS is not altered. (d) Summary diagram of the effects of des-acyl ghrelin on the gastroduodenal motility and brain mechanism mediating its action.

I.c.v. and i.v. injections of des-acyl ghrelin disrupted fasted motility in the antrum but not in the duodenum [7] (Figure 3(a)). The frequencies of fasted motility in the antrum were decreased to 58.9% and 54.5% by des-acyl ghrelin injcted i.c.v. and i.v., respectively, [7]. However i.c.v. and i.v. injections of des-acyl ghrelin did not alter fed motor activity in both the antrum and duodenum [7]. These data indicate that the dominant role of exogenous des-acyl ghrelin affects fasted motility in the antrum but not in the duodenum. The results showed that capsaicin treatment did not alter the disruptive effect of i.v. injection of des-acyl ghrelin on fasted motility in the antrum [7]. These results were consistent with electrophysiological studies, which showed that peripheral administration of ghrelin suppressed firing of the vagal afferent pathways, whereas des-acyl ghrelin had no effect on vagal afferent pathways [27]. Difference in the involvement of vagal afferent pathways in the action of ghrelin and des-acy ghrelin was confirmed by c-*Fos* expression in the NTS. I.p. injection of ghrelin significantly increased the density of c-*Fos*-positive cells in the NTS (Figure 2(c)), while i.p. injection of des-acyl ghrelin induced no change in the density of c-*Fos*-positive cells in the NTS compared with vehicle-injected controls [7] (Figure 3(c)). Taken together, these results suggest that peripherally administered des-acyl ghrelin may cross the blood-brain barrier (BBB) and act directly on the brain receptor and disrupt the fasted motility in the antrum (Figure 3(d)).

The centrally administered CRF type 2 receptor antagonist, but not the CRF type 1 receptor antagonist, blocked the effects of centrally and peripherally administered des-acyl ghrelin on gastric motility [7] (Figure 3(b)). Between

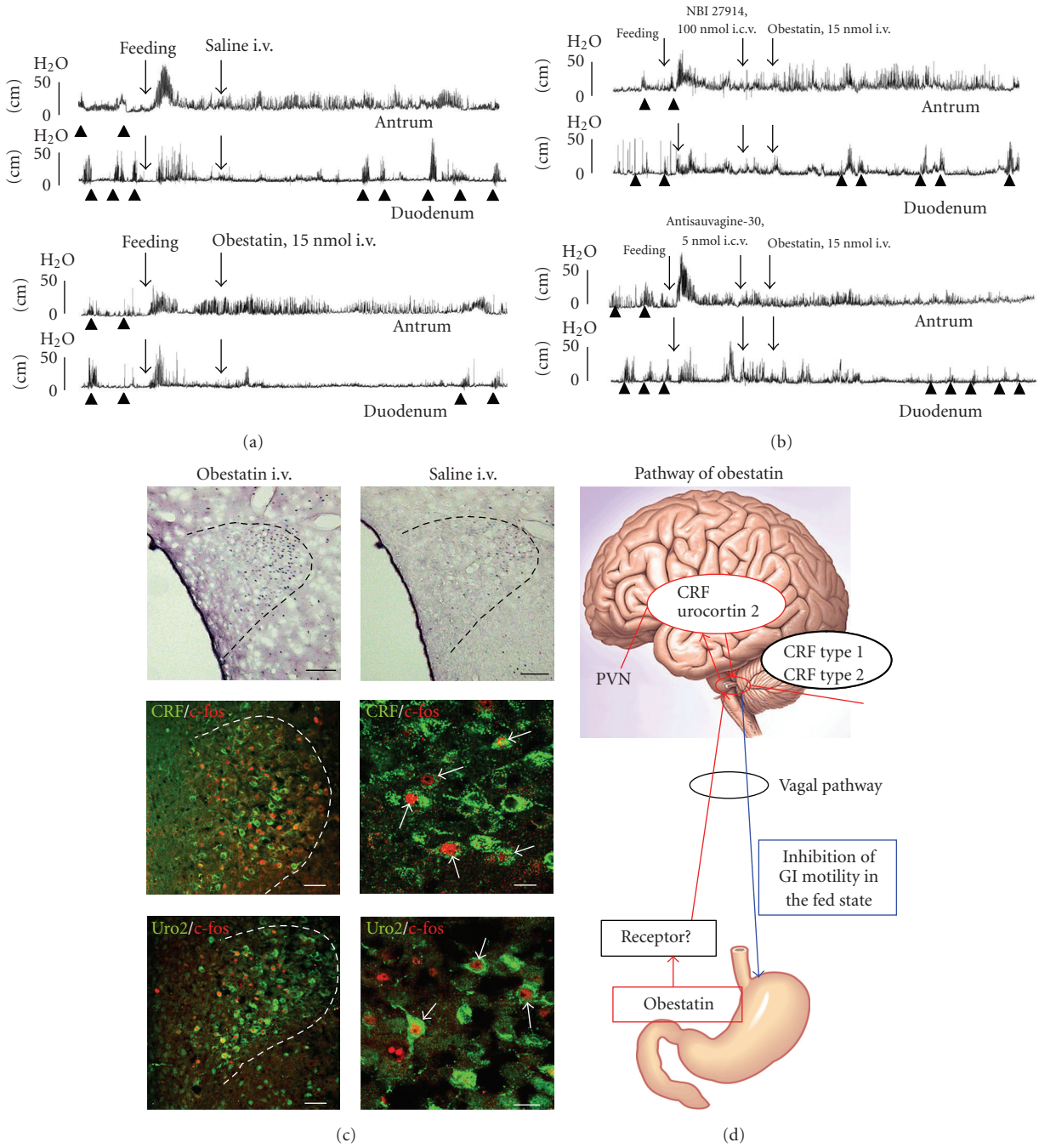


Figure 4: Effects of obestatin on the gastroduodenal motility. (a) Effects of i.v. injection of obestatin on the fed motor activity of the antrum and duodenum. I.v. injection of obestatin prolongs the time between the initiation of phase III-like contractions and injection of obestatin in the duodenum. (b) The elongation of the time between injection of obestatin and initiation of phase III-like contractions in the duodenum induced by i.v. injection of obestatin is reversed by i.c.v. injection of selective CRF type 1 receptor antagonist NBI-27914 and also by selective CRF type 2 receptor antagonist antisauvagine-30. (c) The density of c-*Fos*-positive cells in the PVN is increased by i.v. injection of obestatin compared to saline-injected control. CRF-positive or urocortin 2-positive neurons are overlapped with c-*Fos*-positive neurons in the PVN. (d) Summary diagram of the effects of obestatin on the gastroduodenal motility and brain mechanism mediating its action.

two CRF receptor subtypes, CRF type 1 receptor is highly involved in anxiety-related behavior and CRF type 2 receptor is involved in regulating food intake and peripheral functions such as gastric acid secretion or gastric emptying. CRF is a relatively selective ligand for CRF type 1 receptor, whereas urocortin 2 is a ligand more selective for CRF type 2 receptor [28, 29]. The density of c-*Fos*-positive cells in the PVN was significantly increased by i.p. injection of des-acyl ghrelin compared to vehicle-injected controls [7] (Figure 3(c)). These data suggest that peripherally administered des-acyl

ghrelin may activate neurons in the PVN by crossing the BBB and exert inhibitory effects on the antral motility via CRF type 2 receptor in the brain (Figure 3(d), Table 1).

6. Obestatin and Gastroduodenal Motility

Zhang et al. first reported that i.p. injection of obestatin suppressed cumulative food intake, decreased body weight gain, and inhibited gastric emptying and jejunal muscle contraction in mice [2]. Since then, however, the inhibitory effects of obestatin on food intake and gastrointestinal motility have remained controversial [8–13]. Most of the previous studies which showed the negative effects of obestatin on the gastrointestinal motility have only measured the gastric emptying or MMC cycle time as indices for motor activity. In our recent study, for more precise analysis, motor activity in both fed and fasted states was quantified by the %MI, and we measured the time taken to the initiation of phase III-like contractions in the antrum and duodenum of conscious rats [14].

We showed that motor activity in the antrum and duodenum was inhibited when obestatin was given i.v. to conscious rats in the fed state but not when it was given in the fasted state [14]. I.v. injection of obestatin decreased the %MI of fed motility in the antrum and prolonged the time before the return of fasted motility in the duodenum [14] (Figure 4(a)). Such inhibitory actions were the opposite of those obtained with ghrelin [16]. The results showed that the inhibitory action of obestatin appeared 30–90 minutes after i.v. injection [14], which is consistent with the timing of the effects of i.v. injection of ghrelin (~30 minutes) on gastroduodenal motility [16]. I.v. injection of obestatin induced a significant increase in the number of c-*Fos*-positive cells in the PVN compared to saline-injected controls [14] (Figure 4(c)). Immunofluorescence overlap staining showed that the PVN neurons activated by i.v. injection of obestatin contain CRF or urocortin 2 [14] (Figure 4(c)). The involvement of CRF type 1 and type 2 receptors in the action of obestatin on the gastroduodenal motility was examined [14]. Results showed that the inhibitory actions of i.v. injection of obestatin on the motor activities in the antrum and duodenum were blocked by i.c.v. injection of CRF type 1 and type 2 receptor antagonists, suggesting that both types of CRF receptors in the brain may mediate the action of peripherally injected obestatin on gastroduodenal motility [14] (Figure 4(b)). The results showed that vagal afferent nerve blockade by capsaicin reverses the inhibitory effects of obestatin on duodenal motility but does not alter the inhibitory effects of obestatin on antral motility [14]. These results suggest that vagal afferent pathways might be involved partially, but not entirely, in the action of obestatin. Involvement of vagal afferent pathways was confirmed by the finding that the number of c-*Fos*-positive neurons in the NTS was increased by i.v. injection of obestatin [14]. In addition to vagal afferent pathways, it is possible that circulating obestatin acts on brain targets directly by crossing the BBB, because a previous study has shown that there is a rapid influx of i.v.-injected ^{125}I-labeled obestatin from the blood to the brain [30]. Therefore the lack of effects of obestatin on antral motility during capsaicin treatment might be explained by direct action of peripherally injected obestatin on brain targets by crossing the BBB, similar to what has been observed for des-acyl ghrelin. We further examined whether obestatin can antagonize the stimulatory effects of ghrelin on gastroduodenal motility [14]. We found that obestatin failed to antagonize the ability of ghrelin either to stimulate the %MI in the antrum or to accelerate the initiation of fasted motility in the duodenum when administrated in the fed state [14]. These results were consistent with previous studies in which obestatin failed to antagonize the ability of ghrelin to stimulate gastric emptying or to shorten the MMC cycle time [8].

GPR39 was initially proposed as the receptor for obestatin [2], and GPR39 expression has been detected in peripheral organs such as the duodenum and kidney but not in the pituitary or hypothalamus [4]. However recent publications indicate that obestatin is unlikely to be the endogenous ligand for GPR39 on the basis of a lack of specific binding of obestatin to GPR39 receptor-expressing cells [2, 4, 5, 31]. Nevertheless, although binding of obestatin to the receptor GPR39 remains controversial, the functional effect of obestatin on gastrointestinal motility has been clearly demonstrated in our study.

Our study indicates that obestatin inhibits gastroduodenal motility in the fed state but not in the fasted state of conscious rats. In the brain, CRF- and urocortin 2-containing neurons might be activated by i.v. injection of obestatin, and at the level, CRF type1 and type2 receptors might be involved in the inhibitory action of obestatin on antral and duodenal motility (Figure 4(d), Table 1). Vagal afferent pathways might be involved partially, but not entirely, in these actions of obestatin (Figure 4(d), Table 1).

7. Conclusion

Although ghrelin, des-acyl ghrelin, and obestatin are derived from a common prohormone, originating from endocrine cells in the stomach, their roles on the gastrointestinal motility are quite different each other. Ghrelin stimulates the gastroduodenal motility in both fed and fasted states, des-acyl ghrelin inhibits the stomach motility in the fasted state, and obestatin inhibits the gastroduodenal motility in the fed state of animals (Table 1). Different hypothalamic peptides are involved in these actions, NPY Y2 and Y4 receptors may mediate the action of ghrelin, CRF type 2 receptor may mediate the action of des-acyl ghrelin, and CRF type 1 and type 2 receptors may mediate the action of obestatin (Table 1). The regulatory roles of ghrelin, des-acyl ghrelin, and obestatin on the gastrointestinal motility might give us the therapeutic strategies for the functional disorders of the gastrointestinal tracts

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Review Article

Ghrelin and Functional Dyspepsia

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The majority of patients with dyspepsia have no identifiable cause of their disease, leading to a diagnosis of functional dyspepsia (FD). While a number of different factors affect gut activity, components of the nervous and endocrine systems are essential for normal gut function. Communication between the brain and gut occurs via direct neural connections or endocrine signaling events. Ghrelin, a peptide produced by the stomach, affects gastric motility/emptying and secretion, suggesting it may play a pathophysiological role in FD. It is also possible that the functional abnormalities in FD may affect ghrelin production in the stomach. Plasma ghrelin levels are reported to be altered in FD, correlating with FD symptom score. Furthermore, some patients with FD suffer from anorexia with body-weight loss. As ghrelin increases gastric emptying and promotes feeding, ghrelin therapy may be a new approach to the treatment of FD.

1. Introduction

Dyspepsia is associated with a variety of organic and functional disorders. The organic causes of dyspeptic symptoms include peptic ulcers, cholelithiasis, reflux disease, and malignancy. In patients initially presenting with dyspepsia, approximately 33% to 50% have an underlying organic disease [1]. Routine clinical evaluation and procedures do not reveal the cause of symptoms in the majority of patients with dyspepsia. If symptoms persist for more than three months with symptom onset of at least six months prior to the diagnosis, affected patients are diagnosed with functional dyspepsia (FD).

Despite an absence of organic disease in patients with FD, abnormalities in gastric emptying, dysregulation of gastroduodenal motility, and visceral hypersensitivity are often associated with FD. As ghrelin affects gastric motility/emptying [2–7] and secretion [8, 9], this peptide may play a pathophysiological role in FD. It is also possible that the functional abnormalities in FD may affect the production of ghrelin by the stomach. Indeed, plasma ghrelin levels are reported to be altered in FD [10, 11], frequently correlating with FD symptom score [11, 12]. Some patients with FD also suffer from anorexia with significant weight loss, frequently leading to diagnosis of eating disorders. As yet, no treatment guidelines for patients with FD or eating disorders have been established. Given its role triggering eating behaviors, ghrelin might be an appropriate treatment for FD, potentially improving food intake by influencing gastric emptying and motility. In this article, we discuss FD and the potential role of ghrelin in this disease.

2. Functional Dyspepsia

Functional dyspepsia (FD), a functional gastroduodenal disorder (FGDD) [13, 14] and one of the functional gastrointestinal disorders (FGIDs) [15], was previously known as nonulcer dyspepsia (NUD), essential dyspepsia, or idiopathic dyspepsia [16, 17]. The original Rome I criteria, which established the classification and diagnosis of FD in 1994 [18], have since been updated twice as Rome II in 2000 [19] and Rome III in 2006 [15].

The Rome II (Table 1) [19] defines FD as persistent or recurrent pain or discomfort centered in the upper abdomen. Discomfort refers to a subjective, negative feeling

Table 1: Diagnostic criteria and classification of FD in Rome II.

Diagnostic criteria
At least 12 weeks, which need not be consecutive, in the preceding 12 months of
(1) Persistent or recurrent symptoms (pain or discomfort centered in the upper abdomen)
(2) No evidence of organic disease (including at upper endoscopy) that is likely to explain the symptoms
(3) No evidence that dyspepsia is exclusively relieved by defecation or associated with the onset of a change in stool frequency or stool form (i.e., not irritable bowel).
Classification
(B1a) Ulcer-like dyspepsia
Pain centered in the upper abdomen is the predominant (most bothersome) symptom.
(B1b) Dysmotility-like dyspepsia
An unpleasant or troublesome nonpainful sensation (discomfort) centered in the upper abdomen is the predominant symptom; this sensation may be characterized by or associated with upper abdominal fullness, early satiety, bloating, or nausea.
(B1c) Unspecified (nonspecific) dyspepsia
Symptomatic patients whose symptoms do not fulfill the criteria for ulcer-like or dysmotility-like dyspepsia.

Table 2: Dyspeptic symptoms defined by Rome III. This table is adopted by permission from Elsevier Limited [13, 15].

Symptom	Definition
Postprandial fullness	An unpleasant sensation akin to the prolonged persistence of food in the stomach
Early satiation	A feeling that the stomach is overfilled soon after starting to eat. This feeling is out of proportion to the size of the meal and results in the patient being unable to finish the meal
Epigastric pain	Pain located between the umbilicus and sternum in the midline of the torso. The pain is a subjective and unpleasant feeling, but difficult to describe. Some patients may describe feelings of tissue damage or chest pain
Epigastric burning	Pain located in the epigastrium that has a burning quality, but does not radiate to the chest

Table 3: Rome III criteria for functional gastroduodenal disorders. This table is adopted by permission from Elsevier Limited [13].

B			Functional gastroduodenal disorders
	B1		Functional dyspepsia (for application in clinical practice but not otherwise useful)
		B1a	Postprandial distress syndrome
		B1b	Epigastric pain syndrome
	B2		Belching disorders
		B2a	Aerophagia
		B2b	Unspecified excessive belching
	B3		Nausea and vomiting disorders
		B3a	Chronic idiopathic nausea
		B3b	Functional vomiting
		B3c	Cyclic vomiting syndrome
	B4		Rumination syndrome in adults

characterized by or associated with non-painful symptoms such as upper abdominal fullness, early satiety, bloating, or nausea. For a diagnosis, symptoms must have persisted or recurred for at least 12 weeks over a 12-month period. A dyspepsia subgroup classification was also proposed for research purposes, based on the predominant (most bothersome) symptom, (a) ulcer-like dyspepsia features pain (from mild to severe) as the predominant symptom, while (b) dysmotility-like dyspepsia exhibits discomfort (not pain) as the predominant symptom.

The Rome III system radically reformulated the classification of FD (Table 2) [14]. The description of symptoms centered in the upper abdominal area was refined by the Committee to pain in the precise epigastric region, while other key symptoms (early satiety and fullness) must be meal-related. According to Rome III, discomfort was abandoned as a key concept, replaced by post-prandial fullness and early satiety. The Rome III criteria moved away from subcategorization using the predominant symptom (dysmotility-like and ulcer-like dyspepsia of Rome II), although similar entities, (a) postprandial distress syndrome and (b) epigastric pain syndrome, are subsumed under the FD section (Table 3). The Committee recognized that there are no one symptoms present in the majority of patients previously labeled with a diagnosis of FD. The time course for diagnosis was made less restrictive for all functional disorders; diagnosis was acceptable for symptoms originating six, not 12, months prior to diagnosis when currently active (i.e., meet criteria) for at least three months [13]. For research purposes, the term FD was abandoned in favor of a new classification system described in Table 3.

Although the etiology of FD remains unclear, a number of factors may play a role in the development of symptoms [1] (Table 4). Visceral hypersensitivity is thought to be critical in causing FD by enhancing perception and processing of gastrointestinal neural inputs [20]. In a study of experimentally induced gut distention, the majority of patients with FD developed greater discomfort than matched healthy controls. As increased gut sensitivity may affect gut motility, acid secretion, and gastric distension, hypersensitivity may

Table 4: Postulated mechanisms leading to the development of dyspeptic symptoms in patients with functional dyspepsia. This table is adopted by permission from Elsevier Limited [1].

Visceral hypersensitivity
(a) Increased perception of distention
(b) Impaired or altered perception of acid
(c) Visceral hypersensitivity secondary to chronic inflammation
Motility disorders
(a) Postprandial antral hypomotility
(b) Reduced relaxation of the gastric fundus
(c) Decreased or impaired gastric emptying
(d) Changes of the gastric electric rhythm
(e) Gastro-esophageal reflux
(f) Duodeno-gastric reflux
Changes in acid secretion
Hyperacidity
Helicobacter pylori infection
Stress
Psychological disorders and abnormalities
Genetic predisposition

underlie many of the symptoms of FD. Patients with FD often have concomitant GI motility disorders and psychiatric illnesses. Whether these disorders are an epiphenomenon or related to the underlying disease remains unclear. Symptom development and exacerbation of FD are often linked to stressful life events, causing the patient to seek medical help at that time. Thus, patients seeking care for FD symptoms are more likely to have active life stressors than those who remain undiagnosed, leading to bias in the sample population. External stressors may also affect intestinal function, which in connection with visceral hypersensitivity, may lead to increased symptom perception.

3. Relationship of Ghrelin to the GI Tract and Feeding Behaviors

Neural and hormonal communication between the gut and the brain modulate appetite, feeding, and digestion [21, 22]. In the integrated gut-brain-energy axis, gastrointestinal (GI) motility, gastric acid secretion, digestion, and defecation are coordinated with appetite, satiation, and metabolism. Both organic and functional GI disorders are associated with alterations of the physiologic factors regulating the gut-brain-energy axis.

Multiple hormones are secreted by the gut and adipose tissues during feeding, digestion, and fasting, each of which can profoundly affect the GI tract [22]. A subset of these hormones directly affects secretory function along the GI tract (e.g., gastrin stimulates acid secretion by parietal cells); major sites of hormone action also include enteric and vagal neurons, the area postrema (AP) within the medulla, and the hypothalamic arcuate nucleus (HAN). The AP and the median eminence, which has a close relationship with the HAN, have greatly reduced or absent blood-brain barriers. Impulses from the AP travel along nerve projections to the adjacent nucleus tractus solitarius (NTS) and higher regions of the brain, while those from the HAN do to other parts of the hypothalamus. Several hormones can cross the blood-brain barrier, including those with specific transporters such as leptin, insulin, and ghrelin [23]. Hormones released from the gut act on both the enteric nervous system (ENS) to contribute to the migrating motor complex (MMC) cycle and the CNS to promote the gradual re-establishment of appetite. The plasma concentrations of leptin and glucagon-like peptide 1 (GLP1) are low during fasting, while those of ghrelin and orexin are high. Significant research has focused on hormones whose plasma concentrations increase during fasting, as it is hypothesized that these hormones strongly affect hunger and energy expenditure. In addition to systemic effects, fasting-associated hormones also strongly affect a myriad of GI functions, possibly preparing the GI tract for food reception. Such a role has been proposed for ghrelin, the first orexigenic hormone identified that is produced in and released from the stomach. Ghrelin has a well-established role in increasing appetite and food intake [24, 25] and in stimulating gastric emptying and acid secretion [26, 27]; these functions are mediated, at least in-part, via vagal nerve pathways [9, 28]. The details concerning relationship of ghrelin to the GI tract function and food intake are also described in several manuscripts of this special issue (see, e.g., "Ghrelin's effect on hypothalamic neurons", "Ghrelin-NPY axis", "Gastric ghrelin and leptin", "Motilin and ghrelin in the dog," and "Ghrelin and stress in GI tract").

4. Plasma Concentrations of Ghrelin in FD

The gut and brain are highly integrated, communicating bidirectionally through neural and hormonal pathways [29]. Psychosocial factors can significantly influence digestive function, symptom perception, disease presentation, and outcome. Functional GI disorders, in return, can alter the physiologic factors regulating digestion and feeding [22].

The circulating levels of hormones related to appetite regulation are altered in dyspeptic disorders. Patients with dysmotility-like dyspepsia have higher serum concentrations of leptin, which is associated with gastritis and *H. pylori* infection [30]. Given that leptin is produced within the stomach to activate vagal-nerve terminals, reduce appetite, and increase mucin secretion, leptin may have a protective role in the upper gut during states of injury [22]. Plasma ghrelin levels in patients with FD are, however, controversial. While the total ghrelin levels were reported to be significantly higher in patients with FD (32 dysmotility-like and 7 ulcer-like FD patients) [31], they were significantly lower in patients with dysmotility-like FD [10, 32]. Concerning the active ghrelin levels, they were decreased in patients with postprandial fullness and/or early satiation defined by the Rome III classification [33], whereas similar between dysmotility-like FD patients and healthy controls [10]. The reason for discrepancy in plasma ghrelin levels remains uncertain, warranting further investigation.

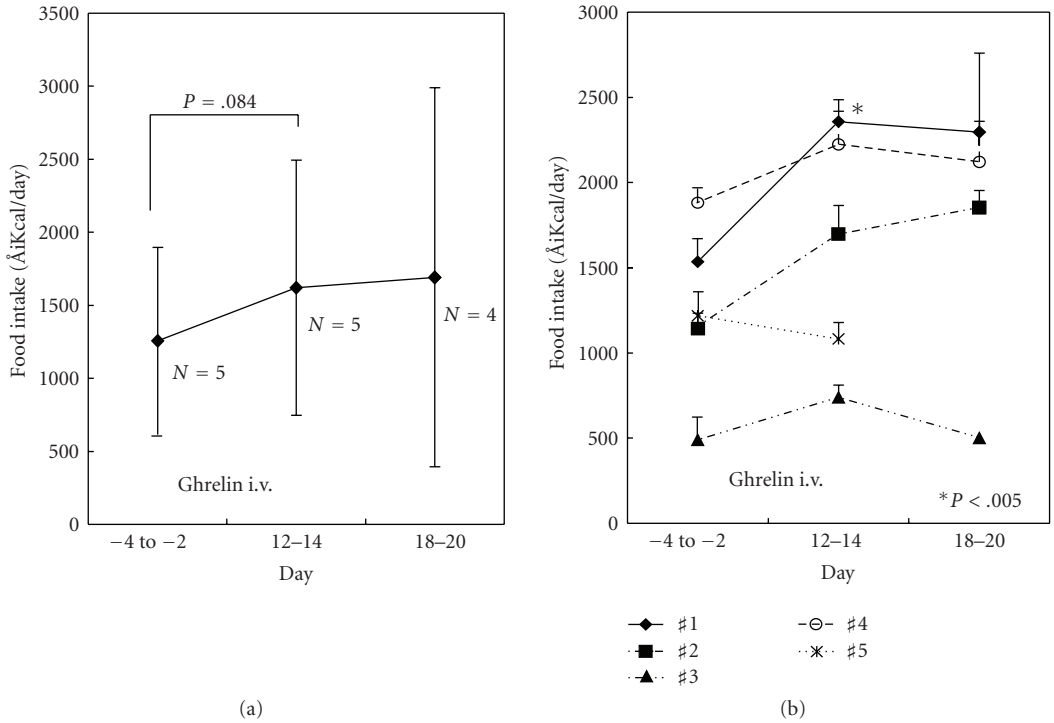

Figure 1: Daily food intake was measured before ghrelin injection (days −4 to −2), at the completion of treatment (days 12 to 14), and one week after injection (days 18 to 20) in five subjects who completed two weeks of ghrelin treatment. (a) Mean and 95% confidence interval (CI) for five subjects; (b) three-day means and SD of daily food intake for each subject.

In terms of pathogenetic implication of these alterations in plasma ghrelin levels, the relationship with gastric emptying time in these patients was investigated. Significant correlation between the active ghrelin levels and the T_{max} value [33] and delayed gastric empyting in the majority of patients with abnormally low total ghrelin levels [32] were observed. In addition, elevations in the acylated form of ghrelin (active ghrelin) were significantly associated with subjective symptom score in FD patients [12]. Increased ghrelin concentrations are also seen in patients with duodenal and gastric ulcers, suggesting a possible relationship to mucosal injury [34].

5. Ghrelin Administration to Patients with FD

The therapies currently available for the treatment of dyspepsia, and for FD specifically, target the underlying hypothesized pathophysiology, including increased gastric acid sensitivity, delayed gastric emptying, and *H. pylori* infection. Only a small proportion of patients, however, experiences symptomatic relief using these treatments [35]. New treatment modalities targeting impaired gastric accommodation, visceral hypersensitivity, and central nervous system dysfunction are currently under development.

In dysmotility-type FD, which comprises the largest subset of patients, abnormalities in gastrointestinal motility and sensitivity are thought to underlie the development of symptoms. Some patients with FD suffer from anorexia with weight loss, frequently leading to diagnosis with eating disorders. No treatment guidelines for patients with FD or eating disorders have been established. As ghrelin increases gastric emptying [2–6] and promotes feeding [36, 37], we investigated the ability of repeated ghrelin administrations to increase appetite and food intake in patients with FD [38]. We administered ghrelin by intravenous infusion (3 μg/kg) twice a day before breakfast and dinner for two weeks to five patients. Ghrelin administration tended to increase daily food intake by approximately 30% in comparison to levels before and after completion of treatment, although this difference did not reach statistical significance ($P = .084$) (Figure 1(a)). Increases in food intake were maintained even one week after treatment (days 18–20). Although the precise mechanism is not known, the acute effects of ghrelin on gastric function may lead to sequential improvements in gastric mucosa and/or function. In addition, improvement in food intake may result from decreased anxiety or increased confidence concerning food intake in these patients. On an individual basis, food intake increased in four of the five subjects tested, decreasing in Patient #5 (Figure 1(b)). Food intake in Patient #1 was significantly elevated at the end of ghrelin treatment (days 12–14) from levels observed before treatment ($P < .005$). Hunger sensation was significantly elevated following a drip infusion ($P < .0001$). No severe adverse effects were observed. These results support the therapeutic potential of ghrelin in patients with FD. Additional studies, including larger placebo-controlled trials, will be necessary to confirm the usefulness of ghrelin in FD treatment.

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Review Article

Rikkunshito and Ghrelin

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Rikkunshito is a popular Japanese traditional medicine that is prescribed in Japan to treat various gastrointestinal tract disorders. In a double-blind controlled study, rikkunshito significantly ameliorated dysmotility-like dyspepsia and brought about a generalized improvement in upper gastric symptoms such as nausea and anorexia when compared with a control group. Several studies in rats have shown enhanced gastric emptying and a protective effect on gastric mucosa injury with rikkunshito administration. In addition, rikkunshito in combination with an anti-emetic drug is effective against anorexia and vomiting that occur as adverse reactions to chemotherapy in patients with advanced breast cancer. However, the mechanism by which rikkunshito alleviates gastrointestinal disorders induced by anticancer agents remains unclear. It has recently been shown that rikkunshito ameliorates cisplatin-induced anorexia by mediating an increase in the circulating ghrelin concentration. Moreover, Fujitsuka et al. found that decreased contractions of the antrum and duodenum in rats treated with a selective serotonin reuptake inhibitor were reversed by rikkunshito via enhancement of the circulating ghrelin concentration. These findings show that rikkunshito may be useful for treatment of anorexia and may provide a new strategy for improvement of upper gastrointestinal dysfunction.

Rikkunshito is one of the few traditional Japanese medicines for which a double-blind study has been conducted. This trial was performed in patients with dysmotility-like dyspepsia [1, 2] based on a report that rikkunshito showed efficacy against non-ulcer dyspepsia, which is an old diagnostic classification. In a subsequent comparative clinical study, rikkunshito was found to be more effective than cisapride against undefined gastrointestinal complaints such as chronic gastritis [3]. Rikkunshito is prepared by compounding eight herbal medicines listed in the Japanese Pharmacopoeia: *Atractylodis Lanceae Rhizoma*, *Ginseng Radix*, *Pinelliae Tuber*, *Hoelen*, *Zizyphi Fructus*, *Aurantii Nobilis Pericarpium*, *Glycyrrhizae Radix* and *Zingiberis Rhizoma*. It has recently been shown that oral administration of rikkunshito stimulates secretion of the orexigenic peptide, ghrelin, from the stomach [4, 5]. In this section, the effects of rikkunshito are introduced, with main focus on the action of rikkunshito as an enhancer of ghrelin secretion.

Anorexia is commonly seen in gastrointestinal diseases, although it is not a specific symptom. Anorexia is particularly common in chronic gastritis and gastric cancer but also occurs in acute hepatitis, hepatic cirrhosis, chronic pancreatitis, and chronic cholecystitis. Harasawa et al. [1] showed that rikkunshito promotes improvement of anorexia in a double-blind study in patients with functional dyspepsia (FD). A combination of rikkunshito plus serotonion (5-hydroxytriptamine, 5-HT)and 3 receptor antagonist (an anti-emetic agent) reduced anorexia and vomiting induced as adverse reactions after chemotherapy in patients with advanced lung cancer, compared with administration of the anti-emetic agent alone [6]. Similarly, administration of a selective serotonin reuptake inhibitor (SSRI), fluvoxamine, in combination with rikkunshito for eight weeks resulted in a significant reduction in the number of patients who complained of adverse events, especially retching, compared with SSRI administration alone [7]. The gastrointestinal symptom rating score also significantly improved within two weeks of starting coadministration of the SSRI with rikkunshito [7]. These findings suggest that rikkunshito suppresses the onset of adverse reactions to frequently prescribed drugs that cannot be treated adequately by adjuvant therapy with current Western medicines.

Cisplatin has been shown to cause a significant decrease in plasma ghrelin and food intake in rodents [4], and intravenous injection of exogenous acylated ghrelin inhibited the decrease in food intake after cisplatin administration.

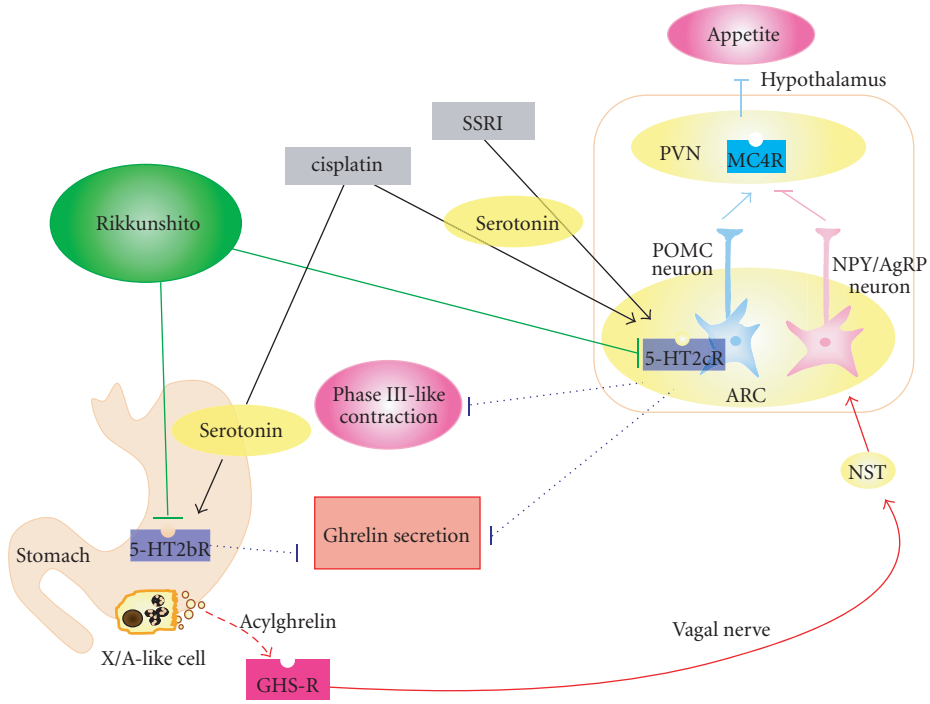

Figure 1: Schematic diagram of action mechanisms of rikkunshito on appetite.

Rikkunshito also inhibited the decrease in circulating ghrelin concentration and ameliorated the decrease in food intake caused by cisplatin. Interestingly, coadministration of a ghrelin receptor antagonist, [D-Lys^3]-GHRP-6, with rikkunshito abolished this effect. These findings suggest that the mechanism of improvement of anorexia by rikkunshito may involve ghrelin receptor activation via stimulation of ghrelin secretion from the stomach into the plasma. Heptamethoxyflavone, an active ingredient flavonoid in rikkunshito, has been shown to have a pivotal effect on stimulation of ghrelin secretion. In addition, P388-bearing mice showed a tendency for improved survival with rikkunshito treatment, and survival was further improved by treatment with cisplatin in combination with rikkunshito, although the difference was not significant [8]. These results show that administration of rikkunshito has no adverse effect on the anticancer action of cisplatin itself.

Fujitsuka et al. showed that oral administration of rikkunshito restores disturbed motor activity in the gastrointestinal tract and improves anorexia in rats administered SSRIs [5]. Intraperitoneal administration of fenfluramine or fluvoxamine shifted fasted rats from a fasted-like motor pattern in the antrum and duodenum to fed-like motor activities similar to those seen after feeding. A significant decrease in the plasma concentration of acylated ghrelin, delayed gastric emptying, and decreased food intake were also observed after administration of the SSRI. Concomitant oral administration of rikkunshito with an SSRI suppressed the decrease in plasma acylated ghrelin, changed the fed-like motor activity to fasted activity, improved anorexia, and enhanced gastric emptying. These effects were abolished by coadministration of a ghrelin receptor antagonist with rikkunshito.

Cisplatin and SSRIs are widely used in clinical practice. 5-HT is a key factor in adverse reactions to these drugs, since both stimulate production of excess 5-HT and suppress 5-HT metabolism in vivo. Thus, it is of interest that involvement of the 5-HT2 receptor in appetite control has recently been shown [9]; specifically, appetite is suppressed when the 5-HT2B receptor in gastric smooth muscle and the 5-HT2C receptor in the central nervous system are activated by receptor agonists. 5-HT produced during treatment with cisplatin or SSRIs binds to various receptor subtypes and is likely to stimulate the 5-HT2B and 2C receptors. A decrease in plasma ghrelin is suppressed by administration of antagonists for these receptors, leading to improvements in food intake and gastrointestinal dysmotility [4, 5]. Isoliquiritigenin, heptamethoxyflavone and hesperidin are ingredients of rikkunshito that have been shown to antagonize 5-HT2B and 2C receptors [4]; thus, these ingredients are considered to play an important role in the improvement of appetite by rikkunshito. Administration of hesperidin reverses the decrease in plasma ghrelin in cisplatin-treated rats and shifts the fed-like motor pattern induced by SSRI administration to a fasted pattern. Thus, 5-HT2C antagonism by active components in rikkunshito may lead to the improvement of anorexia.

More recent report demonstrated that administration of rikkunshito improve anorexia of aging via inhibiting a reduced hypothalamic ghrelin receptor reactivity [10]. The data indicated that aging-associated anorexia is caused by an increase in plasma leptin, which results from disturbed

reactivity of ghrelin in the hypothalamus and regulation of ghrelin secretion. Oral administration of cilostamide, phosphodiesterase type 3 (PDE3) inhibitor improved anorexia in aged mice. The components of rikkunshito (nobiletin, isoliquiritigenin, and heptamethoxyflavone) had inhibitory effects on PDE3 activity. Dysregulation of ghrelin secretion and ghrelin resistance in the appetite control system occurred in aged mice and that rikkunshito ameliorated aging-anorexia via inhibition of PDE3.

In summary, administration of rikkunshito stimulates secretion of ghrelin from stomach via peripheral 5-HT2B and central 5-HT2C receptor antagonism as seen in Figure 1. Moreover, rikkunshito is also an enhancement of ghrelin receptor reactivity via PDE3 inhibition. These studies raise the possibility of using rikkunshito to treat anorexia.

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Review Article

Effect of Ghrelin on Glucose-Insulin Homeostasis: Therapeutic Implications

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Ghrelin is a 28-amino-acid peptide that displays a strong growth hormone- (GH-) releasing activity through the activation of the growth hormone secretagogue receptor (GHSR). The first studies about role of ghrelin were focused on its orexigenic ability, but despite indisputable pharmacological data, the evidence for a physiological role for ghrelin in the control of appetite is much less clear. Mice with targeted deletion of either ghrelin or the GHSR exhibit an essentially normal metabolic phenotype when fed a regular chow diet, suggesting that ghrelin may have a redundant role in the regulation of food intake. RNAs for ghrelin as well as GHSR are expressed in the pancreas of rats and humans and several studies propose that ghrelin could have an important function in glucose homeostasis and insulin release, independent of GH secretion. Low plasma ghrelin levels are associated with elevated fasting insulin levels and insulin resistance, suggesting both physiological and pathophysiological roles for ghrelin. For this reason, at least theoretically, ghrelin and/or its signalling manipulation could be useful for the treatment or prevention of diseases of glucose homeostasis such as type 2 diabetes.

1. Introduction

GH is released from the pituitary gland in a pulsatile manner and it is mainly regulated by episodic changes in two hypothalamic hormones, growth hormone-releasing hormone (GHRH) and somatostatin. GHRH stimulates GH secretion whereas that somatostatin inhibits it [1]. In 1976, it was revealed that modified opioid peptides had low GH secretory activity [2]. Since then, many efforts have been made to develop and improve potential applications of these GH secretagogues (GHSs) [3–7]. GHSs act on the pituitary and hypothalamus to release GH, not through the growth hormone releasing hormone receptor (GHRHR) but through an orphan receptor, the GHSR [8]. These facts indicated that an unknown endogenous ligand for GHSR should exist. In 1999, ghrelin was identified as the endogenous ligand for the GHSR. It is a 28-amino-acid peptide predominantly produced by the stomach that functions as a somatotrophic and orexigenic signal from the stomach [9, 10]. Ghrelin is most abundantly expressed in specialized cells in the oxyntic glands of the gastric epithelium, originally termed X/A-like cells [11]. Approximately 60%–70% of circulating ghrelin is secreted by the stomach, and most of the remainder originates in the small intestine [11]. Nevertheless low-level ghrelin expression also occurs in several tissues outside the gut, including hypothalamus (arcuate nucleus and paraventricular nucleus), pituitary, lung, adrenal cortex, kidney, bone, testis, placenta, and pancreatic islet cells [12]. The GHSR mRNA is expressed as two splice variants encoding the cognate receptor GHSR1a and the apparently nonfunctional receptor GHSR1b [13]. GHSR1a signals via inositol trisphosphate (IP_3) generation and Ca^{2+} release and has constitutive activity [13, 14]. GHSR1b mRNA is as widely expressed as ghrelin, whereas GHSR1a gene expression is concentrated in the hypothalamus-pituitary unit, although it is also distributed in other central and peripheral tissues [15]. Ghrelin circulates in the bloodstream in two different forms: acylated (or n-octanoylated, AG) and

unacylated (or des-octanoylated or des-acylated, UAG) [9]. AG has a unique feature: a posttranslational esterification of a fatty (n-octanoic or, to a lesser extent, n-decanoic) acid on serine residue at position 3 [9]. Recent data showed that Ghrelin O-acyltransferase (GOAT), a membrane-bound enzyme, is responsible for octanoylation of the serine-3 residue of ghrelin [16, 17]. Ghrelin acylation is considered necessary for its actions via GHSR1a, such as its strong GH-releasing activity [9, 18–20]. Normally AG accounts for less than 10% of the total ghrelin in the circulation. The majority of circulating ghrelin is UAG, which does not have effects in GH release, but it is not biologically inactive [19, 21–29]. It binds with high affinity to a receptor, different from GHSR1a and yet unknown [9, 12]. The first studies about ghrelin demonstrated that it increases food intake and adiposity [10]. Moreover, plasma ghrelin levels have been shown to increase prior a meal and during fasting and to decrease after a meal, and they are negatively correlated with body weight [30–32]. All these data suggested a role in the control of energy homeostasis. But the conflicting food intake and body weight data from transgenic and knockout models, which present normal metabolic phenotype, has made difficult defining a key role for endogenous ghrelin in the control of appetite [27, 33–39]. Nevertheless, the data consistently suggest that ghrelin may be important in the control of glucose homeostasis and insulin release.

It was reported that prolonged treatment with GHSs provoked hyperglycemia and hyperinsulinism but this effect was supposed to reflect increased GH secretion [40–43], as GH plays an important role modulating energy homeostasis and metabolism [44]. Particularly, GH exerts both acute and chronic effects on carbohydrate and lipid metabolism [44]. Interestingly, both actions display an opposite pattern, with acute effects showing a transient "insulin-like" action and chronic effects exhibiting an "anti-insulin" action. In this sense, GH administration decreases blood glucose concentration, stimulates glucose uptake by skeletal muscle, and stimulates glucose transport and lipogenesis in isolated adipocyte [44, 45]. However these effects are transitory; after a few hours the chronic anti-insulin effects of GH arise, increase blood glucose concentration, insulin resistance, stimulation of lypolysis, and inhibition of glucose transport. High plasma GH levels induce hyperinsulinemia and insulin resistance [12, 46]. On the other hand, besides its lipolytic action, GH exhibit antilipogenic effects [47, 48] promoting proliferation of lean tissues, while reducing accumulation of fat tissue. Accordingly, GH-deficient states in humans and rodents are characterized by a decrease in lean body mass accompanied by increased adipose tissue [49–53].

Early studies demonstrated that RNAs for ghrelin, as well as GHSR, are expressed in the pancreas of rats and humans [9, 15, 54, 55] and β-cells lines [56, 57] suggesting a possible relation between ghrelin and insulin. Studies using different experimental systems localized ghrelin-immunoreactive cells in rat and human pancreas in the α-cells [54, 57, 58], β-cells [55], PP-cells [57], and other islets cells [57, 59], including those named ε-cells [60]. The first evidences about an interaction between ghrelin and glucose metabolism arose when it was seen that single subcutaneous ghrelin injections induced an increase of the respiratory quotient (RQ), which suggested an augmented utilization of carbohydrate and reduced utilization of fat to meet energy requirements that was congruent with the observed increase in body fat [10]. Another evidence that suggested that ghrelin could affect glucose metabolism was the fact that it stimulated acid secretion through vagal mediation [61, 62] and some studies suggested that the parasympathetic nerves that regulate hormonal control of insulin pass through the cervical vagus and the hepatic branch, and that the hepatic vagus nerve is important for the regulation of hepatic glucose production in the post absorptive state [63, 64]. All these data and numerous studies since 2000 to the present time suggest that ghrelin has an important role in regulating β-cell function and glucose homeostasis. Indeed, the weight of evidences could support even a more physiologically important function in the control of glucose homeostasis than appetite regulation.

In this work we will review the results obtained by different investigators about the relation between ghrelin and glucose metabolism and insulin release as well as its possible therapeutic role in disease states like diabetes.

2. Effects of Exogenous Ghrelin on Glucose and Insulin Levels

2.1. Short-Term Effects. The first studies with ghrelin showed that acute ghrelin treatment induced hyperglycemia and reduced insulin secretion in healthy humans [65] during the first hours of treatment. The time course of glucose modifications occurred with a peak observed before any significant insulin decreased. Subsequently, these findings were confirmed by other authors in human and rodents [58, 66–71]. However, when these experiments were carried out in obese patients, there was no difference in glucose or insulin levels following ghrelin administration [72]. The first hypothesis suggested that ghrelin itself could have a direct effect on glucose metabolism, regulating hepatic glucose output, promoting glycogen breakdown, or decreasing peripheral glucose uptake; consistent with this view were the findings that ghrelin receptors are expressed in normal human liver [73, 74].

In order to discard a secondary effect due to increased GH secretion, human subjects were treated with a GH receptor blockade, pegvisomant [43], and in this situation a ghrelin mimetic induced increases in glucose and insulin levels. Suggesting ghrelin mimetic-mediate GH-independent insulin resistance, moreover several studies have demonstrated that interference with ghrelin signalling by use of GHSR antagonist decreases blood glucose in wild-type mice as well as GH-deficient lit/lit mice [58, 75]. To diminish the influence of GH, Vestergaard et al. investigate the effects of prolonged ghrelin infusion (not a unique dose) on insulin sensitivity [76] which decreased after few minutes of ghrelin infusion and outlasted both the infusion period and the postinfusion interval. As the reduced insulin sensitivity remained after normalization of both GH and glucose levels, this work supports that ghrelin effect was

caused by the ghrelin infusion per se [77]. In a posterior work the same investigators studied, for the first time, the concomitant effects of exogenous ghrelin and a pancreatic clamp on glucose metabolism in humans; they used a prolonged ghrelin infusion in addition to a somatostatin infusion to avoid GH secretion. Ghrelin infusion decreased basal as well as insulin stimulated glucose disposal and induced peripheral insulin resistance but did not affect hepatic glucose production [71]. When they studied the effect of exogenous ghrelin in hypopituitary men (in the absence of GH and cortisol secretion), in a randomized double-blind, cross-over design, ghrelin treatment acutely decreased peripheral, but not hepatic, insulin sensitivity independently of GH and cortisol [78].

There are data that suggest a relation between ghrelin and glucose-stimulated insulin secretion (GSIS) via the hepatic portal system and the vagus nerve. Gastrectomy and truncal vagotomy are operations characterized by hypoghrelinemia [30], glucose intolerance as a result of hyperglucagonemia, insulinopenia, and impaired first phase of insulin secretion [79]. When ghrelin was infused into the portal vein of rats, inhibited glucose-stimulated release of insulin, however when it was infused into the femoral vein, did not induce such an inhibitory effect. All the more hepatic vagotomy or coinfusion with atropine methyl bromide (a muscarinic antagonist) diminished the inhibitory effect of ghrelin on glucose-stimulated insulin secretion [70]. Damjanovic et al. also performed studies with ghrelin and truncal vagotomy, investigating the effects of intravenous (IV) ghrelin infusion on insulin-mediated glucose disposal during a hyperinsulinemic-euglycemic clamp in humans who underwent total gastrectomy and truncal vagotomy [80]. In these patients glucose disposal rate (GDR) decreased during ghrelin infusion; however this difference was not translated into a significant difference in insulin concentration, probably because the exogenous insulin by far overweighs endogenous insulin. Thus, there cannot be ruled the possibility that diminished glucose utilization after ghrelin administration is partly explained by the decrease in endogenous insulin secretion, although this was not detected in the study [80]. It appears that acute ghrelin administration might be involved in the negative control of insulin secretion and glucose consumption in gastrectomized patients [80].

In summary, short-term effects of exogenous ghrelin induces hyperglycaemia and hypoinsulinism in health humans and rodents in a GH independent fashion.

In Table 1 are summarized the results obtained after acute ghrelin treatment in several models and situations.

2.2. Long-Term Effects. Generally long-term ghrelin treatment induced an increase in plasmatic values of glucose, whereas plasmatic insulin levels, unlike short-term effects, did not change or enhanced after ghrelin treatment. But long-term effects of exogenous ghrelin on glucose and insulin levels are not conclusive; there are differences inter-experiments which could reflect different doses, administration way, and/or species used. In long term studies essentially there are two way of administration: those in which the administration of ghrelin was systemic: intraperitoneal (IP) or subcutaneous (SC), and central: when the hormone was administered directly in a cerebral region.

2.2.1. Systemic Administration. Involves treated IP with ghrelin during 4 days, plasma glucose concentrations increased. At the same time, the authors measured body glycogen stores and observed that liver glycogen content was unaffected, but the quadriceps muscle and kidney glycogen stores decreased, indicating them as the possible source of elevated plasma glucose levels [69]. Similar results were obtained by Asakawa et al. with mice; they examined the effects of repeated administration of IP ghrelin on glycaemic control under a high fat diet (HFD). In these conditions insulin levels were increased by the treatment and blood glucose concentration displayed a moderate increase but did not reach statistical significance [75].

When Barazzoni et al. administrated subcutaneous ghrelin during four days to normal rats, they found hyperglycemia; nevertheless plasma insulin levels did not change [85, 86]. The treatment increased transcript levels of the key enzyme of the gluconeogenic pathway, glucose-6-phosphatase (G6Pase) in liver. For these reasons the authors suggested that enhanced gluconeogenesis in liver would contribute to increase circulating glucose in ghrelin-treated animals [85].

2.2.2. Central Administration. In others studies the animals received ghrelin treatment intracerebroventricularly (ICV). 6-day ICV ghrelin infusion provoked an increase on insulin-stimulated glucose utilization during euglycemic-hyperinsulinemic clamps in epididymal and inguinal white adipose tissue (WAT) as well as brown adipose tissue (BAT), but not in soleus muscle. During the clamps, hepatic glucose production was comparably suppressed by hyperinsulinemia in all groups. The treatment did not change plasma glucose or insulin levels [87]. Comparable results were obtained by Kamegai et al. administering repeated injections of ghrelin into the lateral ventricle of rats during 72 hours, without changes in plasma glucose and insulin concentrations, although there was a trend toward higher levels [88]. However, in another study, ICV ghrelin injections every 24 hours during five days to adult male rats clearly increased serum insulin levels without evoking changes in blood glucose levels [89].

Although the results obtained by ghrelin treatment in the long term are not enough clear, it seems to exist a tendency toward an increase in both plasma glucose and insulin levels. These data could indicate a role for ghrelin in worsening insulin sensitivity.

In Table 2 are summarized the results obtained in plasma glucose and insulin levels after prolonged treatment with ghrelin.

2.3. Studies In Vitro and Perfusion. Besides the experiments carry out in vivo, there are works with cellular cultures and pancreatic perfusion that contribute to our knowledge about ghrelin role on glucose and insulin metabolism, pointing to

Table 1: Acute effects of ghrelin administration on glucose-insulin homeostasis in different species and metabolic situations. IV: intravenous; O: oral.

Species	Treatment	Dose	Food before experiment	Treatment duration	Plasma glucose or GIR	Plasma insulin	Reference
Health humans	1 IV AG injection versus 1 IV placebo injection	1 μg AG/kg	Fasting overnight	3 hours	Enhanced	Decreased	[65]
Health humans	1 IV AG injection versus 1 IV placebo injection	3.3 μg AG/kg	Fasting overnight	3 hours	Enhanced	Decreased	[68]
Health humans	1 IV AG injection versus 1 IV placebo injection	1 μg AG/kg	Fasting overnight	2 hours	Enhanced	Decreased	[67, 81]
Health humans	1 IV AG injection + O-GTT versus O-GTT	1 μg AG/kg + 100 g glucose	Fasting overnight	2 hours	Not change	Not change	[81]
	1 IV AG injection + FFA versus FFA	1 μg AG/kg + 10% FFA	Fasting overnight	2 hours	Not change	Decreased	
	1 IV AG injection + arginine versus arginine	1 μg AG/kg + 0.5 g arginine/kg	Fasting overnight	2 hours	Enhanced	Decreased	
Health humans	1 IV AG injection versus 1 IV placebo injection	1 μg AG/kg	Fasting overnight	2 hours	Enhanced	Decreased	[67]
	1 IV UAG injection versus 1 IV placebo injection	1 μg UAG/kg	Fasting overnight	2 hours	Not change	Not change	
	1 IV AG injection + UAG versus 1 IV placebo injection	1 μg AG/kg + 1 μg UAG/kg	Fasting overnight	2 hours	Not change	Not change	
Health humans	IV AG infusion versus IV placebo infusion	5 pmol AG/kg/min	Fasting overnight	3 hours	Enhanced	Enhanced	[77]
Health humans	IV AG infusion versus IV placebo infusion Both with pancreatic clamp + hyperinsulinemic-euglicemic clamp + glucose adjustable	5 pmol AG/kg/min	Fasting overnight	5 hours	During clamp GIR diminished with ghrelin	Not change	[71]
Hypopituitary humans	1 IV AG or UAG injection versus 1 IV placebo injection	1 μg AG or UAG/kg	Fasting overnight	2 hours	Enhanced	Not change	[82]
	1 IV AG + UAG injection versus 1 IV placebo injection	1 μg AG/kg + 1 μg UAG/kg	Fasting overnight	2 hours	Not change	Diminished	
Hypopituitary humans	IV AG infusion versus IV placebo infusion Both with hyperinsulinemic-euglicemic clamp	5 pmol AG/kg/min	Fasting overnight	5 hours	Basal period enhanced, during clamp GIR diminished	Not change	[78]

Table 1: Continued.

Species	Treatment	Dose	Food before experiment	Treatment duration	Plasma glucose or GIR	Plasma insulin	Reference
Gastrectomized humans	IV AG infusion versus IV placebo infusion Both with hyperinsulinemic-euglycemic clamp	5 pmol AG/kg/min	Fasting overnight	5 hours	Diminished GIR	Not change	[80]
Normal rats	IV AG infusion versus IV placebo infusion Both with IV-GTT infusion	1 ng AG/kg/h + 13.3 mg glucose/kg/min	24-hour fasting	40 minutes	Not change	Not change	[70]
	IP AG infusion versus IV placebo infusion Both with IV-GTT infusion	1 ng AG/kg/h + 13.3 mg glucose/kg/min	24-hour fasting	40 minutes	Enhanced	Diminished	
	IV AG infusion versus IP placebo infusion Both with IP-GTT infusion	1 ng AG/kg/h + 13.3 mg glucose/kg/min	24-hour fasting	40 minutes	Not change	Not change	
	IP AG infusion versus IP placebo infusion Both with IP-GTT infusion	1 ng AG/kg/h + 13.3 mg glucose/kg/min	24-hour fasting	40 minutes	Enhanced	Diminished	
Normal rats	1 IV UAG injection + IV-GTT versus IV-GTT	30 nmol UAG/kg + 1 g glucose/kg	Fasting overnight	50 minutes	Not change	Enhanced	[83]
	1 IV AG injection + IV-GTT versus IV-GTT	30 nmol UAG/kg + 1 g glucose/kg	Fasting overnight	50 minutes	Not change	Not change	
Rats with hepatic vagotomy	IP AG infusion versus IP placebo infusion Both with IP-GTT infusion	1 ng AG/kg//h +13.3 mg glucose/kg/min	24-hour fasting	40 minutes	Not change	Not change	[70]
Mice ddY	1 IP AG injection versus 1 IP placebo injection Both with IP-GTT	1 and 10 nmol AG/kg + 1 g glucose/kg	Fasting overnight	2 hours	Enhanced	Decreased	[58]
	1 IP AG injection versus 1 IP placebo injection	1 nmol/kg	Fasting overnight	2 hours	Enhanced		
C57BL/6J mice	1 IV AG injection + IV-GTT versus IV-GTT	50 nmol AG/kg + 1g/kg	3-hour fasting	50 minutes	Not change	Diminished	[84]
GH-deficient little mice	1 IP AG injection versus 1 IP placebo injection	1 nmol AG/kg	Fasting overnight	30 minutes	Enhanced		[58]
Obese humans	1 IV AG injection versus IV placebo injection	1 μg AG/kg	Fasting overnight	2 hours	Not change	Not change	[72]

Table 2: Chronic effects of ghrelin administration on glucose-insulin homeostasis in different species.

Species	Treatment administration	Dose	Food during experiment	Duration treatment	Plasma glucose levels	Plasma insulin levels	Reference
Mice ddy	1 IP AG injection/12-h	3 nmoles AG/ mouse/injection	*Ad libitum* HFD	5-day	Not change	Enhanced	[75]
Tundra vole	1 IP AG injection/day	10 μg AG/kg/day	*Ad libitum* SCD	4-day	Enhanced		[69]
Sprague-Dawley rats	1 ICV AG injection/12-h	1 μg AG/rat/injection	*Ad libitum* SCD	3-day	Not change	Not change	[88]
Wistar rats	1 ICV ghrelin injection/day	1 μg AG/rat/day	*Ad libitum* SCD	5-day	Not change	Enhanced	[89]
	ICV ghrelin infusion	2.5 nmol AG/rat/day	*Ad libitum* SCD	6-day	Not change	Not change	[87]
	1 SC AG injection/12-h	0.2 ug AG/injection	*Ad libitum* SCD	4-day	Enhanced	Not change	[85]

a role for ghrelin in the pancreatic islet. The perfused rat pancreas is a suitable model to characterize the pancreatic hormone secretory pattern elicited by ghrelin in the short term. Egido et al. dissected and perfused in situ the pancreas of rats fed *ad libitum*; the addition of ghrelin to the perfusate did not significantly modify basal insulin release but markedly inhibited the insulin response to increasing glucose concentrations, arginine, and carbachol [90]. It was observed that the glucose-induced insulin release from the rat-perfused pancreas was markedly enhanced by blockade of GHSR and immunoneutralization of endogenous ghrelin. Furthermore, GHSR blockade increased plasma insulin concentrations in gastrectomized and normal rats to a similar extent [91]. The results obtained with perfused rat pancreas support a role for ghrelin inhibiting insulin release. These results were confirmed in studies with isolated islets from normal rats [58, 92] and MIN 6 cells [93], where ghrelin inhibited the insulin response to increasing glucose concentrations. But when ghrelin was coincubated with GHSR antagonists or antiserum against acylated ghrelin, this effect was blocked [58, 92]. Moreover, in islets from ghrelin-null mice, glucose treatment enhanced insulin release [91]. On the contrary, in another study, it was observed that ghrelin (1 pmol/l) stimulated insulin release and increased $[Ca^{2+}]$ in rat islet β-cells in the presence of a stimulatory (8.3 mmol/l) but not basal (2.8 mmol/l) glucose concentration [54]. However, the same authors, in a subsequent study, examined the dose-dependent effects of ghrelin and they found that ghrelin at 1 pmol/l and 0.1 nmol/l modestly potentiated glucose-induced $[Ca^{2+}]$i responses in a little portion of β-cells, but it failed to significantly alter insulin release. This observation that ghrelin is inhibitory at relatively high concentrations of 10 nmol/l, while having little effect at lower concentrations, is consistent with the majority of other reports [58].

Several cell culture studies showed a genetic link between ghrelin and insulin. The Nkx2.2 hoursomeodomain transcription factor is required for islet cell development and differentiation. In this way high levels of Nkx2.2 are necessary to specify or maintain the islet β cell fate [94]. Nkx2.2 null mice completely lack insulin-producing β-cells and have reduced numbers of α-cells. In normal islets, a population of glucagon-expressing α-cells coexpress ghrelin, but approximately two-thirds of ghrelin-expressing cells define a new endocrine islet, ε cell population. In addition, in the Nkx2.2 mutant islet, the ghrelin-producing ε cell population has been drastically expanded at the expense of insulin- and glucagon-producing cells. [60]. Similar to the wild-type islet, ghrelin producing cells in the Nkx2.2 mutant embryonic mouse islets do not coexpress insulin, somatostatin, or PP. However, unlike its expression in wild-type islets, none of the ghrelin-producing cells in the Nkx2.2 mutant coexpress glucagon [60].

On the other hand, insulinoma-associated protein (IA)-2β is a β-cell autoantigen for type 1 diabetes. It is localized in secretory granules in pancreatic β-cells or neuroendocrine cells [95]. Stable overexpression of IA-2β inhibited GSIS in MIN6 cells when performed in medium containing glucose. Doi et al. observed that ghrelin inhibits GSIS in MIN6 cells and that the concentrations of ghrelin inhibiting GSIS were very close to those of ghrelin enhancing IA-2β expression, suggesting that ghrelin may inhibit GSIS via enhancement of IA-2β expression [93]. Incubation of cultured MIN6 cells with increasing doses/times of ghrelin showed that ghrelin induced IA-2β RNA and protein expression dose dependently. The blockage of IA-2β expression with siRNA provoked that the inhibitory effects of ghrelin or overexpression of IA-2β on GSIS were ameliorated, providing direct evidence of the links between ghrelin, IA-2β, and GSIS; changes in insulin content in the cell lysates or in insulin mRNA expression were not observed. [93].

Some of the results obtained with this type of techniques are displayed in Table 3.

2.4. Unacylated Ghrelin. Acylated ghrelin accounts for less than 10% of the total ghrelin; the majority of circulating ghrelin is unacylated. Although UAG does not possess GH releasing activity, it is not biologically inactive. Several studies demonstrated a clear metabolic role for UAG; it is able to share with ghrelin antiproliferative effects on human breast and prostate cancer lines [97, 98], has negative inotropic effects on papillary muscle [99], and can stimulate bone marrow adipogenesis [28]. These effects of UAG could

Table 3: Results obtained with cellular cultures and pancreatic perfusion that contribute to data about ghrelin role on glucose and insulin metabolism.

Cellular type/Perfusion	Treatment	Dose	Insulin release	Glucose output	Reference
Islets from normal rats	AG + glucose versus glucose	10^{-12}M AG + 2.8 mM glucose	Not change		[54]
	AG + glucose versus glucose	10^{-12}M AG + 8.3 mM glucose	Enhanced		
Islets from normal rats	AG + glucose versus glucose	10^{-8}M AG + 2.8 mM glucose	Not change		[58]
	AG + glucose versus glucose	10^{-8}M AG + 8.3 mM glucose	Diminished		
Islets from normal rats	AG + glucose versus glucose	10 nM AG + 20 mM glucose	Diminished		[92]
	UAG + glucose versus glucose	1 μM + 20 mM glucose	Not change		
	AG + glucose + YIL-781 versus glucose	10 nM AG + 20 mM glucose + 1 μM YIL-781	Not change		
	Glucose + YIL-781 versus glucose	20 mM glucose + 1 μM YIL-781	Not change		
Islets from normal rats	GHRP-6 versus placebo	1 μM GHRP-6	Enhanced		[58]
	SPA versus placebo	1 μM SPA	Enhanced		
Ghrelin KO mouse islets	Glucose ghrelin KO versus glucose wildtype	8.3 mM and 16.7 mM glucose	Enhanced		[91]
Min 6 cells	AG + glucose versus glucose	1–10 nM AG + 22.2 mM glucose	Diminished		[93]
Hepatocytes from pigs	AG versus placebo	100 nM AG		Enhanced	[96]
	UAG versus placebo	100 nM UAG		Diminished	
	UAG + AG versus AG	100 nM AG + 100 nM UAG		Diminished	
Pancreas of rat perfused in situ	Ghrelin + glucose versus glucose	10 nM ghrelin + 5.5 mM glucose	Not change		[90]
	Ghrelin + glucose versus glucose	10 nM ghrelin + 9 mM glucose	Diminished		
Pancreas of rat perfused in vitro	Ghrelin + glucose versus glucose	10 nM ghrelin + 8,3 mM glucose	Diminished		[91]
	GHRP-6 + glucose versus glucose	1 μM GHRP-6 + 8.3 mM glucose	Enhanced		
	UAG + glucose versus glucose	10 nmol/l UAG + 8.3 mM glucose	Not change		

not be antagonized by administration of synthetic GHSR1a antagonists [28] as UAG is unable to bind the classical GHSR1a, which recognizes ghrelin in its acylated form only [9]. The signal transduction mechanism(s) for effects of UAG has not been determined. Evidences that UAG is an active peptide implies the existence of GHSR subtypes that recognize and bind ghrelin independently of its acylation. These binding sites have already been demonstrated in the cardiovascular system and in the pancreas [21, 98, 100]. Besides the effects above mentioned, several studies suggested a role of UAG on glucose metabolism. Broglio and colleagues suggested that ghrelin could have a dualistic effect on glucose homeostasis; its effect on insulin secretion and sensitivity could depend on its state of acylation. They observed that in healthy humans, the administration of UAG alone did not induce any change in glucose and insulin levels compared to placebo. Nevertheless UAG counteracts the effects of AG on glucose and insulin levels, but not its stimulatory action on GH, PRL, ACTH, and cortisol levels, indicating that UAG has metabolic impact, being able to antagonize the effects of AG on insulin and glucose levels, while it is really inactive from the neuroendocrine point of view [67].

Similar results were obtained in humans with pituitary insufficiency. In these patients both AG and UAG immediately increase glucose and insulin levels, when AG and UAG were injected together; this combination prevents the acute hyperglycaemic and hyperinsulinemic effects of AG and UAG when injected alone. Moreover, this combination of AG and UAG improves insulin sensitivity for many hours when compared with placebo administration and even more markedly with the aggravation of insulin sensitivity of AG administration [82].

As both AG and UAG are secreted into the portal circulation before they reach the systemic circulation, and the above reported effects of AG and UAG on glucose and insulin levels in vivo are based on measurements of systemic blood samples. Gauna et al. hypothesized that, concerning insulin secretion, assessment of insulin concentration in the portal vein might be more informative than that in the systemic circulation. They demonstred in anesthetized rats that UAG acted as a secretagogue of insulin in the portal vein. Moreover, this UAG-induced increase in insulin levels was abolished by the coadministration of AG. This study showed that UAG potently and dose-dependently enhances the insulin response to an intravenous glucose load in vivo [83]. This insulin secretagogue effect of UAG was marked in the portal vein, whereas it was scarcely detectable in the systemic circulation, suggesting that UAG plays an important role in glucose metabolism in the liver. Gauna et al. estimated that UAG slightly increased the fraction of insulin cleared by the liver, thus contributing to the augmentation of the portal-peripheral gradient of insulin [83]. Furthermore several studies support the possibility that ghrelin has a direct peripheral action on liver [73, 96]. Recently ghrelin levels have been found decreased in liver failure patients [101], a clinical condition with altered nutrition and glucose homeostasis. When Gauna et al. studied the effects of AG and UAG on primary hepatocytes; they confirmed that ghrelin in vitro induces a rapid increase of glucose output by primary hepatocytes, which suggests that AG modulates glucose homeostasis at least by acting directly on the liver. It was found that UAG itself exerts an inhibitory effect on glucose output and; as was seen in normal subjects in vivo, it is able to counteract the inductive effect of AG on glucose release [96]. The results obtained by different authors appear to indicate that the administration of UAG in humans might improve insulin sensitivity and secretion in subjects with relative or absolute GH deficiency and in the presence of GH.

These effects of UAG in the regulation of glucose metabolism might be of therapeutic interest for those pathological conditions characterized by insulin resistance and impaired insulin release.

3. Effects of Endogenous Ghrelin on Glucose and Insulin Levels

3.1. Studies In Vivo with GHSR Antagonists. In order to study the effects of endogenous ghrelin on glucose and insulin metabolism, many investigators used GHSR antagonists like modified GHRP-6 or YIL-781. In normal mice blockade of endogenous ghrelin by intraperitoneal injection of modified GHRP-6 markedly lowered fasting glucose concentrations in a few hours. Similarly during the intraperitoneal glucose tolerance test (IP-GTT), plasma glucose elevation was attenuated and insulin response was enhanced, showing a physiological role for endogenous ghrelin in the regulation of insulin release and blood glucose [58]. On the other hand YIL-781 did not affect fasting blood glucose levels. But, upon IP-GTT, the compound as well as modified GHRP-6 caused a decrease in the glucose excursion relative to the vehicle-treated animals. During an insulin tolerance test (ITT), YIL-781 did not alter the effect of insulin on blood glucose levels. This result, in combination with the effect of the compound on insulin secretion, demonstrates that, at least acutely, the GHSR1a antagonist YIL-781 improves glucose tolerance by promoting insulin release rather than enhancing insulin sensitivity. To evaluate whether GHSR1a antagonists could improve glucose tolerance in a disease model, YIL-781 was tested in the insulin-resistant diet-induced obesity (DIO) rat. In this model an oral dose of YIL-781 causes a reduction in glucose excursion. [92]. The data obtained by Esler et al. provide evidence that GHSR1a antagonists had no apparent effect on insulin sensitivity but improved glucose tolerance by stimulating insulin secretion. When ob/ob obese mice, which are a known genetic model of obesity and diabetes with insulin resistance, were peripherally administered with modified GHRP-6 during several days, plasma glucose levels diminished. This reduction in glucose levels was accompanied by a moderate decrease in serum insulin levels, suggesting that GHSR antagonists ameliorated insulin resistance in the long term [75].

The data obtained with GHSR1a antagonists (summarized in Table 4) suggest that these drugs could improve glucose tolerance and ameliorate insulin resistance in the long term and hence may be promising targets for pharmacological intervention in the treatment of type 2 diabetes.

Table 4: Effects of GHSR antagonists on glucose and insulin levels.

Species	Treatment Administration	Dose	Feeding	Measurement blood samples	Plasma glucose levels	Plasma insulin levels	Reference
Mice ob/ob	1 IP GHSR antagonist injection/12 hours versus 1 IP placebo injection	200 nmol GHRP-6/mouse	*Ad libitum* SCD	Endpoint 6-day	Diminished	Diminished	[75]
Mice ddY	1 IP GHSR antagonist injection versus 1 IP placebo injection	10 μmol GHRP-6/kg	Fasting overnight	Time course 2 hours	Diminished	Enhanced	[58]
	1 IP GHSR antagonist injection versus 1 IP placebo injection	1 μmol SPA/kg	Fasting overnight	Time course 2 hours	Diminished	Enhanced	
	1 IP GHSR antagonist + ghrelin injection versus 1 IP ghrelin injection	1 μmol GHRP-6/kg +10 nmol ghrelin/kg	Fasting overnight	End point 0.5 hours	Diminished		
Normal rats	1 IP GHSR antagonist injection versus 1 IP placebo injection	10 μmol GHRP-6/kg	Fasting overnight	End point 0.5 hours		Enhanced	[91]
Gastrectomized rats	1 IP GHSR antagonist injection versus 1 IP placebo injection	10 μmol GHRP-6/kg	Fasting overnight	End point 0.5 hours		Enhanced	
Normal rats	Oral GHSR antagonist + IP-GTT versus IP-GTT	10 mg YIL-781/kg +2 g glucose/kg	Fasting overnight	Time course 6 hours	Diminished	Enhanced	[92]
	Oral GHSR antagonist versus placebo	30 mg YIL-781/kg	Fasting overnight	Time course 6 hours	Diminished		
DIO rats	Oral GHSR antagonist +IP-GTT versus placebo + IP-GTT	3 mg YIL-781/kg +2 g glucose/kg	Fasting overnight	Time course 6.5 hours	Diminished		

3.2. Glucose and Insulin Levels in GHSR-, Ghrelin-, and Double-Knockout Animals. The knockout (KO) animals represent a good opportunity to study endogenous ghrelin functions. Plasma ghrelin concentration is inversely correlated with body weight and body fat [102]. Moreover, considering that one of the main characteristics of exogenous ghrelin is to increase food intake, body weight, and % body fat [10, 87] it was expectable that the null animal for ghrelin and/or GHSR had marked differences in the ingestion and/or body composition; however the results obtained did not show that. It seems that the type of diet, its duration, age, and nutritional status of the animals are key factors to understand the function of the hormone in the energetic metabolism as well as its effect in the homeostasis of glucose and insulin. The results obtained in rats in relation to the metabolism of glucose and insulin in knockout animals are shown in Table 5.

3.2.1. GHSR Knockout. Some investigators reported that GHSR knockout animals, in comparison with wild-type controls, had only a modest decrease in body weight when maintained on standard chow and similar levels of insulin in both fed and fasted states [106]. However GHSR null mice to 50% caloric restriction (CR) or fasting conditions on standard diet had lower blood glucose and insulin levels than standard diet fed wild-type (WT) mice suggesting enhanced insulin sensitivity [34]. These results were supported by other authors. Zigman et al. also observed that GHSR-null male mice showed lower blood glucose levels when maintained on a standard chow diet (SCD), and corresponding insulin levels were lower, although not always reached statistical significance [104].

It was observed that GHSR null mice had mean body weight and body composition comparable to those of their same-sex wildtype littermates when measured 1 week

Table 5: Glucose and insulin levels in GHSR-, ghrelin-, and double-knockout animals.

Null mice	Treatment	Dose	Food before/during experiment	Measurement blood samples	Plasma glucose levels	Plasma insulin levels	Reference
Ghrelin	KO versus wildtype		SCD 4–20 weeks old	Endpoint	Not change	Not change	[35]
Ghrelin	KO versus wildtype		SCD 4–10 weeks of age	Endpoint	Not change	Not change	[103]
Ghrelin	IP-GTT, KO versus wildtype	2 g glucose/kg	SCD, fasted	Time course-2 hours	Diminished	Enhanced	[91]
	KO versus wildtype		SCD, fed	Endpoint	Endpoint	Not change	
	KO HDF versus KO SCD		HFD 8–12 weeks old	Endpoint	Not change	Enhanced	
	IP-GTT, KO HFD versus KO SCD	2 g glucose/kg	HFD 8–12 weeks old	Time course-2 hours	Not change	Enhanced	
Ghrelin	IP-GTT, KO versus wildtype	2.5 g glucose/kg	SCD 8-week old	Time course-2 hours	Diminished	Enhanced	[36]
Ghrelin	KO versus wildtype		SCD, 6 hours fast	Endpoint	Not change	Not change	[37]
Ghrelin	AG versus saline	2.5 g glucose/kg + 1 IP injection of 150 nmol AG/kg	SCD 8-week old, 18 h fast	Time course-2 hours	Enhanced	Diminished	[36]
	ITT, KO versus wildtype	0.75 U/kg	SCD 8-week old, 8h fast	Time course-2.5 hours	Diminished		
	Hyperinsulinemic-euglycemic clamp, KO versus wildtype		SCD 8-week old		GIR enhanced during clamp		
	KO versus wildtype		SCD 12-week old	Endpoint	Not change	Not change	
	KO.ob/ob versus wildtype.ob/ob		SCD 12-week old	Endpoint	Diminished	Enhanced	
	KO.ob/ob versus wildtype.ob/ob		SCD 12-week old, 24 hours fast	Endpoint	Diminished	Not change	
Ghrelin	IP-GTT, KO versus wildtype	2 g glucose/kg	SCD, 6 hours fast	Time course-2 hours	Not change	Not change	[37]
	ITT, KO versus wildtype	1 U/kg	SCD, 6 hours fast	Time course-2 hours	Not change	Not change	
Ghrelin	O-GTT, KO DIO versus wildtype DIO	1 g glucose/kg	HFD 8–23 weeks old, 16 hours fast	Time course-2 hours	Not change	Diminished	[33]
Ghrelin	KO versus wildtype		10-week SCD + 40 days on 50% caloric restriction with SCD	Time course every 2 days	2–16 day diminished		[34]
Ghrelin	KO DIO versus wildtype DIO		HFD 8–23 weeks old	Endpoint	diminished	Diminished	[33]
	IPGTT, KO DIO versus wildtype DIO	1 g glucose/kg	HFD 8–23 weeks old, 16 hours fasted	Time course-2 hours	Not change	Diminished	
	Hyperinsulinemic-euglycemic clamp, KO DIO versus wildtype DIO	10 mU insulin/kg + constant infused insulin 5 mU/kg/min + infused 20% glucose	HFD 8–23 weeks old, 16 hours fast	Time course-1.5 hours	GIR enhanced		
	Hyperglycemic clamp KO DIO versus wildtype DIO	20% glucose at rates that stabilized blood glucose at 300 mg/dl	HFD 8–23 weeks old, 16 hours fasted	Time course-1.5 hours		Diminished	

Table 5: Continued.

Null mice	Treatment	Dose	Food before/during experiment	Measurement blood samples	Plasma glucose levels	Plasma insulin levels	Reference
	KO versus wildtype		24-week SCD	Endpoint	Not change	Not change	
	KO versus wildtype		24-week SCD/18 h-fasting	Endpoint	Diminished	Diminished	
GHSR	KO versus wildtype		10-week SCD + 40 days on 50% caloric restriction with SCD	Time course every two days	2–28 day diminished		[34]
	KO versus wildtype		14-week SCD +10-week HF + 18 h-fasting	Endpoint	Not change	Not change	
	KO versus wildtype		14-week SCD +10-week HF	Endpoint	Not change	Not change	
	IP-GTT KO versus wildtype	2 g glucose/kg	SCD, 6 hours-fasting	Time course 2 hours	Not change	Not change	
GHSR	ITT KO versus wildtype	1 U/kg	SCD, 6 hours-fasting	Time course 2 hours	Not change	Not change	[37]
	KO versus wildtype		SCD, 6 hours-fasting	Endpoint	Not change	Not change	
GHSR	KO versus wildtype		SCD 4–19 weeks old	Endpoint	Diminished	Diminished	[104]
GHSR	KO versus wildtype		SCD 8-week old	Endpoint	Diminished	Diminished	[105]
Ghrelin + GHSR	IP-GTT dKO versus wildtype	2 g glucose/kg	SCD, 6 hours-fasting	Time course-2 hours	Not change	Not change	
	dKO versus wildtype		SCD, 6 hours-fasting	Endpoint	Not change	Not change	[37]
	ITT dKO versus wildtype	1 U/kg	SCD, 6 hours-fasting	Time course-2 hours	Not change	Not change	

after weaning or exposure to standard chow. However, several weeks of exposure to HFD after weaning resulted in significantly less accumulation of both body weight and body fat content in GHSR null mice, as compared with littermate controls, and these animals presented resistance to diet-induced obesity [33, 104]. Interestingly, these differences are masked in HFD fed mice only in their adult stage; in this situation the deletion of GHSR does not prevent DIO or weight gain after weight loss [34].

Once more, in GHSR null mice fed with HFD, several measures of greater insulin sensitivity were observed, including lower fasted blood glucose and plasma insulin, lower insulin levels during glucose tolerance tests, and improved performance in hyperinsulinemic-euglycemic and hyperglycemic clamp studies [33].

On the other hand, the results obtained in RQ for GHSR null mice are discrepant. The knockout created by Nakano et al. presented decreased RQ during long-term HFD study that represents a shift in metabolic fuel preference toward the utilization of fat as an energy substrate [104]. On the contrary, Longo's animals have higher RQ, indicating a preference for carbohydrate as fuel regardless of gender or diet. These data could suggest that ghrelin's effects on metabolic fuel preference are transient and may not have a significant effect throughout the lifespan. Perhaps adult GHSR null mice are subject to metabolic adaptations especially in regard to energy intake and expenditure. However the range of RQ values was wider in knockout mice, indicating greater metabolic flexibility in these animals [33].

3.2.2. Ghrelin Knockout. When ghrelin KO animals and WT controls were exposed to prolonged and earlier HFD (after weaning), ghrelin KO mice showed mean body weight and mean body fat percentage that were lower than those of similarly treated wild-type controls [107]. This diet produced glucose intolerance and insulin resistance in wild type mice [91, 107]. By contrast, ghrelin knockout mice fed with HFD showed close to normal glucose responses and markedly enhanced insulin responses to IP-GTTs compared with control ghrelin knockout mice fed with SCD [91]. As a possible underlying mechanism Dezaki et al. suggested that lack of ghrelin and its insulinostatic activity may raise the maximal capacity of glucose-induced insulin release and enable islets to secrete more insulin to meet an increased demand associated with HFD–induced obesity, thereby achieving normoglycemia [91]. Moreover Ghr KO mice on the HFD presented lower levels of glucose and insulin as well as lipids compared with wild-type on this diet; hence ghrelin as well as GHSR null mice exposed to HFD after weaning

exhibit greater glucose tolerance. The results of GTTs and ITTs were similar to those previously observed for the same authors with pharmacological blockade of ghrelin action [58], reinforcing the concept that endogenous ghrelin serves as a regulator of insulin release and of glycemia. However, when ghrelin null mice and wild type mice were subjected to acute exposure to HFD late in life, slight differences in body composition between ghrelin KO animals and wild-type controls were reported, and no change in glucose and insulin levels [103]. Comparable results were obtained in animals fed with standard chow, where insulin and glucose levels did not display changes [35]. Moreover these animals did not display differences in cumulative food intake on standard chow or body weight change and food intake in response to reexposure to food following a fast [35]. However, Sun et al. realized several studies where they observed, that compared to WT, ghrelin KO mice exhibited significantly lower glucose levels after IP-GTT and correspondingly higher levels of insulin. In addition, the initial insulin response at 15 minutes was significantly higher in the ghrelin KO compared to WT mice [36]. When ghrelin KO mice were subjected to 50% caloric restriction, they had lower blood glucose levels than their WT littermates suggesting that ghrelin would be involved in providing a counterregulatory glucose response during negative energy balance [34].

In another line of ghrelin knockout mice, glucose levels were monitored in lean mice (wild-type and ghrelin KO) and obese mice (wild type ob/ob and ghrelin KO ob/ob) at different ages [36]. The lean mice were euglycemic; as expected, glucose and insulin levels were elevated both in ob/ob and ghrelin KO ob/ob mice. However blood glucose was elevated at age 4 weeks in ob/ob mice and at 6 weeks in ghrelin KO ob/ob mice, and although obesity was as severe as in ob/ob mice, ghrelin KO ob/ob exhibited lower glucose levels and their blood glucose normalized upon fasting. Hence, ablation of ghrelin markedly improved glucose homeostasis in ob/ob mice [36]. The improvement in glucose homeostasis in ghrelin KO ob/ob mice was accompanied by increased serum insulin levels. Remarkably, compared to ob/ob mice, ghrelin KO ob/ob mice displayed reduced blood glucose concentrations after IP-GTT, which was accompanied by increased insulin secretion [36]. When ghrelin KO mice, maintained from weaning on regular chow, were subjected to IP-GTT, ghrelin treatment produced higher blood glucose and markedly lowers insulin levels, showing that ghrelin acutely suppresses insulin release, suggesting that the improved glucose tolerance which was observed in ghrelin KO ob/ob mice fed with HFD during IP-GTT could be a consequence of ghrelin-ablation. Moreover, ghrelin ablated mice presented greater reductions in glucose levels 30 minutes following ITT suggesting increased insulin sensitivity. When the authors subjected WT and ghrelin KO mice to euglycemic hyperinsulinemic clamp studies, basal hepatic glucose production rate was the same in both genotypes. But during the low-dose insulin clamp, suppression of glucose production was higher in ghrelin KO mice, proposing once more that the liver of ghrelin KO mice was more sensitive to insulin. Furthermore, an increase in glucose infusion rate (GIR) and an increase in GDR were detected, indicating that besides increasing glucose-induced insulin secretion, ghrelin ablation increased peripheral insulin sensitivity and improves glucose tolerance [36].

Wortley et al. found a trend toward decreased weight and leaner body composition in male ghrelin knockout mice after 6 weeks on the HFD, which could be explained by a decrease in RQ observed only in these animals; therefore the constitutive absence of ghrelin causes a distinct shift toward lipid metabolism during consumption of an HFD [103].

3.2.3. Ghrelin/Ghrelin Receptor Double Knockout (dKO) Mice. Pfluger and colleagues created ghrelin/ghrelin receptor double knockout mice. Plasma glucose and plasma insulin levels did not differ between aged WT and dKO mice after an overnight fast. An IP-GTT overall failed to reveal significant differences in glucose tolerance between genotypes. Mice deficient in either ghrelin, GHSR, or both showed lower glucose peak levels at a single time point (15 minutes after the injection) suggesting a slightly faster release of insulin. Mice were subjected to an ITT; in ghrelin KO mice glucose levels were similar to WT mice. In dKO and GHSR KO mice, glucose levels, however, dropped more rapidly. In general, glucose levels of dKO and GHSR mice tended to remain lower throughout the 120 minutes of the ITT, compared with WT mice. However, although integrated glucose levels in both GHSR KO and dKO mice tended to be lower compared with WT control mice, the deficiency of ghrelin, its receptor, or both did not seem to have a major impact on overall insulin sensitivity or the overall regulation of glucose homeostasis. They observed substantial but mostly insignificant trends in glucose tolerance and insulin sensitivity. Importantly, all these data were obtained from mice maintained on normal standard chow diet [37].

Pfluger et al. speculated that their mouse mutants still may exhibit some level of ghrelin signaling, although by definition they genetically deleted ghrelin [9], its putative ghrelin associated peptide [108], ghrelin splice variants [109], and the constitutively active ghrelin receptor GHSR [110]. For this reason the authors suggested that the existence of both additional ligand and additional receptor, coded for by genes other than the ghrelin and the GHSR gene, could explain why the dKO mouse shows a phenotype that still has to be categorized as very mild.

In summary, the results obtained with knockout animals seem to indicate that ghrelin is not a critical orexigenic factor. Nevertheless, the ghrelin/GHSR pathway plays a role in glucose homeostasis by regulating insulin sensitivity and glucose sensing. If it was confirmed that ghrelin ablation restores the first-phase of insulin secretion, as observed in ghrelin knockout ob/ob mice, [36] this could have clinical relevance, because in humans the loss of first-phase insulin secretion is predictive for the development of type 2 diabetes [111]; therefore, in subjects at risk for type 2 diabetes, treatment with a ghrelin antagonist may prove beneficial. Kelley and colleagues proposed a central pathophysiological construct to describe the altered metabolism associated with insulin-resistant and glucose-intolerant states: the concept of

Table 6: Relation between overexpression of ghrelin in different tissues or cellular types and glucose-insulin levels.

Transgenic animals	Ghrelin levelstransgenic versus wildtype	Treatment	Food before/during experiment	Treatment duration	Plasma glucose levels transgenic versus wildtype	Plasma insulin levels transgenic versus wildtype	Reference
Ghrelin is overexpressed inadipose tissue	AG: not change UAG: enhanced	Nothing	*Ad libitum*	Endpoint		Enhanced	[26]
		IP-GTT	16-h fast	2.5 hours	Diminished		
		IP-ITT	16-h fast	2.5 hours	Diminished		
Ghrelin is overexpressed in stomach and hypothalamus	AG: enhanced UAG: enhanced	IP-GTT	18-h fast	2.5 hours	Enhanced	Diminished	[113]
		IP-ITT	4-h fast	2.5 hours	Not change	Not change	
Ghrelin is overexpressed in pancreas	AG: not change UAG: enhanced	Nothing	Overnight fast	Endpoint	Not change	Not change	[39]
		IP-GTT	Overnight fast	2 hours	Not change	Diminished	
		IP-GTT	Overnight fast	2 hours	Not change	Diminished	
		ITT	4-h fast	3 hours	Not change	Not change	
Ghrelin is overexpressed in hypothalamus, cortex and liver	AG: enhanced UAG: enhanced	IP-GTT	20-h fast	2 hours	Enhanced	Not change	[114]
Ghrelin is overexpressed in hypothalamus, cortex and liver	AG: not change UAG: enhanced	IP-GTT	20-h fast	2 hours	Not change	Not change	
Ghrelin is overexpressed in wide variety of tissues	AG: not change UAG: enhanced	Nothing	*Ad libitum*	Endpoint	Not change	Not change	[27]

"metabolic inflexibility" [112]. Metabolically normal people can adapt to the discontinuities in fuel availability and utilization present in daily life, whereas diabetic people cannot. Metabolic inflexibility means that insulin-resistant individuals are unable to efficiently increase carbohydrate utilization, even when carbohydrates are plentiful. This is a rewording of the essence of impaired glucose tolerance (and insulin resistance). The results obtained in RQ of GHSR KO mice seem to indicate greater metabolic flexibility and hence improve glucose tolerance. When null mice were fed with either SCD or HFD, their body weights were not different from that of their WT littermates on the same diet. However ghrelin and GHSR null mice were resistant to DIO when were fed with HFD immediately after weaning. But ablation of the ghrelin/GHSR signal does not prevent DIO raised on SCD and then fed with HFD as adults. Considering these data it would be possible to conclude that the loss of ghrelin signalling protects against several fatty diet-induced features of metabolic syndrome and improves insulin sensitivity. But all these results should be taken with caution, considering that the age of exposition and the type of diet seem to be key factors to observe the effect of ghrelin on glucose and insulin metabolism.

3.3. Glucose and Insulin Levels in GHSR and Ghrelin Transgenic Animals. There are some studies realized with ghrelin transgenic mouse with overexpression of ghrelin in different tissues or cellular types (Table 6). Many of them presented plasma UAG levels higher than those of their nontransgenic littermates whereas that plasma acylated ghrelin levels did not change [27, 38, 39]. Hence these models can serve to study the role of desacyl as well as acylated ghrelin in the regulation of glucose metabolism and insulin release.

Iwakura and colleagues developed and analyzed rat insulin II promoter-ghrelin transgenic mice (RIP-G Tg) in which pancreatic ghrelin concentration was higher than that of nontransgenic littermates; moreover in control mice ghrelin was not detected in β-cells by immunohistochemistry. Ghrelin transgene driven by RIP was considered to be expressed in β-cells, although higher expression levels of ghrelin mRNA were also found in the brain of RIP-G Tg compared with that of nontransgenic littermates. When these animals were subjected to IP-GTT, plasma insulin levels were significantly lower in Tg mice than those in nontransgenic littermates, although there was no significant difference in plasma insulin levels between RIP-G Tg and nontransgenic littermates on the fasting state [39]. The glucose-stimulated insulin secretion of RIP-G Tg was decreased without changes in glucose levels, but there were no abnormalities with the arginine-induced insulin secretion, pancreatic histology, pancreatic insulin mRNA levels, and insulin content in the RIP-G Tg. When the authors did several tests from isolated islets of RIP-G Tg, they found that insulin secretion as well as immunoreactivity of glucose transporter in the pancreatic β cell, in RIP-G Tg β cells, was indistinguishable from that of nontransgenic littermates, indicating that insulin secretion was not affected by overexpression of ghrelin transgene in vitro, although it was affected in vivo [39]. When these animals were subjected to ITT, they showed a tendency to lower blood glucose levels. Considering the results, the authors suggested that the suppression of insulin secretion of RIP-G Tg is likely due to the effect of desacyl ghrelin on insulin sensitivity [39]. Nevertheless these results do not agree with others studies.

Reed et al. created mice with ghrelin overexpressed in neurons using the neuron-specific enolase (NSE) promoter

sequences and mouse ghrelin cDNA (NSE-ghrelin). Ghrelin expression in NSE-ghrelin brain tissues was increased compared with wild-type mice; it was also increased to a much smaller extent in liver of these mice, but in stomach or duodenum did not differ from wild-type mice. They worked with two lines of NSE-ghrelin mice: one line with increased circulating AG and UAG (L43) and one line with only UAG (L73). In both lines young NSE-ghrelin mice had normal glucose tolerance; however, L43 NSE-ghrelin mice, but not L73 mice, developed glucose intolerance at 32 week of age. Despite the impaired glucose tolerance in L43 mice, insulin levels did not differ from those of wild-type mice [114]. However, unlike the studies from Iwakura et al. plasma insulin levels did not change after IP-GTT in those animals with high levels of UAG (L73). The differences between both studies can be the consequence of several factors like age or others. In another line of transgenic mice, Zhang et al. created animals in which the ghrelin gene is overexpressed in adipose tissue via the fatty acid-binding protein-4 (FABP4) promoter. Transgenic mice overexpressing the ghrelin gene in adipose tissue demonstrated significant increases in plasma concentrations of UAG, whereas ghrelin remained unchanged. Overexpression of ghrelin from the FABP4 promoter reduced the weight of white adipose tissues and resistance to HFD-induced obesity [26]. Alterations in glucose tolerance and insulin sensitivity tests were detected in FABP4-ghrelin transgenic mice. When these animals were subjected to IP-GTT, glucose levels were significantly lower than in controls; however FABP4-ghrelin transgenic mice had a greater hypoglycemic response to insulin administration than control animals. It seems that UAG improves glucose tolerance and insulin sensitivity, providing more evidences that UAGs play a role in the regulation of glucose metabolism. These data are strengthened by the observation that plasma insulin levels are elevated in transgenic mice [26].

Recently, Bewick et al. generated a mouse model with increased ghrelin expression and production in stomach and brain. Ghrelin transgenic mice exhibited increased circulating AG and total ghrelin which was associated with hyperphagia and increased energy expenditure. These animals were subjected to IP-GTT and ITT; the animals were glucose intolerant due to an inhibition of glucose-stimulated insulin release but without change in insulin sensitivity [113].

4. Mechanism of Action

In order to understand how ghrelin can modify glucose and insulin homeostasis, it is important to study the mechanism of action exerted by ghrelin in tissues implied in carbohydrate metabolism.

4.1. Liver. *De novo* synthesis of glucose in the liver from precursors such as lactate, gluconeogenic amino acids, and glycerol is a central mechanism to provide the organism with glucose in times of starvation [115], a natural situation in which ghrelin levels are increased [10, 30]. On the other hand, when glucose is directly available from external resources, gluconeogenesis is dispensable and consequently needs to be shut off. Integration of these events is complex and occurs through various hormonal and nutritional factors. The principal parameters affecting hepatic glucose output are the concentrations of the available glucogenic substrates and the activity of a few regulatory enzymes. The activity of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase is regulated by transcriptional and nontranscriptional mechanisms, whereas the third key enzyme fructose-1,6-bisphosphatase (FBPase) is also regulated through competitive inhibition by fructose 2,6-bisphosphate. Insulin is the most important hormone that inhibits gluconeogenesis, through the activation of the insulin receptor (IR). It acts predominantly by suppressing the expression of the genes for the key gluconeogenic enzymes PEPCK and G6Pase [116]. In the H4-II-E-cells (rat hepatoma cell line) and HepG2 cells (human hepatocellular carcinoma cell line) ghrelin was shown to stimulate insulin receptor substrate 1^{o}(IRS1) and its downstream molecules, including growth factor receptor-bound protein 2 (Grb2) and mitogen-activated protein kinase (MAPK). Whereas on the other hand, it diminished phospho protein kinase B (pAKT) and phospho-glycogen synthase kinase (pGSK) levels in both cell lines and upregulated gluconeogenesis in H4-II-E-cells by attenuating the effect of insulin on the expression on PEPCK [73].

AKT is a key protein kinase downstream of the insulin receptor [117] and its activation plays a key role in suppressing hepatic gluconeogenesis [118, 119], since GSK-3, which phosphorylate glycogen synthetase (GS) is inhibiting, is phosphorylated by AKT, and this phosphorilation inactivates GSK-3 kinase activity, suppressing hepatic gluconeogenesis resulting in enhanced glycogen deposition [118].

Forkhead box O1 (FOXO1) and peroxisome proliferator activated receptor-γ-coactivator (PGC)-1α are two transcriptional components that are targets of insulin signalling and that can activate the process of gluconeogenesis in liver. FOXO1 has been shown to bind directly to the promoters of gluconeogenic genes and activate the process of glucose production [120–122]. It is directly phosphorylated by AKT. This phosphorylation results in exclusion of FOXO1 from the nucleus. A second transcriptional component controlled by insulin and having a role in gluconeogenesis is the coactivator PGC-1α. It is induced in the liver during fasting and is elevated in several models of diabetes or deficiency in insulin signalling. Notably, expression of PGC-1α at physiological levels turns on the entire program of gluconeogenesis [123]. PGC-1α hepatic transcription has been reported to be downregulated by AKT activation through forkhead transcription factor FOXO1 phosphorylation and nuclear exclusion [124].

Barazzoni et al. observed that in rats sustained ghrelin administration reduced hepatic phospho/total-AKT (P/T-AKT) and P/T-GSK [86]. These changes in AKT-GSK signalling were associated with enhanced PGC-1α expression. Reduced liver AKT signaling could potentially contribute to concomitant blood glucose increments, preserving hepatic glucose production in calorie-restricted status [86].

The routes that have been modified after treatments with ghrelin in liver and which could modify plasma glucose levels are represented in Figure 1.

4.2. Pancreas. Insulin secretion is accurately linked to blood glucose levels in the physiological range. The role of the β-cells is to sense an increase in the concentration of nutrients in the blood and to synthesize, package, and release insulin to control blood glucose homeostasis. Various agents as amino acids (particularly arginine and leucine) and fatty acids can increase the secretion of insulin, but only in the presence of facilitating concentrations of glucose (above 3 mM), whilst nonmetabolizable analogues of glucose such as galactose or fructose are inactive as secretagogues [125]. The above fuel secretagogues are initiators of secretion, but there are also other agents including neurotransmitters, glucagon-like peptide (GLP-1), gastric inhibitory peptide (GIP), and pituitary adenylate cyclase-activating polypeptide (PACAP) that act as "potentiators", enhancing secretion only at permissive concentrations of fuel secretagogues. These molecules usually act via G-protein coupled receptors and the generation of classical second messengers such as cAMP and Ca^{2+} [126]. The first studies about stimulus-secretion coupling in β-cells early concluded that glucose must be metabolized by β-cells to induce insulin secretion, Ca^{2+} has an essential role in insulin secretion, and pancreatic β-cells are electrically excitable [125].

Islet β-cells are equipped with high-capacity glucose transporters located at the plasma membrane that are known as glucose transporters-2 (GLUT-2) [127]. GLUT-2 is required for efficient glucose-stimulated insulin secretion, as demonstrated by studies in transgenic mice [128, 129]. β-cells contain a high Km glucokinase (glucose-phosphorylating hexokinase, GK), which displays strongly cooperative kinetics and has thus been termed the β-cell "glucose sensor." The reduction in β-cell GK levels was associated with reduced capacity to secrete insulin in response to glucose [130]. Glucose stimulation of insulin secretion involves two pathways: the triggering of ATP-sensitive K^+channel- (KATP-) dependent pathway, and the amplifying of KATP channels-independent pathway. The rise in blood glucose induces an increase in β-cell glucose metabolism, resulting in increased production of ATP from several sources: glycolysis, mitochondrial glucose oxidation, and active shuttling of reducing equivalents from the cytosol to the mitochondrial electron transport chain. The resultant increase in ATP/ADP ratio inhibits KATP channels, depolarizing the plasma membrane, leading to opening of the voltage-dependent calcium channels (VDCCs), which allows calcium influx. The resultant intracellular calcium concentration ($[Ca^{2+}]i$) rise triggers exocytosis of the insulin-containing granules (reviewed in [131]). However, an expanding bulk of data also makes it apparent that this KATP-channel dependent mechanism of glucose-stimulated insulin secretion does not fully describe the islet glucose response, and signals other than changes in ATP: ADP ratio have been increasingly implicated as important regulators of insulin secretion in recent years. The voltage-dependent K^+ channels (Kv) [132] are thought to repolarize glucose-stimulated action potentials and inhibit Ca^{2+} entry through voltage-gated Ca^{2+} channels; therefore, Kv channels serve as negative regulators of insulin secretion, and Kv channel antagonists are insulinotropic in a glucosedependent manner. Kv channels are comprised of the pore-forming α subunits (Kv2.1 is thought to be the predominant isoform in islet β-cells) and regulatory β-subunits, analogous to the pore-forming and regulatory subunits of the KATP channel complex. Kv channel β–subunits are proposed to act as intracellular redox sensors, and an increase in cytosolic NADPH : NADP ratio in patch-clamped-cells was shown to be associated with an increased rate of inactivation of the Kv channel [133]. Inhibition of Kv channels by NADPH, derived from pyruvate cycling, could serve as a logical complementary mechanism to ATP regulation of KATP channel activity, since suppression of Kv channels would slow membrane repolarization, allowing the effects of KATP channel inhibition to be sustained through a second phase of insulin secretion (Figure 2). But this model is not fully established (reviewed in [134]).

In rat isolated islets, several works showed that endogenous and exogenous ghrelin suppressed glucose-induced insulin release [58, 91, 92, 135]. Dezaki et al. presented ghrelin signalling in β-cells. They observed that in rats ghrelin of both endogenous and exogenous origin resulted in pertussis toxin- (PTX-) sensitive decrease in plasma insulin concentrations, contrasting with PTX-insensitive increase in GH levels by ghrelin [58, 136]. PTX catalyzes the ADP-ribosylation of the α subunits of the heterotrimeric G proteins G_i, G_o, and G_t. This prevents the G proteins from interacting with G protein-coupled receptors on the cell membrane, thus interfering with intracellular communication. Since the Gα subunits remain in their GDP-bound, inactive state, they are unable to inhibit adenylyl cyclase, thus keeping levels of adenylyl cyclase and cAMP elevated [137]. In intact cells, PTX inhibited a number of insulin-stimulated cellular events, such as glucose transport and its metabolism. The function of endogenous ghrelin was assessed by the effects of GHSR antagonist in vivo and in rats treated with ghrelin and PTX. In addition, studies with isolated islets from ghrelin-KO mice observed that modified GHRP-6 increased plasma insulin concentrations after IP administration, indicating suppression of insulin levels by endogenous ghrelin. The insulinostatic effect of ghrelin was unaltered by pretreatment with phospholipase C (PLC) inhibitor. However the effects of endogenous and exogenous ghrelin on insulin levels were not observed in PTX-treated rats. In islets isolated from ghrelin-KO mice, glucose-induced insulin release was greater than those from wild-type mice. This enhancement was blunted by pretreatment with PTX. They observed that ghrelin increased Kv currents and that tetraethylammonium (TEA), a Kv channel blocker, eliminated the ability of ghrelin to suppress insulin release. Furthermore, ghrelin treatment-inhibited glucose induced $[Ca^{2+}]i$ increases in β-cells. All the effects of endogenous and exogenous ghrelin on Kv and $[Ca^{2+}]i$ as well as insulin release were blunted in the presence of PTX. This finding suggests that glucose-induced insulin release in islets is markedly decreased by endogenous

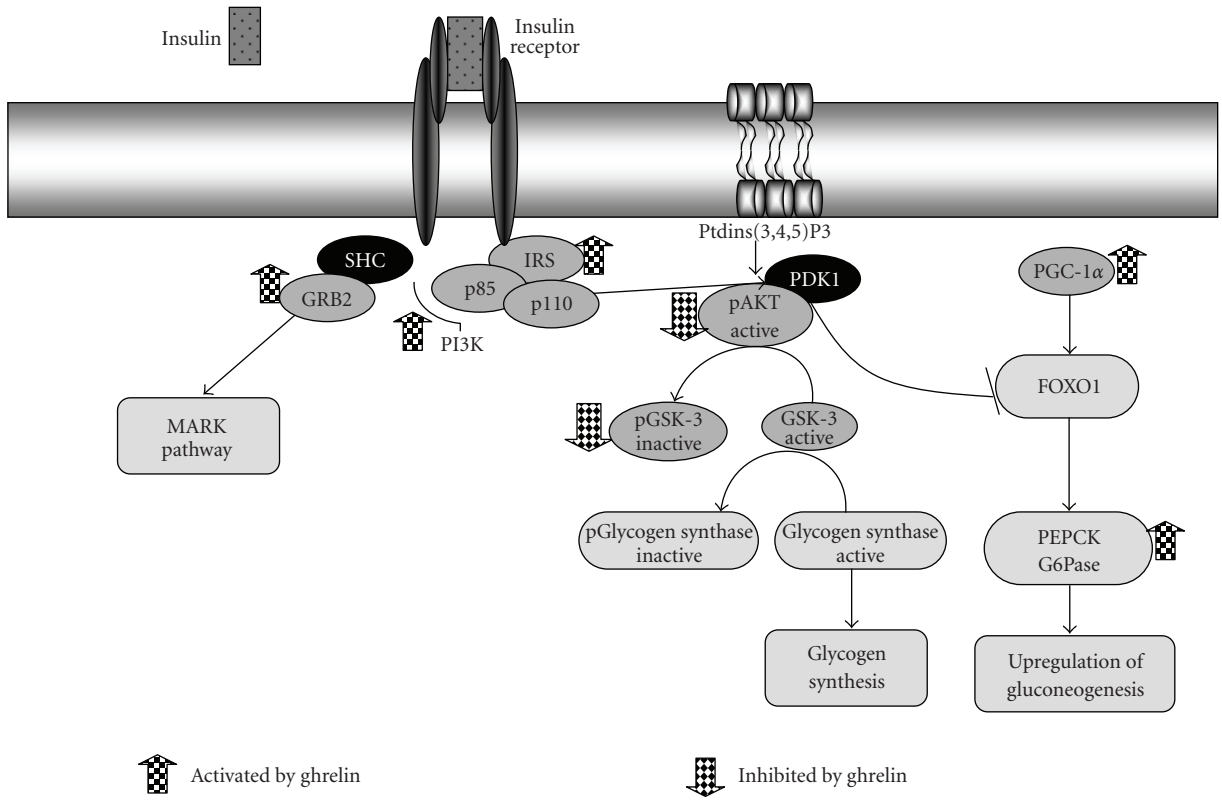

Figure 1: Regulation of hepatic gluconeogenesis and glycogen synthesis by ghrelin. Insulin activates the insulin receptor tyrosine kinase (IR), which phosphorylates and recruits different substrate adaptors. AKT is a key protein kinase downstream of the insulin receptor and its activation plays a key role in suppressing hepatic gluconeogenesis, since GSK-3, which phosphorylate glycogen synthetase (GS) is inhibiting, is phosphorylated by AKT suppressing hepatic gluconeogenesis, resulting in enhanced glycogen deposition. Sustained ghrelin administration in rats reduced hepatic AKT-GSK activation and enhanced PGC-1a expression, suggesting upregulation of gluconeogenesis and downregulation of glyconeogenesis.

ghrelin. Endogenous ghrelin in islets restrict glucose-induced insulin release via the following mechanism: ghrelin directly acts on the β-cell GH secretagogue receptor and via PTX–sensitive mechanisms attenuates glucose-induced $[Ca^{2+}]i$ signalling, partly through enhancement of TEA-sensitive delayed outward K^+currents [58, 136]. When the islet β-cells were treated with the antisense oligonucleotide specific for $G\alpha_{i2}$-subunit of G proteins, the effects of ghrelin on $[Ca^{2+}]i$ and insulin release were abolished (Figure 2). These findings demonstrate that ghrelin suppresses glucose-induced insulin release via $G\alpha_{i2}$- and Kv channel–mediated attenuation of Ca^{2+} signalling in β-cells [136].

All these data reveal that endogenous ghrelin in islets acts on β-cells to restrict glucose-induced insulin release, at least partly via attenuation of Ca^{2+} signaling, and that this insulinostatic action may be implicated in the upward control of blood glucose. These unique signaling mechanisms and molecules mediating the insulinostatic action of ghrelin on β-cells provide potential therapeutic targets for the prevention and treatment of type 2 diabetes and hyperinsulinemia [58, 136].

4.3. Adipocytes. The insulin stimulation of glucose uptake in adipose and muscle tissue occurs through a complex and as yet incompletely defined signalling pathway acting through the insulin receptor tyrosine kinase. The primary effect is to promote the movement of the GLUT-4 protein from intracellular storage sites to the plasma membrane. In the basal state, GLUT-4 is localized to a morphologically defined "tubulovesicular system" present in the intracellular compartment, while in the presence of insulin, GLUT-4 is immunolocalized to the plasma membrane of fat cells [138]. The rate-limiting step at which insulin stimulates uptake of glucose in fat is the translocation of GLUT-4 to the plasma membrane [139]. At least two discrete signalling pathways have been implicated in insulin-regulated GLUT-4 translocation. The first involves the lipid kinase phosphatidylinositol 3-kinase (PI3K) [140, 141], and the second involves the proto-oncoprotein c-Cbl [142, 143]. When insulin binds to its receptor induces a conformational change in the receptor and leads to activation of its tyrosine-kinase domain. On activation, the receptor phosphorylates several proximal substrates, including members of the IRS and c-Cbl. Tyrosine-phosphorylated IRS proteins, which are thought to be held in close proximity to the plasma membrane through association with the underlying cytoskeleton, recruit more effectors molecules, such as PI3K, to this location. Two important targets of PI3K in muscle and fat cells that have

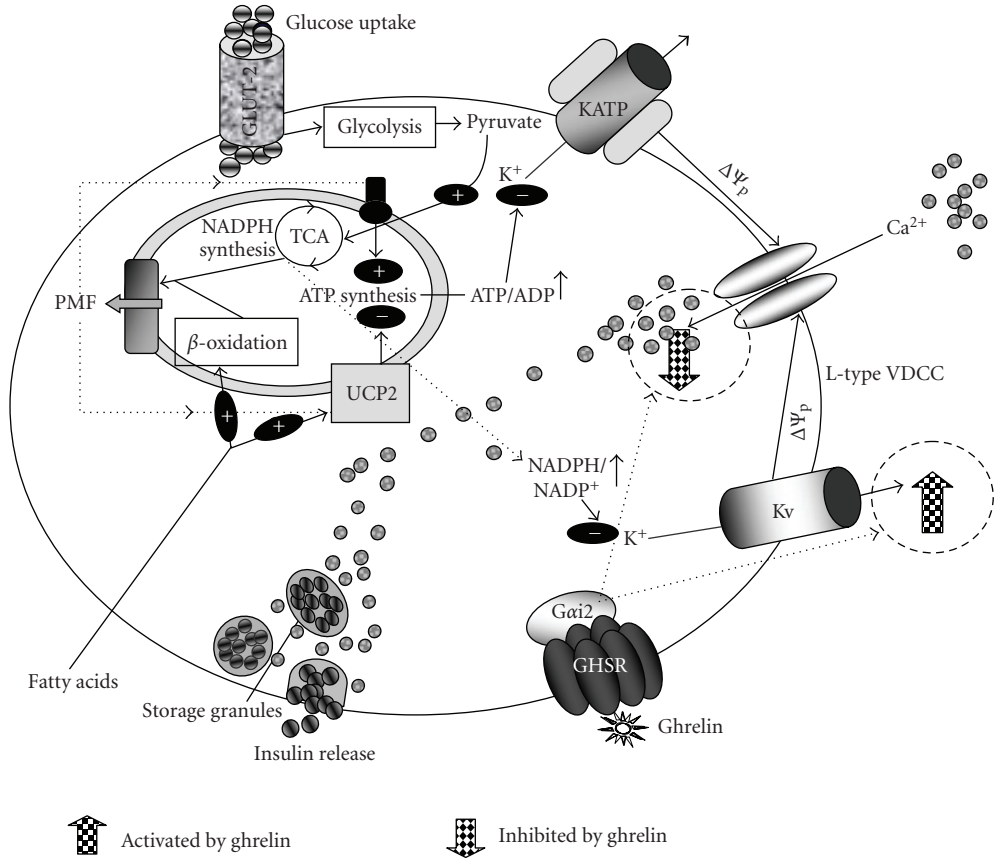

Figure 2: β-cell mechanisms of insulin release and its regulation by ghrelin. When the plasma glucose concentration rises, β-cells oxidize it. Glucose oxidation establishes a protonmotive force (PMF) that drives ATP synthesis, increasing the ATP/ADP ratio. This causes closure of KATP-channels, depolarisation of the plasma membrane potential ($\Delta\psi$p) and Ca^{2+} flux into the cell, triggering insulin release. UCP2 activity dissipates the protonmotive force, lowering ATP/ADP. Ghrelin directly acts on the β-cell and via PTX–sensitive mechanisms attenuates glucose-induced $[Ca^{2+}]$i signalling partly through enhancement of TEA-sensitive delayed outward K^+ currents resulting in decrease plasma insulin levels. PTX catalyzes the ADP-ribosylation of the α subunits of the heterotrimeric G proteins Gi, Go, and Gt. This prevents the G proteins from interacting with G protein-coupled receptors on the cell membrane thus interfering with intracellular communication. Since the Gα subunits remain in their GDP-bound, inactive state, they are unable to inhibit adenylyl cyclase, thus keeping levels of adenylyl cyclase and cAMP elevated. PTX inhibited a number of insulin-stimulated cellular events, such as glucose transport and its metabolism. Antisense oligonucleotide specific for Gαi2-subunit of G proteins blocks the effects of ghrelin on $[Ca^{2+}]$i and insulin release. Hence ghrelin presumably suppresses glucose-induced insulin release via Gαi2- and Kv channel–mediated attenuation of Ca^{2+} signalling in β-cells.

been shown to have a role in insulin-stimulated GLUT-4 translocation are the AKT and the protein kinase C (PKC). PI3K activates AKT by generating polyphosphoinositides in the inner leaflet of the plasma membrane. This acts as an anchorage site for AKT through its pleckstrin homology domain, thereby bringing it in close proximity to its upstream regulatory kinase, phosphatidylinositol-dependent kinase-1 (PDK-1). The second putative signalling pathway that has been shown to have a role in insulin-stimulated GLUT-4 translocation operates independently of PI3K and involves a dimeric complex that comprises c-Cbl and the c-Cbl-associated protein CAP. Intriguingly, whereas many growth factors trigger the activation of PI3K, AKT, and PKC in many cell types, aspects of the c-Cbl–CAP pathway, including the tyrosine phosphorylation and the expression of CAP, seem to be unique to muscle and fat cells [144]. Patel et al. examined the expression of GHSR1a in discrete adipose tissue depots and while GHSR1a expression was detected in the epididymal and pericardial deposits, it was not found in the perirenal, subcutaneous, and omental deposits. Ghrelin and des-acyl ghrelin did not affect basal deoxyglucose uptake in adipocytes from the epididymal fat deposits. However, treating isolated epididymal adipocytes with ghrelin in the presence of insulin increased insulin-stimulated deoxyglucose uptake. Des-acyl ghrelin had no significant effect on insulin-stimulated deoxyglucose uptake in isolated epididymal adipocytes. Ghrelin had no effect on basal deoxyglucose uptake in isolated perirenal adipocytes, which do not express the GHSR1a mRNA. As expected, insulin increased glucose uptake, but ghrelin in the presence of insulin did not further increase this response. Furthermore, des-acyl ghrelin did not increase insulin-stimulated deoxyglucose uptake in perirenal adipocytes. These data suggest that ghrelin may act synergistically to potentiate

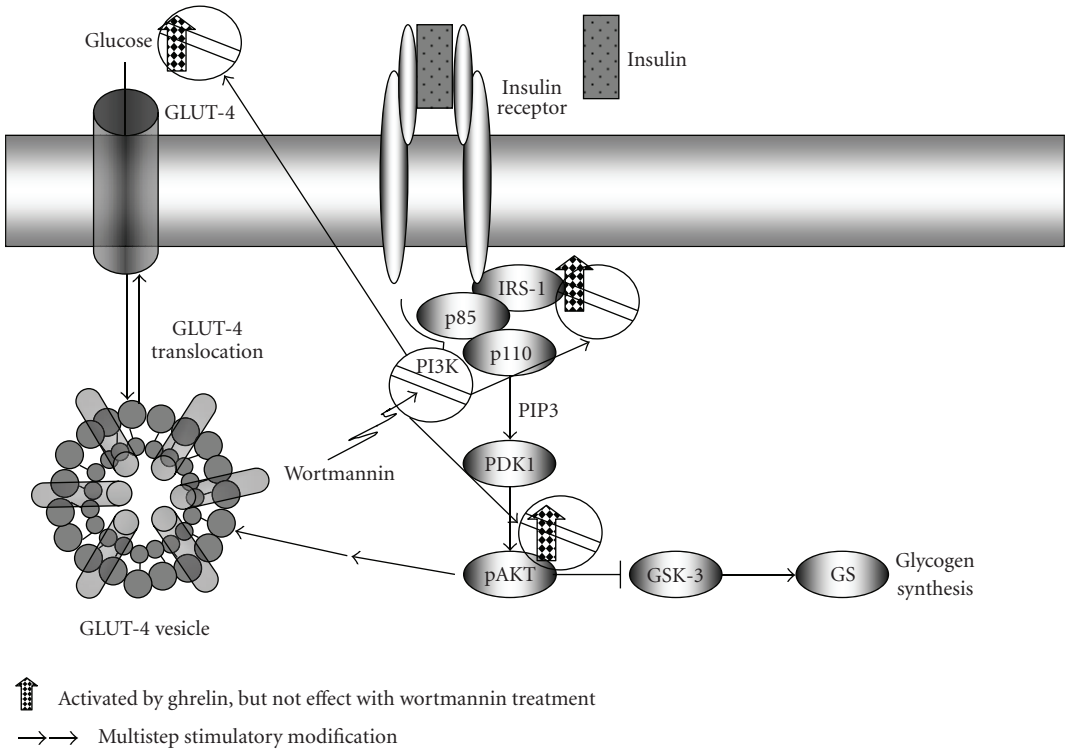

Figure 3: Glucose uptake in adipose tissue and its regulation by ghrelin. Insulin activates the IR, which phosphorylates and recruits different substrate adaptors such as the IRS family of proteins. Tyrosine phosphorylated IRS then displays binding sites for numerous signaling partners. Among them, PI3K has a major role in insulin function, mainly via the activation of the AKT/PKB and the PKCz cascades. Activated AKT induces glycogen synthesis, through inhibition of GSK-3. Insulin stimulates glucose uptake in muscle and adipocytes via translocation of GLUT-4 vesicles to the plasma membrane. GLUT-4 translocation involves the PI3K/AKT pathway. Ghrelin treatment induced increases IRS-1 and AKT phosphorylation, but when the adipocytes were treated with wortmannin, a PI3K inhibitor, completely blocked this ghrelin induced increase in glucose transport and phospho-AKT expression, suggesting that PI3K/AKT activation may mediate the effect of ghrelin on glucose transport in these adipocytes.

insulin-stimulated glucose uptake and may improve insulin sensitivity [145]. Interestingly, ghrelin did not affect insulin-stimulated glucose uptake in perirenal adipocytes, which do not express GHSR1a, and des-acyl ghrelin, which does not bind to GHSR1a, did not influence insulin-stimulated glucose uptake in epididymal adipocytes The effects of ghrelin on adipocyte glucose uptake might be expected to result in fatty acid accumulation and an increase in adiposity in the long term [145]. Kim and colleagues incubated terminally differentiated 3T3-L1 adipocytes with insulin or/and ghrelin overnight and assayed glucose transport. Insulin and ghrelin increased glucose transport and the cotreatment of insulin and ghrelin induced a further increase in glucose transport. In addition, ghrelin treatment induced increases IRS-1 and AKT phosphorylation, but when the adipocytes were treated with wortmannin, a PI3K inhibitor, completely blocked this ghrelin induced increase in glucose transport and phospho-AKT expression [146], suggesting that PI3K/AKT activation may mediate the effect of ghrelin on glucose transport in these adipocytes (Figure 3).

All these data suggest that the direct effects of ghrelin on insulin-stimulated glucose uptake are mediated by the GHSR1a and PI3K/AKT activation.

5. Pharmacological Uses of Ghrelin on Glucose-Insulin Homeostasis

Overt diabetes mellitus is defined clinically by fasting or postprandial hyperglycemia or an abnormally increased glucose excursion in response to a defined glucose load. Insulin resistance, measured as impaired glucose disposal in a hyperinsulinemic-euglycemic clamp study, is one of the earliest detectable disorder and is considered a cardinal pathophysiologic feature [147]. Fasting hyperinsulinemia is also present early in the disease process and is thought to be a compensatory mechanism to maintain euglycemia in the setting of insulin resistance [148]. Even while maintaining a healthy lifestyle, most patients need pharmacological intervention which might consist of one or a combination of the following oral medications: sulfonylureas, glinides, incretin mimetics, α-glucosidase inhibitors, metformin, or thiazolidinediones. However 30%–40% of patients are not adequately controlled by these therapies and require subcutaneous insulin injections intended to restore normoglycemia, but they can inadvertently lead to hypoglycemia, a potentially fatal consequence. Thus, new drugs and novel methods of treatment are needed.

Table 7: Potential therapeutic uses of ghrelin agonists and antagonists on glucose-insulin homeostasis.

Ghrelin agonists	Ghrelin antagonists
Insulinoma	Type 2 Diabetes mellitus
Anorexia nervosa	Metabolic syndrome
Cachexia of malignancy	Obesity

Table 8: Summary of putative ghrelin effect on glucose-insulin homeostasis and related physiological actions.

Ghrelin effects on glucose-insulin homeostasis
Increase glycemia
Decrease insulinemia
Increase food intake
Increase body weight and adiposity percentage
Increase GH secretion

Among diabetic patients, 10%–20% fall into the category of insulin-dependent diabetes mellitus (IDDM) or type 1 diabetes, which generally appears before age 40, frequently in adolescence, and results from autoimmune destruction of insulin producing pancreatic β-cells. Type 1 diabetic patients depend on insulin administration for their survival. Noninsulin-dependent diabetes mellitus (NIDDM) or type 2 diabetes is far more common than IDDM, affecting 80%–90% of diabetic patients. The prevalence of obesity and type 2 diabetes continues to increase at alarming rates [149]. Type 2 diabetes is a prototypic complex, polygenic disease with a strong heritable component, which is also heavily influenced by environmental factors, especially diet and physical activity. It appears that altered communication among tissues and loss of the ability of tissues to adapt to changing metabolic states play a critical role in the altered glucose homeostasis that leads to the development of type 2 diabetes. It is characterized by a combination of factors that affect the organism's ability to respond to insulin. The condition has two hallmark features: (1) insulin resistance and (2) compromised function of the pancreatic β-cell, such that insulin secretion is insufficient to counterpart the degree of insulin resistance. There is general agreement that type 2 diabetes, unlike IDDM, is tightly associated with obesity. Over 80% of individuals with type 2 diabetes are obese. However, only 10% of obese individuals are diabetic. In the prediabetic phase, when insulin resistance has already begun, the β-cell actually hypersecretes insulin despite normal blood glucose levels. What has defied explanation is precisely what causes this insulin resistance in the first place and how it relates in a temporal sense to the accompanying hyperinsulinemia.

Ghrelin receptor modulation could be clinically useful for different situations related with glucose-insulin homeostasis (Table 7). Several works demonstrated that ghrelin concentrations are negatively associated with fasting insulin levels, the prevalence of type 2 diabetes and insulin resistance in humans, regardless of race [102, 150, 151]. The data obtained since ghrelin discovery show that both the acylated and unacylated molecules are actively involved in the acute and long-term control of glucose metabolism and insulin sensitivity in humans, which might enable new treatment modalities for the many disorders in which insulin sensitivity is disturbed. Thus, pharmacological, immunological, and genetic blockade of ghrelin or ghrelin action in pancreatic islets all markedly enhanced glucose-induced insulin release and improve the diabetic condition. Hence ghrelin inhibition could be useful for the treatment of diabetes [152, 153].

On the other hand the ability to efficiently build fat reserves in times of nutritional abundance appears to have resulted from evolutionary pressure to protect against subsequent periods of food scarcity. The tendency to efficiently store fat in times of caloric excess appears to have become paradoxically maladaptive in settings of continuous food availability, as indicated by the present epidemic of obesity in Western societies. The data obtained in the last years seem to indicate that ghrelin may be one of the primary mechanisms by which an individual can sense changes in nutrient availability and trigger biological responses that modulate the efficiency of energy storage (and particularly fat deposition) during periods of fuel overflow or after a period of scarcity of nutrients. At present, ghrelin is the only peripheral orexigenic factor that is effective upon its intravenous administration [81]. Put in this context, the blockade of the route of ghrelin could prove useful in controlling adiposity in human obesity, as blockers of the orexigenic signal from the gastrointestinal tract to the brain, or diminishing the ability to efficiently store fat reserves. Inverse agonists of the ghrelin receptor, by blocking the constitutive receptor activity, might lower the set-point for hunger between meals [110, 154]. All these data suggest that ghrelin-ghrelin receptor modulation has the potential to improve the diabetic condition by promoting glucose-dependent insulin secretion and promoting weight loss.

In contrast, ghrelin may be useful as an orexigenic agent for the treatment of eating disorders such as anorexia nervosa. Administration of ghrelin can stimulate appetite and improve the nutritional status of these patients. However, plasma ghrelin concentrations in anorexia nervosa are high, indicating a situation of ghrelin resistance [100]. In fact, circulating ghrelin levels have been found altered in different clinical situations, like renal failure or hepatic failure [101, 155]. Ghrelin-derived drugs could also be useful in all the clinical situations associated with cachexia, such as malignancy, advanced cardiac failure, renal failure, postoperative patients, and human immunodeficiency virus-lipodystrophy. In Table 8 we summarize putative ghrelin effects on glucose-insulin homeostasis and related physiological actions.

In summary, there are multiple studies suggesting that ghrelin could have an important function in glucose homeostasis and insulin release and probably insulin action. At least theoretically ghrelin and/or its signalling manipulation could be used for the treatment or prevention of diseases of glucose homeostasis such as type 2 diabetes.

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Review Article

Ghrelin in Diabetes and Metabolic Syndrome

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Metabolic syndrome is a cluster of related risk factors for cardiovascular disease, type 2 diabetes and liver disease. Obesity, which has become a global public health problem, is one of the major risk factors for development of metabolic syndrome and type 2 diabetes. Obesity is a complex disease, caused by the interplay between environmental and genetic factors. Ghrelin is one of the circulating peptides, which stimulates appetite and regulates energy balance, and thus is one of the candidate genes for obesity and T2DM. During the last years both basic research and genetic association studies have revealed association between the ghrelin gene and obesity, metabolic syndrome or type 2 diabetes

1. Introduction

A great deal of evidence suggests that ghrelin is involved in the development of metabolic syndrome and type 2 diabetes (T2DM). Ghrelin plays also an important role in cardiovascular system. We have examined ghrelin and its genetic variation with respect to the occurrence of the components of metabolic syndrome and the risk of T2DM. In this paper we give an overview of what is known about the role of ghrelin in obesity, insulin resistance, T2DM, and cardiovascular disease, and how ghrelin is involved in the regulation of glucose, insulin, adipose tissue, and cardiovascular metabolism. We also discuss the putative role of genetic variation in the ghrelin and ghrelin receptor genes in metabolic syndrome and T2DM.

2. Ghrelin Concentrations in Obesity, Insulin Resistance, and Type 2 Diabetes Mellitus

The recent literature suggests that in addition to food intake and energy balance, ghrelin also controls glucose metabolism [1]. Furthermore, current evidence suggests that ghrelin could contribute to the metabolic syndrome [1]. It has been shown that ghrelin concentrations are reduced in different pathophysiological conditions including obesity, type 2 diabetes, and other conditions with metabolic disturbances [2, 3].

Ghrelin is a target for posttranslational modifications, which results in two different forms of circulating ghrelin: unacylated ghrelin (UAG) and acylated ghrelin (AG), in which Ser 3 is octanoylated [4]. A relative excess of AG compared to UAG has been reported in insulin resistance and related conditions [3] raising the possibility that UAG/AG ratio could play a role in development of metabolic syndrome.

Plasma ghrelin concentration has been shown to be lower in obese Caucasians when compared with lean Caucasians [2, 3, 5, 6], and in some studies higher AG concentrations have been reported in obese but otherwise healthy subjects compared to nonobese healthy subjects [3]. In persons with type 2 diabetes the fasting ghrelin concentrations are lower in obese than in lean persons and the similar ratio is with AG concentrations [7]. Circulating ghrelin concentrations are also reduced in healthy offspring of type 2 diabetic patients [8] indicating the presence of possible genetic component in the regulation of ghrelin plasma levels. When the ghrelin concentrations were compared between lean Caucasians and lean Pima Indians, it was found that the concentration was

significantly lower in Pima Indians, the population with high tendency to obesity and type 2 diabetes [5].

There are also differences in fasting and postprandial ghrelin concentrations in nondiabetic populations between lean and obese persons. Postprandial plasma ghrelin is suppressed proportional to meal calorie content in normal weight [9] but not in obese subjects [10], which suggest that food intake fails to suppress ghrelin levels in obese humans [11].

Low ghrelin concentrations are also associated with higher prevalence of the metabolic syndrome with progressively lower ghrelin levels in relation to the number of components of the metabolic syndrome [1, 12]. This is mostly explained by higher BMI in subjects with lower ghrelin levels, because adiposity influences all other features of the metabolic syndrome [1–3, 5, 12–14]. In fact, it has been shown that total plasma ghrelin as well as UAG concentrations are lower in obese patients with metabolic syndrome compared to nonobese counterparts [14]. Furthermore, among obese subjects, plasma ghrelin levels are lower in insulin resistant persons compared to insulin sensitive persons [15]. However, the concentrations of total ghrelin, AG, or UAG separately are not different between insulin sensitive and insulin resistant persons with similar body weight [1]. Among overweight and obese patients, the ratio of AG : UAG is lower in insulin sensitive than in insulin resistant subjects [3, 15].

Polycystic ovary syndrome (PCOS) is associated with adiposity and metabolic changes predisposing to insulin resistance and type 2 diabetes mellitus [16–18]. Obese patients with PCOS have lower levels of ghrelin than BMI matched control subjects [19], although in another study no difference was observed in this regard [20].

3. Effect of Weight Loss on Ghrelin Concentration

It has been suggested that ghrelin is linked to excessive food intake in two ways. Firstly, the attenuated postprandial reduction in ghrelin levels may directly increase the length of time for which the subject feels hungry, and secondly, as a consequence of the elevated ghrelin levels, the speed of gastric emptying may not be reduced, and the resulting feeling of satiety not elicited [11]. Without these feelings of satiety, obese individuals eat more than they need, and thus gain weight [13].

So far, the majority of studies have focused on the effects of diet induced or combined exercise/diet weight loss on total ghrelin concentrations [21–28]. These studies are very diverse, with different interventions, intervention periods, age and number of participants, and also inclusion criteria. Most studies have shown that weight reduction increases ghrelin concentrations in obese subjects [21–23, 29] or the concentration is unchanged in overweight healthy adults or obese children after weight loss [24]. However, during weight maintenance after the weight loss, ghrelin levels tend to decrease back to the levels they were before weight loss [25]. Furthermore, an initial decrease along with weight loss and subsequent increase in plasma ghrelin has been reported [26]. Weight loss is also shown to result in increased ghrelin concentrations in normal weight individuals [27]. Only a few studies have been conducted to investigate the exclusive effect of weight loss through exercise intervention on plasma ghrelin levels. In general, these studies have shown either an increase [28] or no change on ghrelin concentrations [30].

4. Effect of Insulin and Glucose Concentrations on Ghrelin Secretion

Insulin is shown to inhibit ghrelin secretion in healthy normal-weight and overweight persons [15, 31, 32], and both oral and intravenous glucose loads are also shown to regulate ghrelin secretion in humans [33–37]. Insulin and HOMA-IR are associated negatively with total ghrelin and UAG concentrations while AG had positive association [3]. Liu and coworkers developed recently a new reliable sandwich method for detection of AG and UAG separately. With this new assay they showed evidence that ghrelin acylation and secretion are regulated separately. The use of this method may facilitate more reliable detection of different ghrelin forms in future [38]. Physiological increases in insulin levels may play a key role in regulating postprandial plasma ghrelin concentrations, since meal-induced ghrelin suppression is absent in severe insulin deficiency [10]. An increase in insulin after the oral or intravenous glucose administration could contribute to the inhibitory effect of glucose on ghrelin concentrations. However, the administration of a combined pulse of glucose and insulin does not acutely suppress ghrelin levels [36–39]. Reduction in ghrelin after intravenous glucose bolus in subjects with type 2 diabetes suggests that early insulin response does not affect plasma ghrelin [39]. Indeed, during euglycaemic clamp, an increase in insulin levels leads to suppression of ghrelin levels and is remained suppressed during subsequent hypoglycemia and even fell further during following hyperglycaemia. However, another study has shown that hyperinsulinaemia with concomitant hyperglycaemia at concentrations typically seen in insulin-resistant subjects does not affect plasma ghrelin but is decreased only at pharmacological insulin concentrations [40]. However, it is still unclear whether insulin and glucose per se play a direct inhibitory role in ghrelin secretion [16]. The decrease in ghrelin levels after an oral glucose load is modulated by sex, status of obesity, and level of insulin resistance [41]. Ghrelin concentrations are shown to be higher in women than in men. To support of this finding, ghrelin concentrations are shown to correlate positively with testosterone concentrations [41].

5. Effect of Ghrelin on Glucose and Insulin Metabolism

Above we discussed the associations of ghrelin with glucose and insulin levels. However, ghrelin may also participate in the regulation of glucose and insulin metabolism as discussed in this section in more detail. Because glucose and insulin metabolism are tightly connected, their effects are difficult to separate from each other.

A human study conducted recently in morbidly obese nondiabetic persons showed that administration of combination of AG and UAG reduced insulin concentrations significantly without any effect on glucose concentrations, while AG or UAG alone did not have any significant effects [42]. Based on this data, it was concluded that insulin sensitivity was improved in these persons [42]. It has been shown that specifically AG is responsible for improving insulin sensitivity, while UAG has opposite effects [43]. However, in another study conducted with healthy young persons showed that administration of ghrelin impairs insulin and glucose metabolism by increasing glucose concentrations and decreasing insulin levels [44], and a number of other studies have supported these findings [45–48]

Acute ghrelin administration in humans increases plasma glucose levels by downregulation of insulin, and arginine is shown to amplify the hyperglycaemic effect of ghrelin which can be blocked by the administration of the GHS-R agonist D-(Lys^3)-GHRP6. To support these findings, plasma ghrelin levels are shown to correlate negatively with insulin concentrations and are associated with fasting insulin levels, insulin resistance, and obesity [49, 50]. Specifically, AG is shown to be responsible for the decrease in insulin and a consequent rise in glucose levels [49]. It has been suggested that UAG alone has been suggested to be devoid of any endocrine effects but is able to antagonize the effects of AG on insulin secretion [51]. There is evidence that UAG has a specific functional role in insulin signaling since it has been shown to stimulate insulin secretion in pancreatic cell lines [52, 53]. Furthermore, the combination of AG and UAG may improve insulin sensitivity [51]. This has been shown in GH-deficient patients, in whom UAG prevents the rapid rise in insulin and glucose levels when coadministered with AG [51]. The hyperglycaemic effect of ghrelin could be mediated through activation of catecholamine-induced glycogenolysis or directly by acting on hepatocytes; where it may enhance gluconeogenesis [45, 54]. Interestingly, AG has been shown to stimulate glucose output by primary hepatocytes; whereas UAG mediates an inhibitory effect [49]. Moreover, it counteracts the stimulatory effect of AG on glucose release [49].

Ghrelin is shown to be expressed in pancreas both in rodents and humans [55–57], where it may locally modulate insulin secretion. These findings suggest that ghrelin has a pathophysiological role in regulation of insulin release. Ghrelin is found to inhibit insulin release in rodents and in isolated islets in vitro [58–60], to promote survival of both INS-1E β cell line and human islets of Langerhans [52] and to stimulate insulin secretion in pancreatic cell lines [52, 53]. Furthermore, ghrelin is shown to prevent cell death and apoptosis of HIT-T15 pancreatic β cell line [60]. Ghrelin treatment of neonatal rats exposed to streptozotocin attenuates the development of diabetes and is associated with increased islet neogenesis, suggesting that ghrelin might have a proliferative or cytoprotective effect on β cells [60]. In mice, ghrelin has also shown to hamper insulin's capacity to suppress endogenous glucose production; whereas it reinforces the action of insulin on glucose disposal [61]. Furthermore, simultaneous administration of UAG abolishes the inhibitory effect of ghrelin on hepatic insulin action [61].

6. Effect of Ghrelin on Adipose Tissue

Adipose tissue is one of the most important organs mediating metabolic effects by numerous adipokines and cytokines, which are secreted from adipose tissue [62]. There is increasing amount of evidence that also ghrelin may have an important role in modulating function of adipose tissue. Because obesity has a significant role in modulating the expression of ghrelin, it is important to know how ghrelin is involved in the regulation of adipocyte metabolism. Several studies have suggested that ghrelin may play an important role in adipogenesis and storage of energy in adipose tissue [63–65]. Chronic ghrelin administration has been shown to increase body fat content in rodents and humans [63]. In visceral adipose tissue, ghrelin (AG and UAG) is shown to stimulate lipid accumulation by enhancing the expression of adipogenic genes including PPARg, SREBP1, acetyl-CoA carboxylase, fatty acid synthase lipoprotein lipase, perilipin, adipocyte determination and differentiation-dependent factor (ADD)1, and adipose protein 2/fatty acid binding protein (aP2) during adipocyte differentiation [64]. These functions might be mediated via AMPK pathway [66]. It has been demonstrated that infusion AG and UAG simultaneously in rats independently modulates adipocyte metabolism by inhibiting isoproterenol induced lipolysis [67], regulating adipogenesis [68, 69], suppressing noradrenalin release in brown adipose tissue [70], and promoting glucose and triglyceride uptake and antiapoptotic actions [65]. Ghrelin is also shown to stimulate lipogenesis and to inhibit lipid oxidation in white adipocytes; whereas in brown adipocytes central ghrelin infusion results in decreased expression of uncoupling proteins, molecules contributing to energy dissipation [69]. All of these findings strongly support the view that ghrelin may have an "energy saving" effects on adipose tissue.

In addition, Ghrelin has also been shown to stimulate adipogenesis in vitro [71], and both AG and UAG directly promote bone marrow adipogenesis in vivo [69, 93]. However, Zang et al. have shown that ghrelin inhibits adipogenesis by stimulating cell proliferation in mouse adipocyte cell line [93]. Ghrelin also inhibits the expression of adiponectin. It is of note that the reduced concentrations of adiponectin have been implicated in the pathogenesis of insulin resistance and obesity [94]. Furthermore, ghrelin exerts a receptor-mediated stimulatory effect on leptin production of cultured rat white adipocytes [95].

7. Ghrelin and Immunomodulation

Given the wide distribution of functional GHSR on various immune cells, it was hypothesized that ghrelin may exert immunoregulatory effect on immune cell subpopulations [96]. In vitro, ghrelin treatment is shown to inhibit production of proinflammatory cytokines (interleukin IL1β, IL6, and TNFα by PBMCs via a GHSR-specific pathway [96]). It was further reported that ghrelin inhibits IL6 and TNFα mRNA expression in primary human T cells, which

Table 1: Common names of *GHRL* SNPs with their corresponding rs-numbers.

NCBI RefSNP accession ID	Position	SNP location
rs1629816	−4427G>A	Promoter
rs3755777	−1500C>G	Promoter
rs26311	−1062G>C	Promoter
rs26312	−994C>T	Promoter
rs27647	−604G/A	Promoter
rs26802	−501A/C	Promoter
rs696217	Leu72Met	Exon 3
rs2075356	3056T>C	Intron 3
rs4684677	Gln90Leu	Exon 4
rs35684	5179A>G	3′ region
rs2072578	9344G>A	3′ region

suggests a role for ghrelin in the transcriptional regulation of inflammatory cytokine expression [97].

8. Effects of Ghrelin on Cardiovascular System

Ghrelin has diverse cardiovascular effects, which are most probably ghrelin receptor mediated rather than GH mediated, since expression of ghrelin receptor has been reported in the cardiovascular system [99]. Administration of ghrelin in persons with metabolic syndrome is shown to improve endothelial function by preventing proatherogenic changes [100] and improving vasodilatation [101], by decreasing blood pressure (BP) without an increase in heart rate [102], and additional haemodynamic effects by increasing cardiac output [103]. Chronic subcutaneous administration of ghrelin in rats is shown to exert a therapeutic effect in heart failure by improving left ventricular dysfunction and attenuation of the development of cardiac cachexia [104], by improving left ventricular dysfunction and attenuating the development of left ventricular remodeling and cardiac cachexia in rats with CHF [105]. Plasma ghrelin concentrations are shown to correlate positively with carotid artery atherosclerosis [106]. In addition, ghrelin receptor is upregulated in heart muscle of patients suffering from end-stage heart failure [106].

Molecular mechanisms for the cardiovascular activity of ghrelin have been intensively studied in cell culture models [107, 108]. It has been demonstrated that ghrelin stimulates nitric oxide (NO) production both in cultured endothelial cells and in intact vessels [107, 108], while the NO synthesis can be blocked by NO synthase inhibitor (NOS) (N^G nitro-L-arginine methyl ester), by phosphatidylinositol 3-kinase inhibitor (wortmannin) or by antagonist of ghrelin receptor (D-Lys^3) [107]. Furthermore, ghrelin is shown to mediate NO production through phosphorylation of endothelial nitric oxide synthase (eNOS) [108], Akt, one of the main kinases involved also in insulin signaling pathway [107, 108] and AMP-activated protein kinase (AMPK), in endothelial cells and in intact vessels [108]. Based on these findings ghrelin uses partly insulin signaling pathway for production of NO. Furthermore, downregulation of GHSR-1 by siRNA blocks the NO production and phosphorylation of Akt and endothelial NOS indicating that these functions are mediated by GHSR-1 [107].

Togliatto and coworkers [109] studied separately the effects of AG and UAG on mobilization of endothelial progenitor cells (EPCs) in healthy humans, persons with T2DM, and in ob/ob mice. They found that the treatments had no effect in healthy human subjects. However systemic administration of UAG but not AG prevented diabetes-induced EPC damage by modulating the NAPDH oxidase regulatory protein Rac1 and improved their vasculogenic potential both in individuals with T2DM and ob/ob mice [109]. UAG also facilitated the recovery of mobilization of EPC. Crucial to EPC mobilization by UAG was the rescue of NO synthase phosphorylation by Akt. Furthermore, EPCs expressed UAG binding sites, which were not recognized by AG [108].

To support earlier findings above, Tesauro and coworkers [110] conducted a human study in persons with obesity and metabolic syndrome in order to test if exogenous ghrelin could improve the balance between NO and endothelin-1, a vasoconstrictor peptide produced by vascular endothelial cells. In the absence of ghrelin, the vasodilator response to BQ-123, an endothelin A receptor antagonist, was greater in patients than in controls; whereas infusion of NO synthase inhibitor induced smaller vasoconstriction in patients than in controls [110]. Exogenous ghrelin decreased the vasodilator response to BQ-123 and enhanced the magnitude of changes in forearm blood flow induced by NO synthase inhibitor in patients but not in controls [110]. The favorable effect of ghrelin on endothelin A-dependent vasoconstriction was likely related to the stimulation of NO production, because no change in the vascular effect of BQ-123 was observed after ghrelin in persons with metabolic syndrome during continuous infusion of the NO donor sodium nitroprusside. In patients with metabolic syndrome, ghrelin has benefits to normalize the balance between vasoconstrictor (endothelin 1) and vasodilating (NO) mediators, thus suggesting that this peptide has important peripheral actions to preserve vascular homeostasis in humans [110].

9. Ghrelin O-Acetyltransferase (GOAT)

The peptide hormone ghrelin is the only known protein modified with an O-linked octanoyl side group, which occurs on its third serine residue. This modification is crucial for ghrelin's physiological effects including regulation of feeding, adiposity, and insulin secretion [4]. It is no longer than two years ago when an enzyme ghrelin O-acyltransferase (GOAT), which links octanoate to Ser3 of ghrelin, was discovered by two different research groups [111, 112]. Human GOAT is able to acylate ghrelin also with other fatty acids, besides octanoate, ranging from acetate to tetradecanoid acid [112]. Analysis of the mouse genome revealed that GOAT belongs to a family of 16 hydrophobic membrane-bound acyltransferases and is the only member of this family that octanoylates Ser3 position of ghrelin peptide when coexpressed in cultured endocrine cell lines with prepro-ghrelin [111, 112]. Expression levels of gastric GOAT are the highest under ad libitum and are decreased with fasting, showing similar pattern of decrease to that

Table 2: Associations of polymorphisms in the *GHRL* gene.

SNP	Risk allele	Association	Subjects	References
Leu72Met	Met72	Lower age of onset of self-reported obesity	96 obese and 96 normal-weight Swedish women	[72]
Leu72Met	Met72	Higher frequency in Whites than in Blackslower BMI, fat mass, visceral fat, total TG and RQ; higher IGF-1 levels in Blacks	784 French-Canadian subjects (Quebec Family Study) 778 subjects (276 Blacks and 502 Whites; HERITAGE Family Study)	[73]
Arg51Gln	Gln51	Not observed among Blacks	1442 subjects (741 from obese registry, 701 from normal reference population; SOS)	
Leu72Met	Met72	Higher BMI, earlier age of onset of obesity and reduced first phase insulin secretion	70 tall and obese children	[74]
Gln90Leu	Gln90	Higher frequency in obese children, but also in underweight students	215 extremely obese German children and adolescents, 93 normal-weight students,134 underweight students, 44 normal-weight adults	[75]
Leu72Met	Met72	Lower serum creatinine and lipoprotein a levels	258 Finnish Caucasians with T2D and 522 controls	[76]
Arg51Gln	Gln51	Risk allele for hypertension and T2D; predictor of 2-h plasma glucose in OGTT; lower IGF-1 and higher IGFBP-1 concentrations in normotensives; lower AUC insulin	519 hypertensive and 526 normotensive Finnish Caucasians	[77]
Leu72Met	Met72	In obese/overweight: higher neonatal weight-for-age; earlier age at onset of obesity; higher IGF-1 concentration	81 obese or overweight and 96 normal-weight Italian children and adolescents and 72 normal-weight young adults	[6]
4427G>A	G	Diffuse large cell lymphoma	684 healthy controls and 308 North American subjects with non-Hodgkin Lymphoma	[78]
Arg51Gln Leu72Met	Gln51 Met72	Lower MetS frequencyHigher fasting glucose,TG, and frequency of MetS and lower HDL cholesterol	856 Old Order Amish from US	[79]
Leu72Met	Met72	More depressed and anxious in patients with methamphetamine dependence. No association with methamphetamine dependence	118 Koreans with methamphetamine dependence, 144 controls	[80]
Leu72Met		No association with obesity	222 obese Korean children	[81]
−1500C>G −1062G>C −994C>T Leu72Met	C	Lower HDL cholesterol All four SNPs: no association with T2D	760 T2D and 641 nondiabetic Koreans	[82]
Leu72Met −604 >C/T	Leu72 C	Higher TG, fasting insulin and HOMA-IR. Higher fasting insulin and HOMA-IR	1420 Caucasians (500 normal weight and 920 overweight/obese)	[83]
Leu72Met	Met72	Lower allele frequency in diabetic nephropathy with renal dysfunction. Lower total cholesterol levels in patients with diabetic nephropathy with renal dysfunction	138 subjects with diabetic nephropathy, 69 diabetics without nephropathy	[84]
Leu72Met	Met72	Lower creatinine levels in diabetic group. No association with T2D	206 T2D, 80 controls	[28]
−501A>C	A	Higher BMI	1045 Finnish subjects from the Oulu Project Elucidating Risk for Atherosclerosis (OPERA) study	[85]
Leu72Met		No association with weight loss	771 obese Caucasian Europeans	[86]
Leu72Met	Met72	Higher allele frequency in higher BMI group than in normal-weight group. Higher BMI, waist circumference, and change in body weight from age 18	2238 middle-aged and older Japanese people	[87]

Table 2: Continued.

SNP	Risk allele	Association	Subjects	References
Leu72Met	Met72	Lower BMI in CAD patients but no association with CAD, no association with hypertension, T2D, or dyslipidaemia	317 Chinese CAD patients, 323 controls	[88]
Leu72Met	Met72	Higher scores on Drive for Thinness-Body Dissatisfaction subscale	264 Japanese women	[89]
3056T > *C*	C	Higher weight, BMI, fat mass, waist circumference, sum of skinfold thicknesses, self-reported past min and max BMIs and lower HDL chol		
Arg51Gln Leu72Met	Gln51	Higher cholesterol levels over time. Subjects with Gln51and /or Met72 lost body weight faster than patients with Arg51/Leu72	210 hemodialysed patients prospectively followed up to 15 months	[90]
Leu72Met	Met72	Persons with 72Met allele have lower risk to develop T2DM	507 persons with IGT: the Finnish diabetes prevention study	[91]
−604G/A −501A/C Leu72Met GLN90Leu	G A Leu72 GLN90	Persons with the most common genotype combination of the SNPs 604G/-501A/, Leu72/GLN90 have significantly lower systolic and diastolic blood pressure at baseline and during the 3-year follow-up	507 persons with IGT: the Finnish diabetes prevention study	[92]

of ghrelin [112]. GOAT expression is localized in ghrelin producing cells in gastric mucosa [112–114] as well as in pancreas [111, 112]. It has been found that the genetic disruption of the GOAT gene in mice leads to complete absence of AG in circulation [112].

Kirchner and coworkers have recently studied the role of GOAT in regulating of the activity of ghrelin using different animal models [115]. They showed that GOAT functions as a gastric lipid sensor linking selected ingested nutrients with hypothalamic energy balance regulation via endocrine ghrelin system [115]. Animal models have shown that GOAT is required and sufficient to mediate the impact of dietary lipids on body adiposity, and that activation of the GOAT-ghrelin system is triggered by a lipid-rich environment rather than by caloric depreviation [115]. Specifically, sufficient dietary supply of medium chain triglycerides is important for ghrelin acylation [115].

The discovery of GOAT has provided possibilities to develop tools to study specific functional roles of the two different ghrelin forms, UAG and AG, in human health in more detail. For example, modification of its expression provides tools to study the function of different ghrelin forms and makes possible to develop drugs against obesity and related conditions.

10. Therapeutic Potential against Obesity and Insulin Resistance by Targeting GOAT/Ghrelin System

Increasing prevalence of obesity throughout the world is becoming an increasing health burden. Because obesity is a strong risk factor for development of cardiovascular diseases and T2DM, the development of strategies to combat obesity epidemic is urgently needed. AG and UAG forms of ghrelin as well as GOAT are attractive targets to develop pharmacological treatments for obesity and diabetes.

Pharmaceutical companies have started actively to develop drugs that can target orexigenic or obesity related functions of ghrelin, its receptor, or GOAT [116–119]. Ghrelin receptor antagonists are shown to block GH secretion and thus improve the diabetic condition by promoting glucose-dependent insulin secretion and weight loss and suppressing appetite [120]. Peptide inverse agonist DLys3-GHRP6, which blocks GHRP induced GH secretion, is shown to reduce food intake and body weight. Furthermore, vaccination of mature rats or mouse with ghrelin immunoconjugates against AG decreases feed efficiency, adiposity, and body weight gain in relation to immune response elicited against AG [121, 122]. Recently, a new class of L-RNA-based hormone antagonists, the spiegelmers (SPMs), has been developed [123]. SPMs are L-isomer oligonucleotides that are stable in biological fluids, enabling long-lasting peptide neutralization after a single application [123]. This makes these compounds very useful for experimental purposes and possibly as therapeutic agents. Unlike classic hormone antagonists, SPMs do not interact with the receptor but bind with high affinity to their target molecule and prevent binding to the endogenous receptor. The antighrelin Spiegelmer NOX-B11-3 neutralizes the stimulatory effects on GH release and food intake in animal studies [124].

11. Genetic Association Studies of the Ghrelin and Ghrelin Receptor Genes

Several genome-wide scans have suggested that certain areas of the chromosome 3, the same chromosome where ghrelin and ghrelin receptor genes are located, might be linked with obesity or metabolic syndrome [72, 73]. Polymorphisms in the human GHRL gene and the 5′ flanking region have been

intensively studied. The most studied exonic SNPs include the Leu72Met located in exon 3 and Arg51Gln, which is located in exon 3 within the last codon of the mature ghrelin protein and disrupts the recognition site of the endoprotease, leading to proteolytic cleavage of the carboxy-terminal 66 amino acids to produce mature ghrelin [74], Table 1. Most of the association studies are focused on metabolic syndrome and T2DM, which are summarized in Table 2. A number of studies have shown associations between GHRL SNPs and obesity or related traits, although the results are contradictory (see Table 2). The Met72 allele of GHRL has been associated with earlier age at onset of obesity and higher BMI [6, 74, 78, 87, 88, 91, 125, 126], but negative findings have also been reported [6, 74, 77, 78, 85, 89]. The −501A>C in the promoter region of the GHRL gene and the intronic +3056T>C polymorphisms has been shown to associate with obesity and related conditions [79, 81], while some studies have failed to find association with these SNPs [6, 79, 81, 85, 92, 127–129].

In addition, ghrelin variations are also shown also to be associated with blood pressure [129].

Regarding the genetic association studies of *GHSR* SNPs, only a few studies have been reported so far. From these reports at least two have shown an association between GHSR SNPs and features of metabolic syndrome [75, 76], but most of the studies have shown negative results.

12. Take-Home Message

In terms of obesity, metabolic syndrome, and T2DM, ghrelin is very interesting hormone, which plays a crucial role in glucose and insulin metabolism and in development of obesity and insulin resistance. The knowledge on functions of ghrelin in peripheral tissues, such as pancreas, adipose, and vascular tissues has increased during the last few years. The recent discovery, the characterization of ghrelin-O-acyltransferase, GOAT has provided new challenges to develop drugs against obesity and T2D. The modification of GOAT expression provides tools to regulate the AG : UAG ratio and to study the specific roles of different ghrelin forms (AG and UAG) separately in human health. Regarding the positive cardiovascular effects of ghrelin, it is considered as a direct target for prevention of CVD.

Regarding the genetics of ghrelin and its receptors, more studies are needed to show whether and to what extent they are involved in the pathogenesis of metabolic syndrome and T2DM. In Genome wide association studies no confirmation has been achieved in this regard.

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Review Article

Ghrelin and Metabolic Surgery

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Metabolic surgery is the most effective treatment for morbid obesity. Ghrelin has been implicated to play a role in the success of these procedures. Furthermore, these operations have been used to study the gut-brain axis. This article explores this interaction, reviewing the available data on changes in ghrelin levels after different surgical procedures.

1. Introduction

Surgical procedures are currently the most effective therapy for long-term weight loss [1]. Furthermore, some of these operations lead to the rapid remission of type 2 diabetes in a weight loss independent manner [2]. The mechanism that leads to sustained weight loss as well as diabetes remission after bariatric operations remains to be fully elucidated. However, it is becoming evident that these procedures modulate the gut-brain axis by altering the anatomy of the gut and affecting gut hormones [3]. In fact some of these procedures are now considered suitable models to study the gut brain axis. The landmark study of Cummings et al. , which showed a profound suppression of ghrelin levels following Roux-en-Y gastric bypass, has brought the interaction of weight loss operations and the gut-brain axis into focus [4–6].

1.1. Ghrelin. Ghrelin is a 28-amino acid peptide produced from the fundus of the stomach and the proximal intestine [5–7]. It is the only known orexigenic gut hormone. Central and peripheral administration leads to increased food intake [8, 9]. Ghrelin levels increase prior to meals and are suppressed postprandially in proportion to the amount of calories ingested, therefore suggesting a possible role in meal initiation [10, 11]. The 24-hour profile of ghrelin increases following diet-induced weight loss supporting the hypothesis that ghrelin has a role in the long-term regulation of body weight [4]. Obese individuals have lower fasting ghrelin levels, and significantly reduced postprandial ghrelin suppression compared to normal weight individuals [12].

1.2. Metabolic Surgery. Surgical procedures were designed to promote weight loss by reducing stomach volume (laparoscopic adjustable gastric banding (LABG), laparoscopic sleeve gastrectomy (LSG)), malabsorption of nutrients (biliopancreatic diversion (BPD), duodenal switch (DS)), or a combination of both (Roux-en-Y gastric bypass (RYGB)). It is now known that standard proximal Roux-en-Y gastric bypass causes only malabsorption of micronutrients and not of calories, and restriction does not play a major role. A number of studies have shown that changes in gut hormone concentrations may partly explain the weight loss following weight losss surgery.

Furthermore, following these procedures a vast improvement in glycaemic control has been observed. A meta-analysis reported improved glycaemic control and remission of type 2 diabetes in 83.8% of patients following gastric bypass and 47.8% following gastric banding [2]. The fact that these procedures improve manifestations of the metabolic syndrome, often in a weight-loss independent manner has led to the development of the concept of metabolic surgery. In fact a number of scientific societies have changed their name in order to include this term.

2. Ghrelin and Roux-en-Y Gastric Bypass

RYGB is the most common metabolic procedure worldwide [13]. Cummings et al. showed a profound suppression

of ghrelin levels (24-hour profile) following RYGB [4]. However, the data published since are inconclusive. Different studies showed a decrease in fasting and postprandial ghrelin levels [14–21], no change in fasting and postprandial levels [22–32], and an increase in fasting ghrelin levels after RYGB [33–37]. The reason for this heterogeneity has not been elucidated and multiple explanations have been proposed. Differences in the methods used for evaluating ghrelin levels are possible, but unlikely. It has been suggested that even in the studies reporting an increase in fasting ghrelin levels, ghrelin does not increase to the extent reported with diet-induced weight loss or of nonobese individuals [26]. In a study which investigated the intraoperative changes in ghrelin during RYGB, the complete division of the stomach and the formation of a vertical pouch contributed to the decline in the circulating ghrelin [18]. Moreover, an intact vagus nerve appears to be required for ghrelin to have an appetite effect [38]. Technical differences in the procedure in regards to preservation of the vagus nerve may be responsible. Iatrogenic vagal nerve dysfunction caused intraoperatively might also play a role, as shown by a study which demonstrated a decrease in ghrelin levels on the first postoperative day after RYGB, followed by an increase to preoperative levels at 1 month and a further increase at 12 months [35]. An alternative theory has suggested that the different configuration of the pouch might be responsible [39]. In a vertical pouch, ghrelin producing cells are more likely to be excluded, compared to a horizontal pouch [40]. Finally, hyperisulinaemia and insulin resistances are associated with ghrelin suppression in obese individuals [40]. Therefore, preoperative differences as well as differences in the postoperative improvement in these parameters may cause this inconsistency.

3. Ghrelin and Biliopancreatic Diversion

BPD is an operation that does cause malabsorption of calories. A study on patients prior to and 5 days and 2 months after BPD showed a similar response with an initial reduction in fasting ghrelin, followed by a return to the preoperative levels when food consumption resumed to almost preoperative levels [41]. This finding supports the hypothesis that although the primary source of ghrelin is the gastric mucosa, small intestinal nutrient exposure is sufficient for food-induced plasma ghrelin suppression in humans and gastric nutrient exposure is not necessary for this suppression [42]. However, different studies have shown an increase [43–46] or no change [36, 41, 47] in ghrelin levels after BPD.

A decrease in ghrelin levels has been noted after DS [48, 49]. In this procedure, the reduction in the stomach volume is achieved with a sleeve gastrectomy. It is important to stress the anatomical difference between BPD as described by Scopinaro with horizontal gastrectomy where the fundus remains intact and gets in contact with nutrients, whereas in DS type duodenal switch with sleeve gastrectomy the fundus, the main area of ghrelin production, is resected.

4. Ghrelin and Gastric Banding

LAGB has been shown to reduce hunger and increase satiety [50]. No association with ghrelin levels was demonstrated in the same study [51]. Schindler et al. showed an increase in fasting ghrelin accompanied by a paradoxical decrease in hunger after LAGB suggesting that weight loss is independent of circulating plasma ghrelin and relies on changes in eating behaviour induced by gastric restriction [51]. Further studies on patients following LAGB demonstrated both increased fasting ghrelin levels [7, 19, 25, 52–54] and a blunted postprandial suppression of ghrelin [17, 32]. However, we, as well as others, were not able to show any changes in ghrelin levels after laparoscopic gastric banding [22, 55–57].

5. Ghrelin and Sleeve Gastrectomy

LSG is a relatively new bariatric operation which was designed as a restrictive procedure. Recent studies challenge this classification showing accelerated gastric emptying after LSG [58]. However, another study of patients undergoing LSG showed no difference in gastric emptying compared to preoperatively and therefore the controversy remains [59]. The fact that the fundus of the stomach, the main location of ghrelin-producing cells, is excluded in this procedure led to speculation that ghrelin could play a role in the mechanism of action. Three studies confirmed a decrease in fasting ghrelin levels after LSG [54, 57, 60]. A prospective, double-blind study comparing RYGB and LSG confirmed a significant postprandial suppression of ghrelin postoperatively, while there was no change in the RYGB group [30]. In the same study, the marked suppression of ghrelin levels after LSG was associated with greater appetite reduction and excess weight loss during the first postoperative year compared to RYGB [30]. An even more recent prospective randomised comparison of LSG and RYGB confirms that both operations reduce fasting and meal stimulated ghrelin levels, significantly more so after LSG, so resection of the fundus has more impact on the ghrelin levels compared to just bypassing it [61].

6. Conclusion

The role of ghrelin in the success of bariatric and metabolic surgery remains to be further elucidated. Some of the findings of the initial studies have not been confirmed in more recent investigations. Different gut hormones as well as having an incretin effect have been implicated to be key players in appetite control. However, the hypothesis that ghrelin might play a role in the mode of action of metabolic surgery has been crucial in the development of the field. The weight loss as well as the remission of type 2 diabetes experienced after metabolic surgery is not exclusively attributed to pure restriction or malabsorption any more. A lot of research is focused on exploring the hormonal and metabolic changes after metabolic surgery as well as the mechanism of action.

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Review Article

Metabolic and Cardiovascular Effects of Ghrelin

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Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is synthesized as a preprohormone and then proteolytically processed to yield a 28-amino acid peptide. This peptide was originally reported to induce growth hormone release; large evidence, however, has indicated many other physiological activities of ghrelin, including regulation of food intake and energy balance, as well as of lipid and glucose metabolism. Ghrelin receptors have been detected in the hypothalamus and the pituitary, but also in the cardiovascular system, where ghrelin exerts beneficial hemodynamic activities. Ghrelin administration acutely improves endothelial dysfunction by increasing nitric oxide bioavailability and normalizes the altered balance between endothelin-1 and nitric oxide within the vasculature of patients with metabolic syndrome. Other cardiovascular effects of ghrelin include improvement of left ventricular contractility and cardiac output, as well as reduction of arterial pressure and systemic vascular resistance. In addition, antinflammatory and antiapoptotic actions of ghrelin have been reported both in vivo and in vitro. This review summarizes the most recent findings on the metabolic and cardiovascular effects of ghrelin through GH-dependent and -independent mechanisms and the possible role of ghrelin as a therapeutic molecule for treating cardiovascular diseases.

1. Introduction

Ghrelin, a 28-amino-acid peptide hormone mainly secreted by the X/A-like cells in the oxyntic mucosa of the stomach, has been discovered as the endogenous ligand of the orphan receptor growth hormone secretagogues 1a (GHS-R1a) [1]. In addition to its marked growth hormone *(*GH) releasing activity, ghrelin stimulates food intake and is involved in the regulation of energy homeostasis [2]. Furthermore, ghrelin has a variety of cardiovascular activities, including cardioprotective effects against ischemia, enhancement of vasodilation, cardiotropic effects, and regulation of blood pressure [3–5].

Human ghrelin gene is located on chromosome 3 and consists of 4 exons and 3 introns that encode a 117-amino-acid peptide, pre-proghrelin, that is cleaved into the mature 28-amino-acid form (3.3 kDa) secreted into the blood stream [6, 7]. Two major forms of ghrelin are found in tissues and plasma: *n*-octanoyl-modified ghrelin and des-acyl ghrelin [7]; ghrelin is the first known secreted bioactive peptide in mammals modified by an acyl acid on its third serine residues through the recently discovered enzyme ghrelin O-acyl transferase (GOAT) [8–10]. This postranslational modification is essential for binding to the GHS-R 1a and for several ghrelin biological activities, including the GH-releasing capacity and the actions on the endocrine axis, on energy balance and glucose homeostasis [3]. Nonacylated ghrelin, which represents the most abundant form of circulating ghrelin (80%–90%), has been found to be devoid of GH releasing capacity [1, 11, 12], but exerts a variety of physiological activity in the cardiovascular system as well as in lipid and glucose metabolism [13, 14].

Central and peripheral administration of des-acyl ghrelin significantly decreases food intake and decreases gastric emptying in food-deprived mice [15]. In another study, Chen et al. confirmed that des-acyl ghrelin decreases food intake in mice and showed that ghrelin disrupts the fasted motor activity of the antrum in freely moving conscious rats [16]. Moreover, centrally administrated des-acyl ghrelin increases feeding through activation of the orexin

pathway and may act in hypothalamic feeding regulation [17].

Ghrelin plasma levels are mainly regulated by nutritional and metabolic factors; in fact they are increased by energy restriction (such as malnutrition, anorexia nervosa, and cachexia) and decreased by food intake and overfeeding [18]. These notions are in agreement with several studies showing reduced circulating ghrelin in patients with obesity and metabolic syndrome [19, 20]. Furthermore, ghrelin circulating levels increase in obese subjects when they lose weight [21, 22].

While all forms of human obesity have inappropriately low ghrelin levels, the only exception is the Prader-Willy syndrome, a complex genetic disorder characterized by mental retardation, hyperphagia, short height due to GH deficiency, and muscular hypotony [23]. In these patients, excessive appetite causes progressive obesity, which is surprisingly associated with high ghrelin levels in the same range of patients with anorexia nervosa.

In a recent study, Kirchner et al. suggested that the ghrelin/GOAT system acts as a lipid nutrient sensor that informs the central nervous system on the availability of substrates, rather than a meal initiation factor in reply to fasting [10]. It has also been reported that ghrelin is not inhibited by gastric distension due to water intake but is reduced by glucose administration [24]. Moreover, intravenous glucose loads inhibit ghrelin secretion, whereas protein intake seems to increase or to not modify ghrelin levels [25]. By contrast, constant infusion of lipids does not influence ghrelin levels, while oral administration of fats reduces them, even though not as much as glucose administration [26]. Moreover, it has been demonstrated that ghrelin induces an increase in glucose levels and this effect could be related to activation of glycogenolysis, increased liver gluconeogenesis, or stimulation of catecholamine release [27–30].

A negative association has been shown between ghrelin and insulin secretion [31], but the exact mechanisms by which insulin regulates ghrelin secretion need further studies. Hyperinsulinemia, however, has been suggested to act as a feedback mechanism to suppress ghrelin secretion, because several studies have reported reduced plasma ghrelin in association with different insulin resistance states, including hypertension, type 2 diabetes, or polycystic ovary syndrome [32].

A positive correlation between ghrelin and high-density lipoprotein (HDL) cholesterol concentration has also been observed [33, 34] and, in another study, Park et al. have shown that fasting plasma ghrelin levels are negatively correlated with triglycerides and positively correlated with HDL cholesterol in boys but not in girls; the mechanism underlying this sex difference, however, is not well established [35].

Ghrelin secretion seems to be under cholinergic control, since ghrelin levels are raised by cholinergic agonists and reduced by cholinergic antagonists [14]. In addition, Williams et al. showed that fast-induced rise of ghrelin levels is prevented by vagotomy and is reduced by atropine in rats [36]. Interestingly, ghrelin stimulates the differentiation of preadipocytes and antagonizes lipolysis in vitro [37] and promotes bone marrow adipogenesis in vivo [38]. Chronic ghrelin administration increases body weight, adiposity, and the expression of uncoupling protein (UCP) mRNA in brown and white adipose tissue in mice [39].

2. Tissue Distribution of Ghrelin and Ghrelin Receptor

Ghrelin is predominantly produced by the stomach, but it is also widely expressed in different tissues, such as the hypothalamus, pituitary gland, small and large intestine, placenta, pancreas, kidney, testes, ovary, and lymphocytes [40]. Ghrelin has also been found in several human neoplastic tissues and related cancer cells such as gastric and intestinal carcinoids, lymphomas and thyroid, breast, liver, lung, and prostate carcinomas [41, 42].

The ghrelin receptor (GHS-R) is a typical G-protein-coupled, seven transmembrane domain receptor. The gene of the human GHS-R 1a is located on chromosome 3q26.2 [43] and encodes for two different splice forms of the human GHS-R: GHS-R 1a, which binds ghrelin and leads to intracellular calcium mobilization, and GHS-R 1b, which is not able to bind ghrelin [44].

The GHS-R 1a is particularly concentrated in the hypothalamic-pituitary unit but is also present in other areas of the central nervous system and in several endocrine and nonendocrine tissues, including pancreas, lung, liver, kidney, small and large intestines, myocardium, spleen, ovary, testis, adrenal, adipose tissue, stomach, and the neuronal cells of the gut, both in animals and humans [45].

Also the GHS-R 1b has been found in many peripheral organs, including immune cells, skin, myocardium, pituitary, thyroid, pancreas, ileum, colon, liver, breast, spleen, duodenum, placenta, lung, adrenal, buccal mucosa, stomach, lymphonode, atrium, lymphocytes, and kidney [46]. Interestingly, both GHS-R subtypes have been found in tumoral tissues from organs that do not express these receptors in physiological conditions, for example, the breast [47].

In humans, the mRNA encoding GHS-R1a is detected in the cardiovascular system, but its expression in this system is much lower than in the pituitary [46]. Other authors have shown that human endothelial cells express ghrelin and the presence of ghrelin receptor has been reported in human endothelial cells, vascular smooth muscle cells, and the left ventricle [48]. Also GHS-R1b transcripts are highly expressed in human myocardium, but their physiological function is still unknown [46].

Analyzing ghrelin binding sites in the cardiovascular system, Katugampola et al. demonstrated a higher density of receptors in the myocardium of the right atrium than in the left ventricle, whereas aorta and pulmonary artery have more receptors than saphenous vein or coronary artery (Table 1). Moreover, the same investigators noticed a change in receptors density as a consequence of vascular diseases, with upregulation of ghrelin in vessels with advanced intimal thickening [49]. Binding studies have evidenced the existence of a subtype of ghrelin receptor distinct from

GHS-R 1a, with the same binding affinity both for acylated ghrelin and nonacylated ghrelin, in H9C2 cardiomyocites and endothelial cells [50].

3. Cardiovascular Effects of Ghrelin

Studies both in animal models and humans have shown beneficial effects of ghrelin in the cardiovascular system to support the wide expression of its receptors in cardiovascular tissues (Figure 1).

In animal models, ghrelin has been found to improve cardiac contractility in pathological conditions, to reduce the infarct size and to attenuate the reduction in left ventricular function induced by ischemia-reperfusion [51]. In particular, Frascarelli et al. have found that ghrelin administration significantly reduces the infarct size, as estimated by triphenyltetrazolium chloride staining, in a rat ischaemia-reperfusion model [52, 53]. Furthermore, in a group of rats following myocardial infarction, ghrelin administration has shown to increase body weight, cardiac output, and diastolic thickness of the noninfarcted posterior wall, as well as to inhibit left ventricular enlargement [53]. In animal models of heart failure, ghrelin administration improves cardiac contractility and attenuates the development of cardiac cachexia [52, 53].

In normal subjects, intravenous or subcutaneous ghrelin injection increases cardiac output, improves cardiac contractility, and causes a significant decrease in mean arterial pressure, without changing heart rate [54]. In addition to this, abundant evidence demonstrates a therapeutic effect of ghrelin in patients with heart failure. Thus, ghrelin has been reported to improve left-ventricular function and to attenuate left-ventricular remodelling in patients with chronic heart failure; in addition, acute ghrelin administration has shown to decrease systemic vascular resistances and increase cardiac output, cardiac index, and stroke volume index in patients with chronic heart failure [55]. The same authors have noticed that treatment with ghrelin for three weeks increases body weight, lean body mass, and muscle strength [55]. These results suggest that ghrelin could improve muscle wasting in patients with chronic heart failure and cardiac cachexia, a severe catabolic state characterized by weight loss and muscle wasting, resistant to long-term treatment with nutritional supplements.

In keeping with these findings, other studies have emphasized that ghrelin might have a role in patients with end-stage heart failure and cardiac cachexia, by improving cardiac function and increasing appetite [56, 57]. Ghrelin intravenous administration has therefore been proposed as adjuvant therapy in heart failure, due to its capacity to lead to a gain in left ventricular mass, to increase left ventricular ejection fraction, and to decrease left ventricular end-systolic volume [55].

A negative correlation has been noticed between ghrelin plasma levels and blood pressure, which might suggest that ghrelin is also involved in the sympathetic regulation. In fact, ghrelin seems to suppress sympathetic activity and to decrease blood pressure through mechanisms involving the central nervous system [58]. This hypothesis is supported by studies showing that ghrelin administration significantly decreases plasma norepinephrine levels and the ratio between low-to-high frequency spectra of heart rate variability in rats with myocardial infarction [59]. The reduction in peripheral resistance following ghrelin intravenous infusion results in a decrease in blood pressure levels in humans, even though supraphysiological hormone levels are required to produce this effect [54, 60]. Other mechanisms 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 [27, 61].

In vitro studies suggested that, in human mammary artery, ghrelin causes vasorelaxation by antagonizing the endothelin-induced contraction [61]. We have demonstrated that ghrelin reverses endothelial dysfunction in patients with metabolic syndrome by increasing nitric oxide (NO) bioavailability [62]. We have also reported the molecular vascular actions of ghrelin, which stimulates the production of NO using a signaling pathway involving GHS-R 1a, PI 3 kinase, AKT, and eNOS [63]. More recently, we have extended our knowledge regarding the favorable endothelial actions of ghrelin by demonstrating that this peptide normalizes the altered NO/ET-1 balance within the vasculature of patients with metabolic syndrome, thus suggesting an important role of ghrelin in the regulation of vascular homeostasis [64].

4. Antiapoptotic Effects of Ghrelin in the Cardiovascular System

Ghrelin has also shown to act as an antiapoptotic peptide in the cardiovascular system.

In vitro studies suggest that ghrelin stimulates H9c2 cardiomyocyte proliferation and reduces doxorubicin-induced apoptosis in cardiomyocytes and endothelial cells [50, 65]. Iglesias et al. found that ghrelin is synthesized and secreted by isolated murine and human cardiomyocytes and is able to prevent apoptosis induced by treatment with the apoptosis-inducer cytosine arabinoside (AraC) in mouse adult cardyomyocites cell line HL-1 [66]. Moreover, ghrelin treatment of primary cardiomyocytes prevents apoptosis stimulated by anti-FAS agonist antibodies. In addition, ghrelin stimulates tyrosine phosphorylation and activates ERK-1/2 and Akt in cardiomyocytes and endothelial cells; the activation of these two pathways is required for the antiapoptotic effect of ghrelin [50]. Furthermore, Isgaard et al. have found that ghrelin is also able to stimulate proliferation of H9c2 cardiomyocytes (a cardiac cell line that does not express the ghrelin receptor) in a dose-dependent and specific manner by increasing thymidine incorporation; however, they have reported the presence of alternative ghrelin binding sites on cardiomyocytes cell membranes in the absence of GHS-R 1a [67]. In another study, Kola et al. have proposed that ghrelin might modulate intracellular energy balance in a cell specific manner: ghrelin would be able to stimulate the 5′-AMP activated protein kinase (AMPK) that has a central role in regulating energy provisions in cells especially during

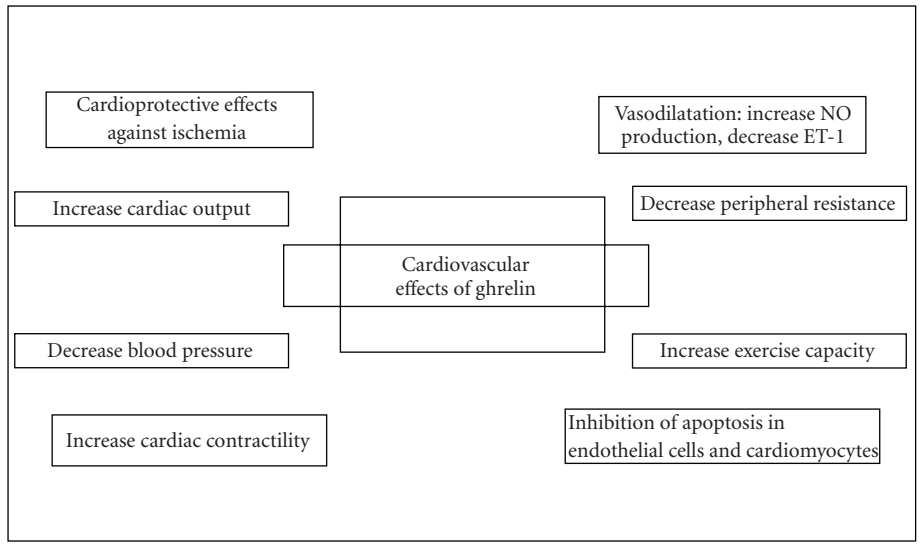

Figure 1: Cardiovascular effects of ghrelin.

Table 1: GHS1 receptors in the human cardiovascular system.

Myocardium
Left ventricle
Aortic endothelium
Mammary artery
Coronary artery
Ventricular cardiomyocites
Vascular smooth muscle
Carotid
Saphenous vein

anaerobic conditions [68]. Ghrelin effect on AMPK activity may be involved in the mechanism of cardioprotection from cellular injury and from ischemia-reperfusion damage [60].

5. Anti-inflammatory Effect of Ghrelin

Recent findings suggest that ghrelin has potent anti-inflammatory effects within the immune system and in human endothelial cells.

Dixit et al. found that ghrelin exerts specific and selective inhibitory effects on the expression of the inflammatory cytokines IL-1β, IL-6, and TNF-α [69]. Moreover, T lymphocytes express both ghrelin and the GHS-R and ghrelin secretion is increased when T lymphocytes are activated [69].

Administration of the GHS.1a agonist GHRP-2 results in decreased IL-6 levels and reduced signs of joint inflammation in arthritic rats [70]. Moreover, DeBoer et al. reported a significant decrease in circulating proinflammatory cytokines in ghrelin-treated rats with chronic kidney diseases (CKD); they also observed an increase in the circulating levels of the anti-inflammatory cytokines IL-10 in CKD rats treated with the ghrelin receptor agonist BIM-28125 [71]. In addition, ghrelin has potent anti-inflammatory effects in human endothelial cells, likely mediated by inhibition of NF-κB, and also inhibits inflammatory cytokines produced by endothelial cells in response to LPS [72]. These anti-inflammatory effects of ghrelin suggest a possible modulatory role of the peptide as a novel strategy in several cardiometabolic disorders associated with chronic systemic inflammation.

Conflict of Interest

The authors declare no conflict of interest.

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Review Article

Chronic Renal Failure, Cachexia, and Ghrelin

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Protein energy wasting is frequently observed in patients with advanced chronic renal failure and end-stage renal disease. Anorexia and reduced food intake are critical contributing factors and negatively impact on patients' survival. Ghrelin is a prophagic peptide produced by the stomach and acting at the hypothalamic level to increase the activity of orexigenic neurons. In patients with chronic renal disease, plasma levels are increased as a likely effect of reduced renal clearance. Nevertheless, patients' food intake is significantly reduced, suggesting inflammation-mediated resistance of hypothalamic nuclei to peripheral signals. A number of forms of evidence show that ghrelin resistance could be overcome by the administration of exogenous ghrelin. Therefore, ghrelin has been proposed as a potential strategy to improve food intake in chronic renal failure patients with protein energy wasting. Preliminary data are encouraging although larger prospective clinical trials are needed to confirm the results and to identify those patients who are likely to benefit most from the administration of exogenous ghrelin.

1. Introduction

The clinical course of chronic diseases is frequently complicated by the progressive deterioration of nutritional status, which significantly impacts on patients' morbidity, mortality and quality of life [1]. Chronic renal failure and end-stage renal disease are not exceptions, and up to 60% of patients undergoing haemodialysis present with malnutrition [2]. It is important to remember that malnutrition in end-stage renal disease is a complex syndrome, which develops not only from reduced energy intake like in simple starvation. Indeed, profound metabolic changes (i.e., increased protein catabolism, reduced muscle anabolism, increased energy expenditure, adipose tissue loss, insulin resistance, etc.) are critical determining factors. Consequently, normalization of energy and protein intake, although it may improve renal patients' clinical outcome [3], does not result in restoration of nutritional status and body composition.

Although the clinical phenotype of malnourished patients with chronic renal failure can be easily recognized and is widely accepted, more uncertainties exist on the terminology to be used to define this syndrome. Indeed, the word "malnutrition" may be misleading since it may generate confusion with the deterioration of nutritional status induced by simple starvation only. The term "cachexia" has been largely used to define disease-associated malnutrition and has been proposed to include malnourished renal patients as well [4]. However, considering the clinical specificities of end-stage renal disease, a number of different terms have been also suggested, including "protein-energy wasting" [5], "malnutrition-inflammation complex (or cachexia) syndrome" [2], and "kidney disease wasting" [6]. For the purpose of this review article and in the attempt to highlight the analogies with other chronic conditions, the term "cachexia" will be used to define the clinical syndrome of weight loss, anorexia, reduced muscle performance, anemia, and so forth, associated with chronic renal failure and end-stage renal disease.

The pathogenesis of cachexia in renal patients is multifactorial, but anorexia and reduced food intake, as well as profound changes in macronutrient metabolism are the driving forces, leading to a practically not reversible catabolic status. The molecular mechanisms prompting these clinical symptoms are increasingly being understood. As recently

reviewed by Muscaritoli et al., increased levels of circulating cytokines, metabolic acidosis, oxidative stress and insulin resistance all appear to be variably implicated, although the individual role of each component in the pathogenesis of chronic kidney disease-related cachexia is still unclear [5]. However, it appears from recent clinical data that inflammation may play a pre-eminent role in triggering the cascade of biochemical events eventually leading to the development of anorexia and muscle wasting, that is, to cachexia.

2. Chronic Renal Failure and Cachexia

As previously mentioned, one of the most important player in the pathogenesis of cachexia in renal patients is the reduction of appetite, that is, anorexia. In a large study involving a cohort of 331 patients undergoing maintenance hemodialysis, the presence of impaired appetite (reported by 38% of the patients studied) was significantly associated with reduced 12-month survival and increased hospitalization rate [7]. Interestingly, anorexia was associated with increased circulating levels of surrogate markers of inflammation, that is, Tumor Necrosis Factor-α and C-reactive protein [7]. These data points to inflammation as the major trigger of the molecular cascade of events eventually leading to anorexia and poor outcome.

Supportive data have been published and indicate that inflammation may also trigger the progressive wasting of skeletal muscles. A number of experimental and clinical studies consistently show that muscle mass wasting is closely related to the presence of inflammation and in particular to inflammation-mediated activation of specific proteases [5, 8, 9]. In uremic rodents and patients, the first step in muscle protein loss is the activation of caspase-3. This cleaves the complex structure of muscle, thereby exposing a characteristic 14 kDa actin fragment in the insoluble fraction of muscle. Then, the ubiquitin-proteasome system is activated, which rapidly degrades proteins released by caspase-3 cleavage of muscle proteins.

In adults and under physiological conditions, muscle protein catabolism and anabolism are in equilibrium and offset each other. During disease, including end-stage renal failure, the activation of the proteolytic pathways is not counterbalanced by a corresponding increase of the anabolic pathways [9]. Indeed, activation of the proteolytic systems occurs when there is suppression of the growth-hormone-(GH-) mediated cellular signaling pathway activated by the insulin/insulin-like growth factor (IGF) 1, the phosphatidylinositol 3-kinase/Akt pathway, the main muscle protein synthesis pathway [9]. It is important to note that renal failure is a state of GH resistance and not GH deficiency [10]. Some mechanisms of GH resistance are: reduced density of GH receptors in target organs, impaired GH-activated post-receptor Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling, and reduced levels of free IGF-1 due to increased inhibitory IGF-binding proteins [11].

End-stage renal disease is associated with a state of insulin resistance [12]. Convincing data show that insulin resistance is associated with increased skeletal muscle protein breakdown [13], and that inflammation represents a determining factor [14]. Oxidative stress could also influence insulin sensitivity of peripheral tissues [14]. Interestingly, inflammation has been demonstrated to derange mitochondrial function thereby favoring leakage of reactive oxygen species [15]. Since mitochondrial dysfunction contributes to the development of insulin resistance in skeletal muscle [16], it appears that inflammation either directly, that is, activating the proteolytic systems, or indirectly, that is, favoring the development of oxidative stress and insulin resistance, is the main mediator of wasting in patients with chronic renal failure. Also, considering its role in anorexia [17], it could be speculated that disease-induced inflammation triggers the cascade of biochemical events leading to cachexia, although the specific phenotype of cachexia of each renal patients is also determined by his/her own genetic profile [17].

Supporting the role of the central nervous system as a preferential target for inflammation in mediating the onset of cachexia in renal patients [17], consistent evidence show that hypothalamic melanocortin signaling triggers anorexia and skeletal muscle wasting in experimental models of uremic cachexia [18], and thus provide a further potential target for the development of effective therapies [19].

3. Ghrelin in the Pathogenesis of Cachexia of Renal Patients

Under physiological conditions, energy homeostasis is tightly controlled by the hypothalamic integration of peripheral signals conveying to the central nervous system information on the metabolic status of peripheral tissues [20]. In the hypothalamus, two populations of neurons are colocalized in the rodents' arcuate nucleus (the infundibular nucleus in humans): the activation of pro-opiomelancortin (POMC) neurons promotes increased energy expenditure and satiety, while activation of Neuropeptide Y (NPY) neurons triggers the onset of appetite [21]. The integrated activities of POMC and NPY neurons are controlled by a complex mechanism: basically, POMC and NPY neurons reciprocally respond to peptides, as well as other signals, produced in peripheral tissues according to specific metabolic conditions, and modulate accordingly energy homeostasis [21].

Ghrelin is a unique hormone with potent orexigenic effects [22]. It is an acylated peptide produced primarily by gastric cells representing the endogenous ligand for the growth hormone secretagogue receptor [23]. In addition to stimulate the release of GH from the pituitary, ghrelin administration stimulates food intake, and carbohydrate utilization, and increases adiposity in rodents, suggesting a role for this hormone in energy balance [24]. Ghrelin influences neuronal activity through its receptor in several areas of the brain governing energy homeostasis, including the hypothalamus (specifically arcuate NPY neurons) [24]. Additionally, orexigenic effects of ghrelin are also mediated by modulation of hypothalamic fatty acid metabolism [25].

Under physiological conditions, acylated ghrelin, that is, the orexigenic form of ghrelin, represents <10% of

circulating ghrelin, the rest being des-acylated ghrelin. Des-acylated ghrelin has been considered as the inactive form of ghrelin, but recent data suggest that it may exert biological functions [26].

Considering its contributory role in determining energy homeostasis, ghrelin has been postulated to be involved in the pathogenesis of renal cachexia. In particular, reduced levels of circulating ghrelin were hypothesized as a pathogenetic mechanism mediating anorexia. Experimental and clinical studies could not support this hypothesis, since ghrelin levels have been consistently found increased in patients with end-stage renal failure, undergoing or not maintenance hemodialysis [27–29]. Supporting the lack of a major role for acylated ghrelin in mediating uremic anorexia, Bossola et al. recently demonstrated that circulating levels are significantly higher in uremic patients with poor/very poor appetite when compared with uremic patients with good/fair appetite [30]. More recently, Zabel et al. showed that in hemodialysis patients, hunger ratings measured with visual analogue scales correlate with markers of inflammation, but no correlation can be found with circulating acylated ghrelin [31]. On the other hand, des-acylated ghrelin has been postulated to suppress food intake, and Muscaritoli et al. demonstrated higher levels of des-acylated ghrelin in anorexic uremic patients undergoing hemodialysis than in non-anorexic patients [32]. However, the role of des-acylated ghrelin in mediating uremic anorexia needs to be further investigated.

The acylation of ghrelin is mediated by a specific enzyme, ghrelin O-Acyltransferase (GOAT), which attaches octanoate to serine-3 of ghrelin. Considering the potential pathogenic role of the ratio in plasma between acylated and des-acylated ghrelin in mediating renal cachexia [33], it would be important to measure the expression and activity of GOAT during chronic renal failure. Unfortunately, such data are not available yet.

The mechanisms responsible for the increase of circulating ghrelin levels during end-stage renal failure are being investigated, and include impaired clearance and/or metabolism of ghrelin in the kidney. In contrast, the etiology of renal failure and hemodynamic parameters do not appear have any effect on plasma ghrelin levels [34]. Quite recently, the concept that ghrelin is a passive bystander influenced by progressive renal failure has been challenged by a series of intriguing results. Wang et al. demonstrated that ghrelin protects against endotoxemia-induced acute kidney injury by a likely inhibition of proinflammatory cytokines [35]. Barazzoni et al. showed in nondiabetic maintenance hemodialysis patients that insulin sensitivity is associated negatively with systemic inflammation and positively with total plasma ghrelin, suggesting a potential novel role of ghrelin in preserving insulin sensitivity in maintenance hemodialysis [36].

The specific role of ghrelin in muscle wasting of renal patients with cachexia remains to be determined. Under physiological conditions, plasma ghrelin levels relate to muscle mass [37], likely because of its orexigenic and GH-releasing effects. More recently, Sheriff et al. showed that a ghrelin receptor agonist attenuates muscle wasting in a model of burn injury-induced proteolysis [38]. It could be postulated that renal cachexia is associated with ghrelin resistance, which in turn may limit the anticatabolic effects of ghrelin, thereby exacerbating muscle proteolysis. This hypothesis needs to be tested in experimental models and in clinical trials.

When considered together, these data indicate that during end-stage renal disease, increased circulating ghrelin levels may represent an attempt to compensate and counteract inflammation, and support the use of exogenous ghrelin as a potential therapy for renal cachexia.

4. Ghrelin in the Therapy of Cachexia of Renal Patients

As previously mentioned, renal failure is a state of GH resistance, and based on the available data, it is a state of ghrelin resistance too. However, as previously mentioned, increased ghrelin levels could be considered as a protective mechanism to counteract the detrimental metabolic effects induced by inflammation. In this regard, exogenous administration of the hormone may overcome ghrelin resistance at target organs, improve metabolic alterations and result in clinical benefit.

In a pilot study testing the effects of a single subcutaneous administration of ghrelin in mild/moderate malnourished uremic patients receiving peritoneal dialysis, energy intake immediately doubled and was not followed by subsequent underswing [39]. These positive preliminary results have been confirmed in a more recent 7-day trial, which confirmed that in malnourished dialysis patients, daily subcutaneous ghrelin administration immediately and significantly increased appetite, with an increase in energy intake noted at the first study meal [40]. More importantly, this effect persisted throughout the week without the occurrence of clinically relevant side effects [40]. Also, energy expenditure, measured with free-living pulse and motion monitors, was unchanged by ghrelin [40]. The mechanisms by which ghrelin administration ameliorate energy intake of uremic patients is currently being investigated. In an experimental model of chronic renal failure, ghrelin treatment resulted in increased food intake and an improvement in lean body mass accrual that was related in part to a decrease in muscle protein degradation [41]. Additionally, circulating inflammatory cytokines were reduced in nephrectomized animals by ghrelin treatment relative to saline treatment. Finally, ghrelin-treated animals showed a decrease in the expression of IL-1 receptor in the brainstem and a decrease in expression of prohormone convertase 2, an enzyme involved in the processing of proopiomelanocortin to the anorexigenic peptide α-MSH [41]. More recently, Barazzoni et al. found that ghrelin treatment normalized low muscle mitochondrial enzyme activities in uremic rats [42]. This effect was associated with a lower muscle triglyceride content and higher AKT phosphorylation. Interestingly, the effects of ghrelin on mitochondria are independent of changes in food intake, while combined ghrelin treatment and higher food intake were needed to enhance AKT phosphorylation [42].

When these effects are considered together, it appears that ghrelin-induced muscle mitochondrial changes and lower tissue triglycerides could favor insulin action and muscle anabolism in the presence of improvement in food intake.

The administration of ghrelin to patients with renal cachexia may yield clinical benefits beyond its anticatabolic effects. In particular, chronic renal failure is frequently associated with a number of cardiovascular alterations, which may be counteracted by exogenous ghrelin. As an example, preliminary data indicate that ghrelin restores endothelial dysfunction in patients with obesity-related metabolic syndrome [43].

5. Conclusion

Cachexia associated with chronic renal failure is a clinically relevant syndrome, which negatively impacts on patients' morbidity and mortality, and impinges on their quality of life. The pathogenesis of this syndrome is quite complex, but inflammation represents a preeminent feature triggering the cascade of biochemical events eventually leading to deterioration of nutritional status. Ghrelin is a unique orexigenic hormone, whose role in cachexia is to counteract the detrimental effects on targeted organs of inflammation and oxidative stress. Although a state of ghrelin resistance appears to develop during end-stage renal failure, exogenous administration of ghrelin appears a promising therapeutic strategy to improve the clinical outcome of uremic patients.

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Research Article

Reconstruction-Dependent Recovery from Anorexia and Time-Related Recovery of Regulatory Ghrelin System in Gastrectomized Rats

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Gastrectomy reduces food intake and body weight (BW) hampering recovery of physical conditions. It also reduces plasma levels of stomach-derived orexigenic ghrelin. This study explored changes in orexigenic ghrelin system in rats receiving total gastrectomy with Billroth II (B-II) or Roux-en-Y (R-Y) method. Feeding and BW were reduced by gastrectomy and subsequently recovered to a greater extent with R-Y than B-II while plasma ghrelin decreased similarly. At postoperative 12th week, ghrelin contents increased in the duodenum and pancreas, plasma ghrelin levels increased upon fasting, and ghrelin injection promoted feeding but not in earlier periods. In summary, gastrectomized rats partially recover feeding and BW, in a reconstruction-dependent manner. At 12th week, ghrelin is upregulated in extra-stomach tissues, plasma ghrelin levels are physiologically regulated, and orexigenic effect of exogenous ghrelin is restored. This time-related recovery of ghrelin system may provide a strategy for promoting feeding, BW, and thereby physical conditions in gastrectomized patients.

1. Introduction

Ghrelin, a 28-amino acid peptide, is produced mainly in the stomach and to a lesser extent in the small intestine, pancreas, and hypothalamus and is the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [1–9]. Serine-3 of ghrelin is acylated with an octanoic acid, the process catalyzed by a recently discovered ghrelin *O*-acyltransferase (GOAT) [10, 11]. The acylation is thought to be required for its biological activity [1], although desacyl ghrelin has been reported to exert several effects [12]. Administration of pharmacological doses of acylated ghrelin (= ghrelin) to intact animals increases food intake, induces body weight gain, and causes obesity [13–18]. The orexigenic and body fat promoting properties of ghrelin and growth hormone (GH) secretagogue (GHS) are thought to be independent of GH and mediated primarily by the hypothalamic neuropeptide Y (NPY) and agouti-related protein (AGRP) systems [14–16, 19–22]. Although pharmacologic effects of ghrelin are well documented, physiological

role of endogenous ghrelin is poorly understood. It has recently been reported that knockout of either the ghrelin gene or ghrelin receptor gene exerts no or minor effects on body weight and body composition [23–25]. However, the lack of phenotypic changes in knockout mice might reflect compensatory mechanisms that are known to operate occasionally.

In humans, gastrectomy results in loss of body weight of about 10% within the first six months after surgery, primarily due to reduced body fat [26]. In addition, gastrectomized patients often complain of loss of appetite, general fatigue, and in some cases impaired bone quality such as osteopenia and osteomalacia [9, 27]. At present there are no satisfactory mechanistic explanation and treatment for any of these symptoms. The effects of gastrectomy on food intake and body composition have been poorly documented in rodents. Loss of ghrelin could be implicated in these symptoms, since as much as 80% of circulating ghrelin is lost following surgical removal of the glandular stomach or the acid producing part of the stomach in rats and humans [4, 28, 29].

In this study, we performed total gastrectomy in rats and examined whether the recovery from gastrectomy-associated anorexia depends on the circulating ghrelin level or other factors and studied temporal changes in the biosynthesis and orexigenic ability of ghrelin following gastrectomy.

2. Materials and Methods

2.1. Animals and Gastrectomy. Male 4-week-old Wistar rats (SLC, Japan) were maintained on a 12-hour light/dark cycle and given conventional food and water for 2 weeks and not deprived of food before gastrectomy. They were operated at 6 weeks of age with body weight around 130–180 g. Rats were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) and a median abdominal incision was made. After the stomach was separated from the greater and lesser omentum, the duodenal bulb was ligated and transected. After the left gastric artery was ligated, the esophagus was clamped above the esophagogastric junction and the stomach was resected. An end-to-side anastomosis between esophagus and jejunum at the 4-5 cm anal side from Treitz ligament was performed with 7–0 monofilament polyglyconate synthetic absorbable string (Maxon; Johnson & Johnson Inc. USA) using interrupted suture (Billroth II (B-II) reconstruction). On the other hand, jejunum was transected at the 2-3 cm anal side from the Treitz ligament. Then, an end-to-side anastomosis between the esophagus and jejunum was performed with 7–0 Maxon using an interrupted suture. Jejunojejuno anastomosis was also done by end-to-side method (Roux-en-Y (R-Y) reconstruction). The abdominal wall and skin closure was made by 5–0 Nylon (Johnson & Johnson Inc. USA) in a running suture. After the operation, the rats were allowed only clear water without food for 3 days, dry milk from the 3rd day, and from the 7th day a conventional pellet diet with free access to water. The care of the animals was in accordance with our institutional guidelines.

2.2. Measurements of Food Intake and Body Weights. After the operation, food intake for 24 hours and body weight were measured once a week for 3 months.

2.3. Preparation of Blood and Tissue Samples. The rats were sacrificed at three months after operation. To measure plasma ghrelin concentrations, blood samples were collected from the inferior vena cava of anaesthetized rats. Duodenum samples of about 3 cm were taken from 1 cm anal side of duodenal stump. Jejunum samples of about 3 cm were taken from 1 cm anal side of esophagojejunostomy. Pancreatic samples were also taken. The tissues were quickly frozen and stored at -80°C until assaing.

2.4. RIAs Using Specific Antiserum and ELISA for Ghrelin. Two kinds of rabbit polyclonal antiserum were used in the present study. One antiserum was raised against the COOH-terminally Cys-extended rat ghrelin (position 1-11) in New Zealand white rabbits (#G606) that was shown to specifically recognize ghrelin with *n*-octanoylated Ser 3 (acylated ghrelin; ghrelin). By the radioimmunoassay (RIA) using this antiserum, designated NH_2-terminal RIA (N-RIA), the concentration of acylated ghrelin was obtained. The other antiserum was raised against the NH_2-terminally Cys-extended rat ghrelin (position 13-28) (#G107) that was shown to recognize both acylated ghrelin and desacyl ghrelin [3]. By the RIA using this antiserum, named as COOH-terminal RIA (C-RIA), the concentration of acylated ghrelin plus desacyl ghrelin was obtained.

These two ghrelin-specific RIAs were used to measure acylated ghrelin and desacyl ghrelin contents in several tissues [3]. The bound and free ligands were separated using a second antibody. "Acylated ghrelin" is expressed as "ghrelin" and "acylated ghrelin plus desacyl ghrelin" is expressed as "total ghrelin" throughout this text.

Plasma concentrations of acylated ghrelin and desacyl ghrelin were measured using ELISA kits (Mitsubishi Kagaku Iatron, Tokyo, Japan).

2.5. Treatment with Ghrelin. Ghrelin (Peptide Institute, Osaka, Japan) or saline was administered subcutaneously to control normal rats (eight-weeks-old) and those received gastrectomy with B-II reconstruction. For ghrelin treatment at 2 and 6 weeks after gastrectomy, the following procedures were used. Control and gastrectomized rats were divided into two groups; one group was treated with ghrelin and the other with saline, and food intake and body weight were measured. The injection of ghrelin (10 nmol/kg body weight) was carried out once a day for seven days, and food intake and body weight were measured. For ghrelin treatment at 12 weeks after gastrectomy, the following procedures were used. Single injection of ghrelin or saline was followed by measurements of food intake for the subsequent 24 hours. In the next week, two groups were changed: one group that had first received ghrelin was injected with saline, and the other group that had first received saline was injected with ghrelin. Food intake after the first injection and that after the second injection were pooled and averaged.

2.6. Statistical Analysis. Data represent the means ± SEM (n = number of rats). Statistical analysis was performed using Student's paired and unpaired t-tests. Values of $P < .05$ were considered statistically significant.

3. Results

3.1. Food Intake and Body Weight in Total Gastrectomized Rats. Food intake for 24 hours and body weight were measured in total gastrectomized and control rats during 12 postoperative weeks. Gastrectomy with B-II ($n = 40$) and R-Y reconstruction methods ($n = 35$) both markedly decreased food intake. In both B-II and R-Y groups, at the 1st week after operation food eaten for 24 hours decreased to a level around 50% of that in control rats (Figure 1(a)). In B-II group the reduced level of food intake continued for 12 weeks after operation. In contrast, food intake in R-Y group increased gradually during 4 postoperative weeks. The daily food intake averaged for postoperative 12 weeks was significantly ($P < .05$) reduced in B-II and R-Y groups (Figure 1(a)). Food intake in R-Y group was significantly ($P < .05$) greater than in B-II group throughout postoperative 12 weeks. Body weight significantly decreased after gastrectomy in both B-II and R-Y groups (Figure 1(b)). Body weight in R-Y group was significantly greater than that in B-II group from the 2nd through 12th postoperative week ($P < .05$).

The correlation between body weight, 24 hours food intake, and plasma ghrelin concentrations at the 12th postoperative week was studied. Food intake and body weight were correlated with each other irrespective of reconstruction methods and were plotted on a single regression line (Figure 2(a)). In contrast, there was no correlation between food intake and plasma concentrations of total ghrelin (Figure 2(b)). We next examined whether body weight correlates with plasma concentrations of ghrelin or total ghrelin, by using N-RIA and C-RIA. Body weight did not correlate with plasma ghrelin and total ghrelin levels (Figures 2(c) and 2(d)). Furthermore, no significant difference between R-Y and B-II groups was observed in averaged ghrelin levels (B-II: 46.3 ± 7.2 fmol/ml ($n = 40$), R-Y: 35.9 ± 4.6 fmol/ml ($n = 35$)) and total ghrelin levels (B-II: 393.0 ± 40.0 fmol/ml ($n = 40$), R-Y: 349.8 ± 23.2 fmol/ml ($n = 35$)), though some rats in B-II groups showed higher levels of ghrelin and total ghrelin. These results indicate that plasma ghrelin levels are significantly reduced in R-Y and B-II groups and that greater food intake in R-Y than in B-II is related to the method of reconstruction but not plasma ghrelin levels.

3.2. Plasma Ghrelin Concentrations and Ghrelin Contents in Duodenum, Jejunum and Pancreas at 12th Postoperative Week. Plasma ghrelin concentrations were markedly reduced in both R-Y and B-II groups to the levels of about 30% of those in control rats (Figure 3(a)). Stomach produces approximately 70% of total ghrelin [3]. Therefore, the reduction of plasma ghrelin levels is due to lack of release of ghrelin from stomach. We examined whether the reduction of ghrelin due to gastrectomy could influence the

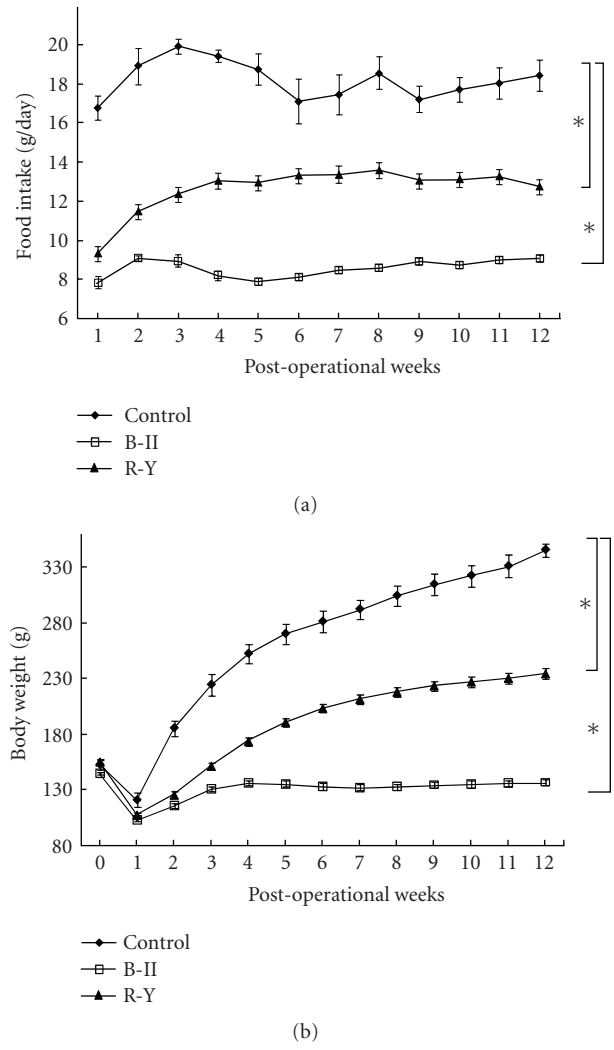

Figure 1: Daily food intake (a) and body weight (b) in rats for 12 weeks after total gastrectomy combined with Billroth II (B-II) or Roux-en-Y reconstruction method (R-Y), and after sham-operation as the control. Gastrectomy was performed at 6 weeks of age. $N = 6$ for control, 40 for B-II and 35 for R-Y. $^*P < .05$.

production of ghrelin in the intestine and pancreas, the tissues known to produce ghrelin. Ghrelin concentrations in the duodenum increased approximately by 100% in R-Y and B-II groups (Figure 3(b)), while those in the jejunum did not change (Figure 3(c)). Ghrelin concentrations in the pancreas were much smaller than those in the intestine, however, they dramatically increased approximately by 500% in B-II and by 400% in R-Y groups (Figure 3(d)). On the other hand, plasma concentrations of total ghrelin were approximately five times higher than those of ghrelin, and they were markedly reduced by gastrectomy in both groups (Figure 3(e)). Contents of total ghrelin in the duodenum and pancreas, but not jejunum, increased in both R-Y and B-II groups (Figures 3(f)–3(h)). The gastrectomy-associated relative changes of total ghrelin in the circulation, duodenum and pancreas were similar to those of ghrelin. These results suggest that gastrectomy-induced reductions in circulating

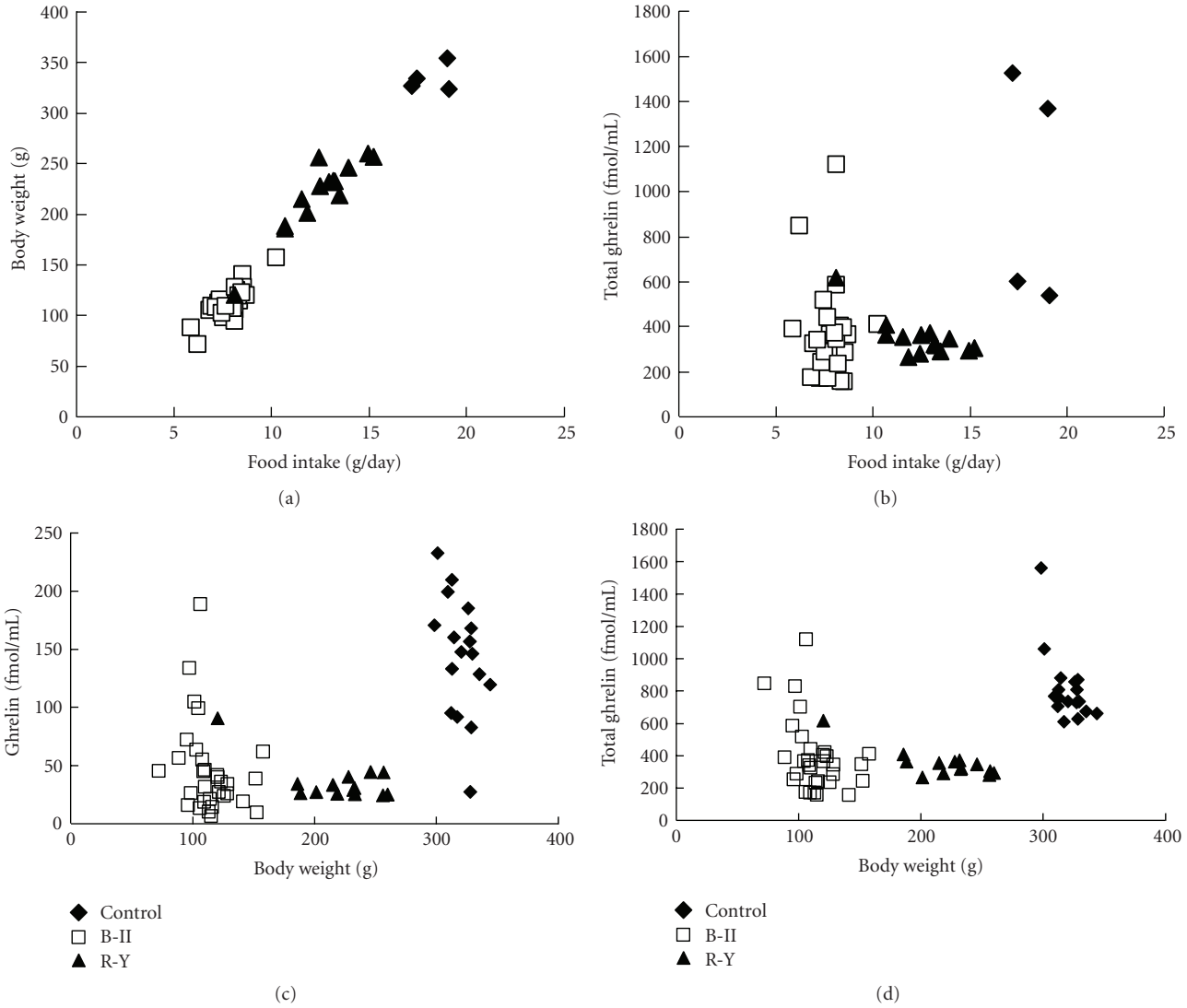

Figure 2: Relationship between daily food intake and body weight (a), between plasma total ghrelin concentrations and daily food intake (b), between plasma ghrelin concentrations and body weight (c), and between plasma total ghrelin concentrations and body weight (d) in rats at 12th week after total gastrectomy combined with B-II or R-Y as compared to the control with sham-operation. Ghrelin concentrations were determined with N-RIA in (c), and total ghrelin concentrations with C-RIA in (b) and (d). Total ghrelin corresponds to the sum of ghrelin and desacyl ghrelin.

ghrelin levels promote synthesis of ghrelin in the duodenum and pancreas, which may have compensatory roles including the restoration of circulating ghrelin levels. In consistence with this inference, plasma levels of ghrelin and total ghrelin that were markedly reduced at 2 weeks after gastrectomy with B-II reconstruction (ghrelin: 25.0 ± 5.3 fmol/ml ($n = 4$), total ghrelin: 295.8 ± 34.6 fmol/ml ($n = 4$)) were significantly ($P < .05$) elevated at the 12th postgastrectomy week (ghrelin: 46.3 ± 7.1 fmol/ml ($n = 40$), total ghrelin: 393.0 ± 40.0 fmol/ml ($n = 40$)).

3.3. Effect of Fasting on Plasma Ghrelin Levels at 12th Postoperative Week. We examined whether reduced levels of plasma ghrelin after gastrectomy could be altered by fasting, the physiological regulator of ghrelin release. At the 12th postoperative week, blood samples were taken from ad-lib fed and 48 hour fasting rats. Effects of fasting on plasma levels of ghrelin and desacyl ghrelin were examined by using ELISA, since RIA did not allow us to determine exact levels of desacyl ghrelin. Plasma ghrelin concentrations were significantly ($P < .05$) elevated by fasting approximately by 3 times in both B-II and R-Y groups (Figures 4(a) and 4(c)). Desacyl ghrelin concentrations were also significantly ($P < .05$) elevated by 3-4 times by fasting in B-II and R-Y groups (Figures 4(b) and 4(d)). Thus, fasting induced similar fold increases in both ghrelin and desacyl ghrelin levels in the circulation in gastrectomized rats.

3.4. Effect of Ghrelin Injection on Food Intake in Control and Gastrectomized Rats. Rats aged 8-weeks were divided

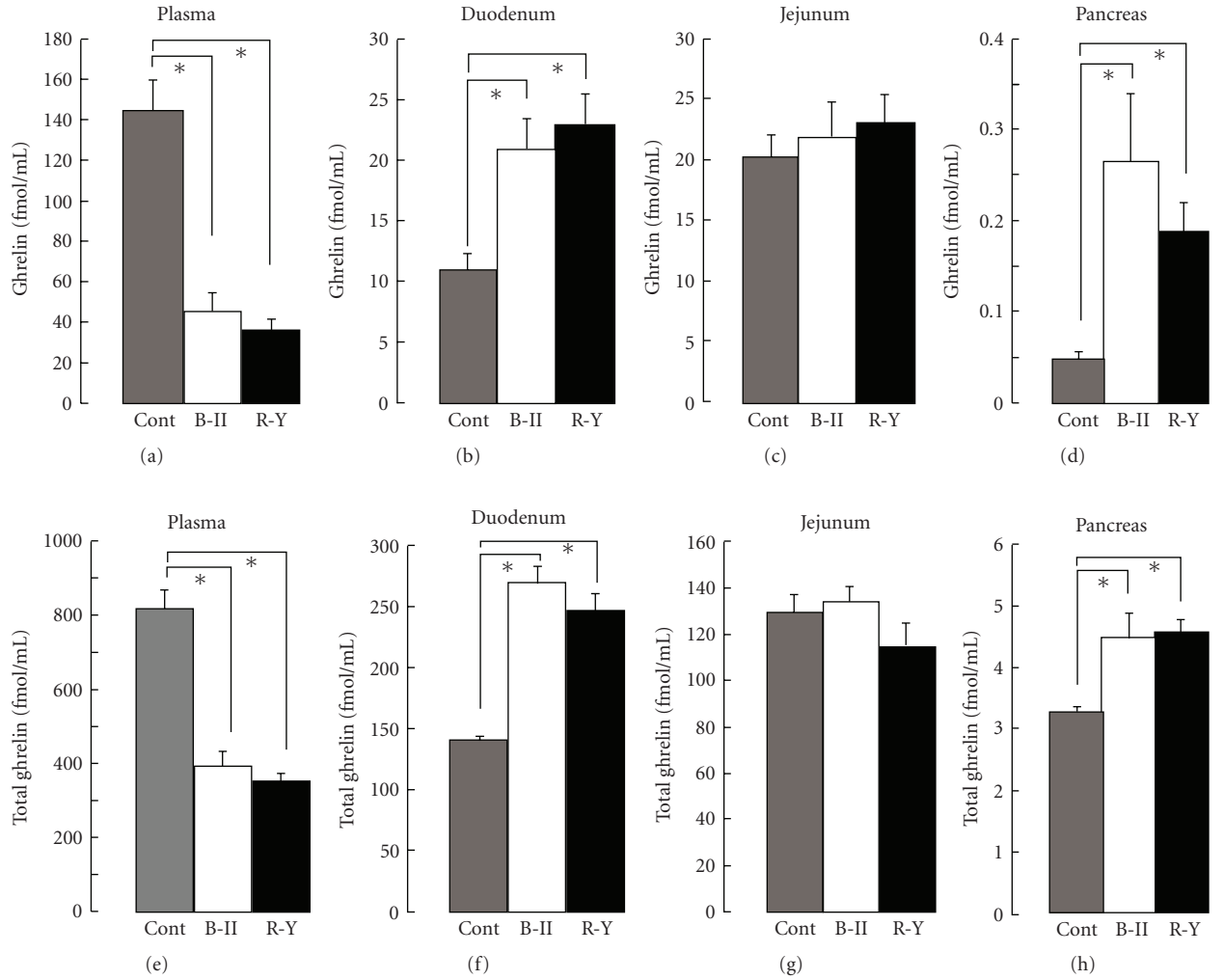

Figure 3: Concentrations of ghrelin and total ghrelin in the plasma, intestine and pancreas after gastrectomy. Ghrelin and total ghrelin concentrations in the plasma ((a), (e)) significantly decreased, while those in the duodenum ((b), (f)) and pancreas ((d), (h)) significantly increased in rats at 12th week after total gastrectomy with B-II or R-Y, compared to the control with sham-operation (cont). In contrast, neither ghrelin (c) nor total ghrelin concentration (g) in the jejunum differed among B-II, R-Y and control groups. Concentrations of ghrelin and total ghrelin were determined by N-RIA and C-RIA, respectively. $N = 17$ for control, 31 for B-II and 14 for R-Y. $^*P < .05$.

into two groups and received subcutaneous injection of either saline or ghrelin once a day for seven continuous days. Injection of ghrelin increased food intake, in which significant ($P < .05$) difference was obtained at the 2nd, 4th and 5th days (Figure 5(a)). The cumulative food intake during the treatment period of 7 days was significantly ($P < .05$) increased by ghrelin (Figure 5(b)).

At 2 weeks after total gastrectomy, rats were divided into two groups and received subcutaneous injection of either saline or ghrelin once a day for 7 continuous days. The 24 hours food intake averaged for the treatment period of 7 days was not significantly different between ghrelin-and saline-treated groups (Figure 5(c)). Essentially the same results were obtained in rats at 6 weeks after total gastrectomy; 24 hours food intake averaged for the treatment period of 7 days was not different between ghrelin-and saline-treated groups (Figure 5(d)).

At the 12th week after total gastrectomy, rats were divided into two groups. One group received daily subcutaneous injection of saline for a week followed by that of ghrelin for the next week. Vice versa, the other group received ghrelin for the first week followed by saline for the next week. The amount of 24 hours food intake in rats injected with ghrelin was significantly ($P < .05$) greater than that with saline (Figure 5(e)). These results indicate that ghrelin injection increases food intake in the later stage of 12 weeks after gastrectomy but not earlier.

4. Discussion

Ghrelin is secreted primarily from the stomach [9, 30], and stimulates food intake and body weight gain. Gastrectomy commonly decreases food intake, body weight, fat mass and bone mass, and is also accompanied by a marked reduction

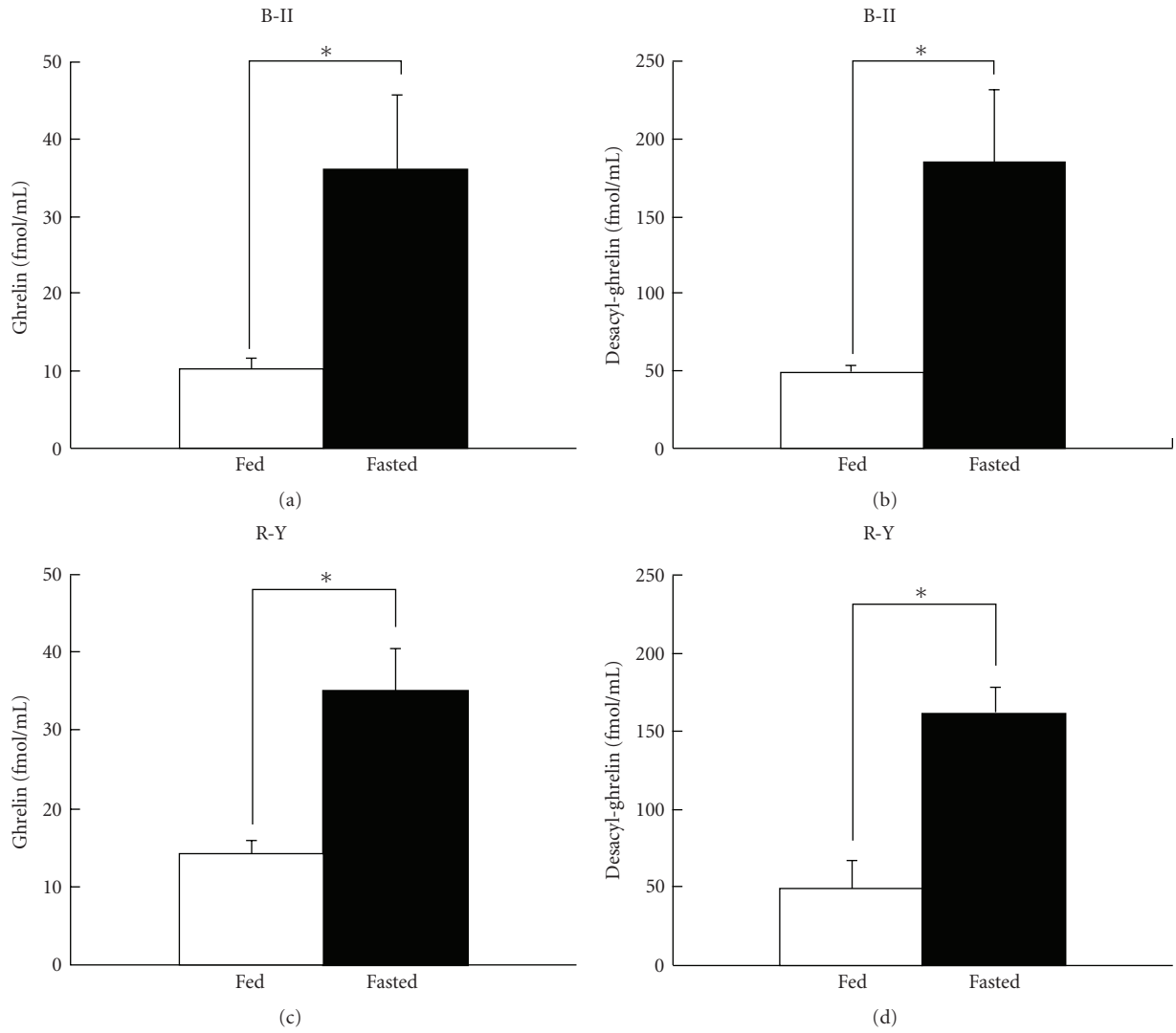

Figure 4: Plasma concentrations of ghrelin and desacyl ghrelin were significantly elevated by fasting. Plasma concentrations of ghrelin ((a), (c)) and desacyl ghrelin ((b), (d)) under fed condition and after 48 hour fasting in B-II group ((a), (b)) and R-Y group ((c), (d)) at 12th postoperative week. Concentrations of ghrelin and desacyl ghrelin were determined by ELISA. $N = 8$ for B-II and 11 for R-Y. *:$P < .05$.

in circulating ghrelin levels [31]. However, whether ghrelin levels are causally related to food intake and/or body weight after gastrectomy is not well understood. In the present study, we produced total gastrectomized rat models and examined postoperative changes in ghrelin levels, food intake and body weight. Plasma ghrelin levels were markedly reduced by gastrectomy irrespective to whether the reconstruction was performed by B-II or R-Y method. However, the recovery from gastrectomy-induced reductions in food intake and body weight was much greater in R-Y than in B-II group. The results indicate that the recovery from reduced food intake and body weight after gastrectomy is not correlated with circulating ghrelin levels but strongly depends on the reconstruction method.

The mechanism for the better recovery of food intake and body weight with R-Y method after total gastrectomy remains unclear. Ghrelin contents in the duodenum, jejunum and pancreas at 12th postoperative week were not different between B-II and R-Y methods. It is speculated that in B-II method bile juice may directly reflux the esophagojejunostomy and consequently decrease food intake. Alternatively, shorter distance between esophagojejunostomy and jejunojejunostomy in R-Y method than in B-II method may yield smaller reflux of bile juice. It is suggested that selecting the reconstruction method with less reflux of bile juice may contribute to better recovery from the gastrectomy-induced anorexia.

At the 12th week after gastrectomy, ghrelin production was markedly enhanced in the duodenum, the organ known to produce the second largest amount of ghrelin [3]. This result was essentially the same between B-II and R-Y groups, suggesting that the enhanced ghrelin production is not related to the reconstruction method but likely due to the removal of gastric ghrelin. It is therefore suggested that the

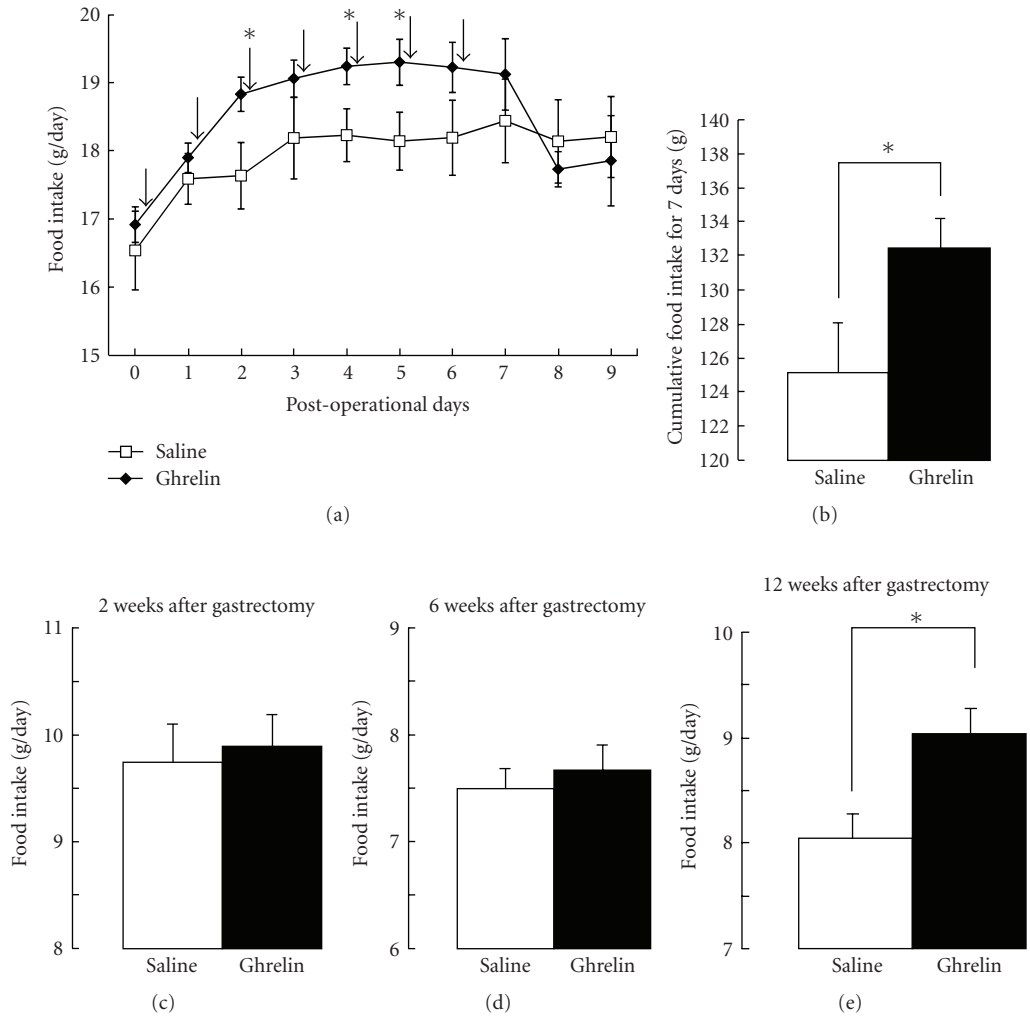

Figure 5: Effect of ghrelin injection on food intake in gastrectomized and control rats. (a) and (b): Ghrelin or saline was injected subcutaneously to control rats once daily for 7 days, and daily food intake was measured for 10 days (a) and expressed by the cumulative food intake for 7 days (b). Arrows indicate the time of injection of ghrelin or saline. $N = 11$ for saline and ghrelin.in (c), (d) and (e): Ghrelin or saline was injected to rats once daily for 7 days at 2 (c), 6 (d) and 12 weeks (e) after total gastrectomy with B-II, and the daily food intake averaged for 7 days is shown. $N = 21$ for saline and 22 for ghrelin in (c). $N = 21$ for saline and 20 for ghrelin in (d). $N = 37$ for saline and ghrelin in (e). $^{*}P < 0.05$.

enhanced ghrelin production in the duodenum could partly compensate for gastrectomy-associated reductions of the circulating ghrelin and its endocrine functions. In another line of experiments, at 12th postoperative week fasting markedly increased plasma ghrelin levels in gastrectomized rats, indicating that the tissue other than stomach, most likely the duodenum, secretes ghrelin in response to fasting, the physiological regulator of ghrelin secretion [9, 32]. Furthermore, the feeding response to peripheral ghrelin administration was once eliminated by gastrectomy but restored at 12th postoperative week. These data suggest that ghrelin can be released and stimulate food intake under fasted conditions at this later period after gastrectomy. Collectively, the markedly upregulated production of ghrelin in the duodenum could contribute to the recovery of food intake and body weight in the later postoperative period.

In this study, ghrelin production was also significantly elevated in the pancreas. Since the ghrelin content in the pancreas and its increment due to gastrectomy are much smaller than those in the duodenum, they may neither significantly contribute to the circulating ghrelin levels nor operate endocrine functions. However, previous studies using ghrelin receptor antagonists and ghrelin-deficient mice have demonstrated that ghrelin in the pancreatic islets inhibits insulin release in an autocrine/paracrine manner and consequently regulate blood glucose levels [33, 34]. The late postprandial dumping syndrome accompanying gastrectomy is characterized by hypoglycemia principally due to excessive insulin release. The mechanism for excessive insulin release remains unclear, lack of the stomach-derived ghrelin could be implicated. If so, it is possible that upregulated pancreatic ghrelin compensates lack of stomach-derived ghrelin and

attenuate insulin release, thereby counteracting the hypoglycemia in the late dumping syndrome. Further study is necessary to elucidate the role and mechanism for the upregulation of ghrelin production in the pancreas after gastrectomy.

It has been well documented that administration of pharmacological doses of ghrelin to intact animals increases food intake, induces weight gain, and causes obesity [9, 13–18]. Ghrelin injection failed to stimulate food intake in the early stages of 2nd to 6th week after operation, but increased it in the late stage of 12th week. The results suggest that some machinery that links ghrelin reception to feeding dysfunctions after gastrectomy but can be restored. A candidate for such machinery is the vagal nerve. It has been reported that the peripheral injection of ghrelin does not increase food intake after cutting vagus nerve [15, 30, 35]. It is thought that peripheral ghrelin signal is transmitted to the feeding center at least partly via afferent vagal nerves [15, 30, 35]. In our study, vagus nerves that were cut by the operation of gastrectomy may be regenerated later. It is speculated that regeneration of vagus nerves takes place not immediately but time-dependently, allowing reception of injected ghrelin and transmission of its signal to the feeding center. In addition, chronic hypoghrelinemia in gastrectomized status was reported to induce hypersensitivity to ghrelin as judged by secretion of growth hormone [36]. If this is also the case for the orexigenic action of ghrelin, the hypersensitivity to ghrelin could make the ghrelin injection more potent and/or efficacious in correcting anorexia after gastrectomy. Collectively, in the certain period when vagus nerves are regenerated and sensitivity to ghrelin is elevated, ghrelin treatment is expected to effectively stimulate feeding. In fact, the fractional increase of food intake in response to ghrelin injection at 12th postoperative week was similar to that in control rats (Figures 5(e) versus 5(a)). Thus, our results raise a possibility that ghrelin replacement therapy given in later stages could be effective in promoting food intake and correcting some symptoms associated with gastrectomy in humans.

Another health problem with dysregulated body weight and feeding is obesity. Obesity often causes hyperglycemia, hypertension and/or dyslipidemia, forming metabolic syndrome. Obesity and metabolic syndrome are major risk factors for cardiovascular disease. The rapidly increasing incidence of obesity has become a serious worldwide health problem [37]. An effective treatment of severe obesity is bariatric surgery, in which gastric bypass is a representative method. Gastric bypass, as well as gastrectomy, markedly reduces appetite and body weight, suggesting that changes in plasma ghrelin levels are involved. However, studies on changes in plasma ghrelin levels after gastric bypass have yielded inconsistent results; decreased, unchanged, or increased [38–40]. In our study, better restoration in feeding and body weight after gastrectomy was obtained with R-Y than B-II method, while plasma ghrelin levels were similar. These observations by us and others collectively suggest that feeding and body weight in the earlier period after gastric surgery, are not associated with plasma ghrelin levels but determined by other factors, possibly reconstruction methods associated with specific histological conditions.

In summary, recovery from gastrectomy-induced reductions in food intake and body weight is not related to plasma ghrelin levels but depends on the reconstruction method in rats. At postoperational 12th week, ghrelin production is increased in the duodenum and pancreas, and circulating ghrelin level is elevated by fasting, suggesting that ghrelin could be released from extra-stomach tissues and play a compensatory role. Ghrelin administration stimulates feeding at postoperational 12th week but not earlier, suggesting that ghrelin treatment in later periods could be effective in treating patients with gastrectomy-induced anorexia and associated symptoms.

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