Physiological significance of ghrelin revealed by studies using genetically engineered mouse models with modifications in the ghrelin system

Hiroyuki Ariyasu and Takashi Akamizu

The First Department of Internal Medicine, Wakayama Medical University, Wakayama 641-8509, Japan

Abstract. Ghrelin, an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R or ghrelin receptor), is a 28–amino acid acylated peptide mainly produced in the stomach. The pharmacological administration of ghrelin is known to exert diverse effects, such as stimulating GH secretion, promoting food intake, and increasing adiposity. In recent years, genetically engineered mouse models have provided important insights into the physiology of various hormones. In this review, we discuss current knowledge regarding the physiological significance of ghrelin on the basis of studies using genetically engineered mouse models with modifications in the ghrelin system.

Key word: Ghrelin

In order to investigate the physiological significance of hormones, genetically engineered mouse models have been widely utilized, and these models are also used as preclinical tools for exploring the novel therapeutic approaches for human disease. In this review, we discuss an outline of the physiological roles of ghrelin on the basis of research using genetically engineered mouse models, such as ghrelin-null, ghrelin receptor–null, ghrelin o-acyltransferase–null, and ghrelin-over-expressing mice.

Secretion of Ghrelin

Date et al. reported that ghrelin-producing cells are X/A-like cells that account for about 20% of the endocrine cell population in rat and human oxyntic glands [16, 17]. Ghrelin mRNA is most abundant in the stomach, small intestine, pancreas, ventricular myocardium, aorta, adipose tissue, and lymphocytes [13-15]. The wide distribution of ghrelin and its receptor suggests that ghrelin might play a range of physiological roles.
anism by which acute or chronic energy status influences circulating ghrelin levels is not fully understood, in vitro cell-based assays and in vivo studies will reveal the factors involved in ghrelin secretion. Ghrelin secretion is influenced by nutrients, hormones, and neural systems [22]. To better understand ghrelin production and secretion in vitro, we developed a ghrelin-producing cell line, MGN3-1 (mouse ghrelinoma 3-1), from a gastric ghrelin-producing cell tumor derived from a ghrelin promoter–SV40 T-antigen transgenic mouse [23, 24]. MGN3-1 cells express the insulin receptor and type 2 and 5 somatostatin receptors (SSTR2 and SSTR5); insulin and somatostatin directly suppress ghrelin secretion via these receptors [24]. Indeed, insulin and somatostatin (or a somatostatin analog) reduce circulating ghrelin levels in vivo [25-27]. By contrast, oxytocin and AVP stimulate ghrelin secretion from MGN3-1 cells. Vila et al. reported that oxytocin suppresses circulating ghrelin levels in normal healthy subjects [28]. However the effects of AVP on ghrelin secretion in vivo remain unknown. In in vitro cell-based assays, other hormones such as glucagon, GLP-1, CCK, gastrin, and leptin failed to simulate ghrelin secretion. Ghrelin-producing cells also express the α1 and β1 adrenergic receptors [29-31], and adrenaline and noradrenaline increase ghrelin secretion via the β1 receptor [29, 30-34].

**Transgenic Mouse Models for the Analysis of Ghrelin**

Genetically engineered mouse models are commonly used to elucidate the physiological significance of hormones or to mimic human diseases. As shown in Table 1, several lines of ghrelin loss-of-function mice have been created, including ghrelin and ghrelin receptor knockouts [35-40]. Ghrelin is an acylated peptide of 28 amino acids in which the serine-3 residue is n-octanoylated. This acyl modification, which is essential for ghrelin’s bioactivity, is mediated by the enzyme ghrelin O-acyltransferase (GOAT) [2, 3]. Consequently, genetic disruption of the GOAT gene in mice leads to the absence of acylated ghrelin in the circulation [2, 41, 42]. Because germline ghrelin-deficient mice may present with developmental adaptations and other compensatory mechanisms that confound the analysis, we and other groups generated transgenic mice that express the diphtheria toxin receptor in ghrelin-secreting cells. In these animals, injection of diphtheria toxin in adulthood ablates ghrelin-secreting cells, resulting in a reduction in circulating ghrelin levels by 80–95% [43-45].

By contrast, it has been challenging to generate ghrelin gain-of-function mouse. Early attempts to create ghrelin-overexpressing mice by standard procedure yielded mice that overproduced des-acyl ghrelin [46, 47]. Therefore, we tried to create Tg mice overexpressing a ghrelin analog that exhibits ghrelin-like activity in the absence of acylation at Ser3 [48, 49]. Because replacement of Ser3 of ghrelin with Trp3 (Trp3-ghrelin) preserves a low level of ghrelin activity, and Trp3-ghrelin can be synthesized in vivo [50], we generated mice overexpressing Trp3-ghrelin under the control of the hSAP (human serum-amyloid-P) promoter. Mice overproducing acylated ghrelin itself were developed by several groups. Bewick et al. used a bacterial artificial transgenic method, and their mice express a bioactive form of ghrelin in the stomach and brain [51]. Reed et al. generated the transgenic mice overexpressing acylated ghrelin in neurons by using the neuron-specific enolase (NSE) promoter sequences [52] Kirchner et al. generated Tg mice simultaneously expressing human ghrelin and GOAT in the liver under the control of the human apolipoprotein E promoter, and these transgenic mice exhibited elevated concentrations of fatty acid–modified forms of ghrelin only when fed an MCT (medium-chain triglyceride)-containing diet [41]. Two different groups, ours and Zhao et al., created ghrelin promoter–SV40 T-antigen transgenic (GP-Tag Tg) mice, in which ghrelin-producing cells proliferate and ultimately developed into ghrelinomas [23, 24, 29]. These mice exhibit elevated plasma ghrelin levels with preserved physiological regulation, at least with regard to feeding status, body weight, and sex difference; plasma ghrelin levels of GP-Tag Tg mice were increased by fasting and decreased by refeeding, and correlated to body weight. The regression coefficient of the regression line of GP-Tag Tg mice was bigger than that of nontransgenic littermates. In addition, Plasma ghrelin levels of female GP-Tag Tg mice were significantly higher than those of male GP-Tag Tg mice [23].

**GH Secretion**

There is now a consensus that administration of ghrelin leads to a marked increase in serum GH levels in rodents and humans [1, 9, 53]. To evaluate the physi-
Likewise, transgenic mice overexpressing a ghrelin analog exhibited no changes in GH/IGF-1 axis [48]. The discrepancies in serum IGF-1 levels can likely be explained by differences in plasma concentrations of circulating ghrelin or ghrelin analog, or alternatively by differences in ghrelin secretion patterns (e.g., continuous vs. pulsatile manner). In any case, however, it is clear that circulating ghrelin is not an essential factor for somatic growth.

Food Intake (FI)

Homeostatic regulatory system
Plasma ghrelin levels rise during fasting and are rapidly suppressed after feeding [4, 56], and peripheral administration of ghrelin induces not only GH secretion but also increased food intake (FI) in both animals and humans [1, 9-12, 57, 58]. With respect to feeding behavior, a dose of ghrelin that stimulates FI results in circulating ghrelin levels similar to those detected after a 24 h fast [59]. Ghrelin receptor is expressed in the hypothalamus, and peripheral administration of ghrelin activates hypothalamic neuropeptide Y (NPY)/Y1 and agouti-related peptide (AgRP) pathways [60-62]. In addition, ghrelin induces FI via the orexin pathway [63]. These results indicate that circulating ghrelin is a physiological meal-initiation factor.

To better understand the physiological roles of ghrelin in FI, several lines of genetically engineered mouse models with modifications in the ghrelin system have been investigated (Table 1, 2). Regardless of the absence of ghrelin in the circulation, feeding behavior such as daily FI, post-fasting FI, forced dark cycle-induced FI, and eating memory in ghrelin-null mice are

### Table 1 Ghrelin Loss-of-Function Mice

<table>
<thead>
<tr>
<th>Model</th>
<th>Serum IGF-I Levels</th>
<th>Food Intake</th>
<th>Insulin Secretion</th>
<th>Gastro-Intestinal Motility</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin k/o</td>
<td>→ or ↓ (No Growth Retardation)</td>
<td>→ (CD) → or ↓ (HFD)</td>
<td>NA</td>
<td>↑</td>
<td>[35][36][37][38][64][80][81]</td>
</tr>
<tr>
<td>Ghrelin receptor k/o</td>
<td>→ or ↓ (No Growth Retardation)</td>
<td>→ (CD) → or ↓ (HFD)</td>
<td>↓</td>
<td>↑</td>
<td>[39][40][72][73]</td>
</tr>
<tr>
<td>GOAT k/o</td>
<td>→ (AF) ↓ (CR)</td>
<td>→ or ↓ (HFD) → or ↓ (MCT)</td>
<td>↓</td>
<td>↑</td>
<td>[41][42][74]</td>
</tr>
<tr>
<td>Mouse ablated ghrelin-producing-cell</td>
<td>→</td>
<td></td>
<td>NA</td>
<td>→</td>
<td>[43][44][45]</td>
</tr>
</tbody>
</table>

CD, Control diet; HFD, High fat diet (Long-chain-triglyceride containing diet); MCT diet, Medium-chain-triglyceride containing diet; AF, adlib fed; CR, Caloric restriction; NA, Not available
natively due to mechanical obstruction of the gastrointestinal tract induced by gastric tumors [23, 29]. To avoid mechanical obstruction, we injected ghrelin-producing cells (MGN3-1) into nude mice subcutaneously [24]. Because MGN3-1 cells secrete substantial amounts of bioactive ghrelin under physiological regulation, mice injected with these cells exhibited elevated circulating ghrelin levels regulated in a physiologically normal manner. These mice exhibited significantly higher FI in comparison with controls. These findings imply that a reduction in circulating ghrelin does not alter FI, whereas elevation in circulating ghrelin in a physiological manner can stimulate FI.

Administration of ghrelin or growth hormone secretagogue reduces fat utilization and induces adiposity in animals, independent of food intake or GH release [12], suggesting that attenuation of ghrelin signals might be an effective treatment for obesity. Consistent with this idea, ghrelin loss-of-function mice increase fat utilization and are resistant to diet-induced obesity [40, 65]. However, some researchers have shown that ghrelin-null and its receptor–null mice are as susceptible as WT mice to diet-induced obesity under high-fat diet conditions [66]. These discrepancies could be due to differences in genetic background or the ages of the mice used in these studies.

Hedonic regulatory system

Energy homeostasis is maintained by both the homeostatic regulatory system and the hedonic regulatory system [67]. The homeostatic regulatory system is controlled by the hypothalamus, whereas the hedonic regulatory system is mainly controlled by the reward system. The mesolimbic dopamine pathway, which projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), is thought to play a

<table>
<thead>
<tr>
<th>Model</th>
<th>Serum IGF-1 Levels</th>
<th>Food Intake</th>
<th>Body Weight</th>
<th>Insulin Secretion</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin Tg Mouse</td>
<td></td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>[51]</td>
</tr>
<tr>
<td>Bacterial artificial Chromosome (BAC)</td>
<td>→</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>[52]</td>
</tr>
<tr>
<td>Neuron Specific Enolase (NSE)</td>
<td>NA</td>
<td>→ (MCT)</td>
<td>↑ (MCT)</td>
<td>NA</td>
<td>[41]</td>
</tr>
<tr>
<td>Mouse with Ghrelinoma (SV40-T-antigen)</td>
<td>↑</td>
<td>−</td>
<td>↓ (due to tumor growth)</td>
<td>↓ [23][29]</td>
<td></td>
</tr>
<tr>
<td>Ghrelin Analogue Tg Mouse</td>
<td>→</td>
<td>−</td>
<td>→</td>
<td>↓</td>
<td>[48]</td>
</tr>
<tr>
<td>Nude Mouse Transplanted with Ghrelinoma Cells</td>
<td>NA</td>
<td>↑</td>
<td>NA</td>
<td>NA</td>
<td>[24]</td>
</tr>
</tbody>
</table>

MCT, Medium-chain-triglyceride containing diet; NA, Not available

indistinguishable from those in their wild-type littermates [35-38, 64]. In addition, ghrelin receptor–null mice and GOAT-null mice did not decrease FI [39-42]. These results imply that ghrelin may not play a critical role in appetite. However, these results with germline ghrelin-null or its receptor–null mice might be explained by compensation in response to lifelong ghrelin deficiency.

Hence, we and McFarlane et al. generated transgenic mice that express the diphtheria toxin receptor specifically on ghrelin-secreting cells [43-45]. When injected with diphtheria toxin in adulthood, ghrelin-secreting cells are ablated and plasma ghrelin levels markedly decrease. Contrary to our expectations, ablation of ghrelin in adult mice did not affect feeding behavior. In these animals, nocturnal and diurnal meal sizes, cumulative FI, post-fasting FI, and adaptation to a negative energy state over a restricted feeding schedule were indistinguishable from those in controls, as in the case of mice who lacked ghrelin for their entire lives (i.e., germline ghrelin-deficient mice) [45]. Consistent with our findings, McFarlane et al. also showed that reduction in circulating ghrelin did not alter FI [44].

Seven studies of ghrelin gain-of-function mice have been published to date (Table 2). Six of them used transgenic mouse models overexpressing the active form of ghrelin, and the other used a ghrelin analog over-expressing mouse. Bewick et al. reported that a transgenic mouse generated by a bacterial artificial chromosome transgenic method overproduced bioactive ghrelin from stomach, resulting in increased FI [51]. However, the other five studies did not observe an increase in FI [23, 29, 41, 48, 52]. The differences between Bewick’s mouse and the others could be due to differences in the sites of ghrelin production, e.g., stomach [51] vs. liver or nervous system [52], or alternatively due to mechanical obstruction of the gastrointestinal tract induced by gastric tumors [23, 29]. To avoid mechanical obstruction, we injected ghrelin-producing cells (MGN3-1) into nude mice subcutaneously [24]. Because MGN3-1 cells secrete substantial amounts of bioactive ghrelin under physiological regulation, mice injected with these cells exhibited elevated circulating ghrelin levels regulated in a physiologically normal manner. These mice exhibited significantly higher FI in comparison with controls. These findings imply that a reduction in circulating ghrelin does not alter FI, whereas elevation in circulating ghrelin in a physiological manner can stimulate FI.

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primary role in the reward system [68], and the mesolimbic DA system is activated by palatable food intake as well as drugs of abuse [69]. Ghrelin receptors are expressed not only in the hypothalamus but also in dopaminergic neurons in VTA, implicating ghrelin in the mesolimbic DA system [70]. Indeed, administration of ghrelin increases the action potential frequency in VTA and dopamine release in the striatum and NAc in WT mice, but not in the ghrelin receptor-null mice [71]. Furthermore, administration of ghrelin increased the consumption of rewarding food and alcohol in WT mice, but not in ghrelin receptor-null mice [72, 73]. Moreover, GOAT-null mice exhibit a decreased hedonic feeding [74]. These reports suggest that the ghrelin system is required for hedonic feeding. In addition to the VTA, ghrelin receptors are also expressed on orexin neurons in the lateral hypothalamus [75]. Lamont et al. reported that both ghrelin-null and ghrelin receptor–null mice had fewer orexin cells and less cFOS expression in the mesolimbic dopamine pathway, relative to WT animals, under a restricted feeding paradigm [76]. These reports suggest that ghrelin increases hedonic energy intake in part by stimulating both the orexin system and the mesolimbic reward system.

**Insulin and Glucagon Secretion**

Exogenous administration of ghrelin raises blood glucose and suppresses insulin release from the pancreas [77-79]. Data from genetically engineered mouse models are essentially consistent with the pharmacological results. Deletion of ghrelin augments glucose-stimulated insulin secretion [80, 81], whereas overexpression of ghrelin suppresses insulin secretion [23, 51, 52]. Several mechanisms by which ghrelin suppresses insulin secretion have been discussed; at the acute scale, ghrelin inhibits insulin release through stimulation of the Gaα2 subtype of GTP-binding proteins, resulting in activation of delayed outward K+ channels (Kv) [82], whereas at the chronic scale, ghrelin upregulates the IA-2β and/or AMPK-UCP2 pathways [83]. Doi et al. showed that administration of ghrelin increased the mRNA expression of IA-2β, a β-cell autoantigen for type 1 diabetes, in insulinoma cell lines; conversely, inhibition of IA-2β expression by RNA interference methods ameliorated ghrelin’s inhibitory effects on glucose-stimulated insulin secretion [84]. Sun et al. demonstrated that ghrelin-null mice exhibited reduced expressions of UCP-2 mRNA in the pancreas, resulting in increased insulin secretion in response to glucose stimulation [81]. Consistent with these results, Wang et al. demonstrated that ghrelin increases UCP2 mRNA expression and AMPK phosphorylation in insulinoma cell lines, and that either overexpression of UCP-2 or treatment with an activator of AMPK attenuates insulin secretion [83].

Ghrelin is produced not only in the stomach, but also in the pancreatic islets [5, 85-90]. We created a transgenic mouse in which mouse ghrelin cDNA and ghrelin O-acyltransferase are overexpressed under the control of the rat insulin II promoter (RIP-GG Tg), and evaluated the physiological role of intrasilet ghrelin in insulin secretion [91]. Although plasma ghrelin levels in the portal veins of RIP-GG Tg mice were unchanged relative to those in control mice, pancreatic ghrelin levels were elevated about 16-fold relative to those in control animals when the mice were fed a medium-chain triglyceride-rich diet. However, glucose tolerance, insulin secretion, and islet architecture in RIP-GG Tg mice were not significantly different from control mice [91]. Therefore, it seems likely that intra-islet ghrelin does not play a major role in the regulation of insulin secretion.

In addition to insulin secretion, ghrelin is also involved in the regulation of glucagon secretion [92]. Administration of ghrelin increase glucagon secretion from α-cells via elevation of intracellular calcium and phosphorylation of ERK. Transgenic mice harboring ghrelinomas have higher levels of plasma glucagon and blood glucose, whereas ghrelin receptor–null mice have lower plasma glucagon and fasting blood glucose [92]. These studies suggest that circulating ghrelin regulates blood glucose by stimulating both insulin and glucagon release from islet cells.

**Gastrointestinal (GI) Motility**

Pharmacological administration of ghrelin increases not only secretion of GH, but also gastric acid, in a dose-dependent manner [93]. The effects of ghrelin on gastric secretion are abolished by pretreatment with either atropine or vagotomy, but not by a histamine H2-receptor antagonist [93]. To date, no study has used ghrelin-null or ghrelin receptor–null mice to evaluate gastric acid secretion.

Administration of ghrelin also induces the migrating motor complex in fasting rat and human and accelerates postprandial gastric motility in healthy humans [94-98]. The half-emptying time of the stomach (T1/2)
pressure by ghrelin is due to a decrease in sympathetic nerve activity rather than a direct effect on blood vessels [112]. Mao et al. reported that the survival rate after myocardial infarction was lower among ghrelin-null mice than among controls, and that the causes of death were malignant arrhythmia and heart failure [113, 114]. An administration of ghrelin in ghrelin-null mice with myocardial infarction reduced the frequency of arrhythmias and the associated mortality [113]. These studies suggest that ghrelin exerts cardioprotective effects by suppressing sympathetic nerve activity.

Conclusions

Since the identification of ghrelin more than 15 years ago, numerous studies have examined the physiological, pathophysiological, and pharmacological roles of ghrelin. The genetically engineered mouse models with modified ghrelin systems that were used in these studies have significantly contributed to our knowledge of physiological importance of ghrelin. On the basis of the findings obtained from these mice and from pharmacological studies, small-scale clinical trials have been performed to assess the utility of ghrelin for the treatment of various disorders including anorexia nervosa [115], cancer cachexia [116, 117], GI disorders [95, 97, 108], and aging-related disorders [119]. In order to make progress toward the clinical application of ghrelin for these diseases, long-term and large-scale clinical trials and the further basic researches are needed. The genetically engineered mouse models with modified ghrelin systems should give us greater and deeper understanding of the physiological and/or pathophysiological significances of ghrelin.

Disclosures

The authors have nothing to declare.

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