Novel and Conventional Receptors for Ghrelin, Desacyl-Ghrelin, and Pharmacologically Related Compounds

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Abstract—The only molecularly identified ghrelin receptor is the growth hormone secretagogue receptor GHSR1a. Its natural ligand, ghrelin, is an acetylated peptide whose unacylated counterpart (UAG) is almost inactive at GHSR1a. A truncated, nonfunctional receptor, GHSR1b, derives from the same gene. We have critically evaluated evidence for effects of ghrelin receptor ligands that are not consistent with actions at GHSR1a. Effects of ghrelin are observed in cells or tissues where the expression of GHSR1a is not detectable or after the Ghsr gene has been inactivated. In several, effects of ghrelin are mimicked by UAG, and ghrelin binding is competitively reduced by UAG. Effects in the absence of GHSR1a and sites at which ghrelin and UAG have similar potency suggest the presence of novel nonspecific ghrelin receptors (ghrelin receptor–like receptors [GRLRs]). A third class of receptor, the UAG receptors, at which UAG, but not ghrelin, is an agonist has been proposed. None of the novel receptors, with the exception of the glycoprotein CD36, which accounts for ghrelin action at a limited number of sites, have been identified. GHSR1a and GHSR1b combine with other G protein–coupled receptors to form heterodimers, whose pharmacologies differ from their components. Thus, it is feasible some GRLRs and some UAG receptors are heterodimers. Effects mediated through GRLRs or UAG receptors include adipocyte lipid accumulation, myoblast differentiation, osteoblast proliferation, insulin release, cardioprotection, coronary artery constriction, vascular endothelial cell proliferation, and tumor cell proliferation. The molecular identification and pharmacologic characterization of novel ghrelin receptors are thus important objectives.

I. The Story of Ghrelin

Two threads of history led to ghrelin, the first being the discovery of novel compounds that cause growth hormone (GH) release, and the second being the hypothesis that there exists a peripheral source of an orexigenic hormone.

In the early 1980s, synthetic peptides that were potent as stimulants of growth hormone release from pituitary cells, but which did not act at the growth hormone releasing hormone receptor, were discovered (Bowers et al., 1984). These were termed growth hormone secretagogues, and substantial effort was made to discover the growth hormone secretagogue receptor, GHSR. This continued until 1996, when a G protein–coupled receptor (GPCR) that responded to growth hormone secretagogues was discovered and cloned (Howard et al., 1996). This receptor protein had two natural forms, a functional 7 membrane spanning receptor, GHSR1a, and a truncated, nonfunctional receptor, GHSR1b. A natural ligand for the receptor was not found until 1999 (Kojima et al., 1999). Surprisingly, this ligand, ghrelin, was extracted from the stomach. Ghrelin, like the earlier discovered artificial growth hormone secretagogues, increased food intake (Nakazato et al., 2001). Much earlier studies had predicted the existence of a signal originating from the stomach or another peripheral source that promotes feeding (Cannon and Washburn, 1912; Carlson, 1913), but the identification of a peripheral orexigenic hormone had eluded researchers. In the few years after its discovery, it was firmly established that ghrelin had physiologic roles in controlling growth hormone release and appetite (van der Lely et al., 2004; Kojima and Kangawa, 2005).

Ghrelin is a 28–amino acid peptide that has an 8–carbon fatty acid side chain on the serine at position 3, whose addition is catalyzed at the precursor stage by the enzyme ghrelin O-acyl transferase (Gutierrez et al., 2008). Although by definition ghrelin has an acyl side chain (Kojima et al., 1999), it is sometimes referred to in publications as acyl-ghrelin. The 28–amino acid peptide without the side chain is referred to in the literature as des-acyl-ghrelin (commonly meaning ghrelin from which the side chain has been cleaved) or unacylated ghrelin (UAG). We have used the abbreviation UAG to refer to this peptide.

Experimental studies, recently reviewed in Pharmacological Reviews (Chen et al., 2009), soon revealed many other effects of ghrelin, effects of other products of the ghrelin gene (ghrelin, UAG, obestatin, and des-Gln14-ghrelin), and effects of small-molecule ghrelin receptor ligands, some of which have been tested for their therapeutic potential (Table 1). It also emerged that not all effects of ghrelin, ghrelin mimetics, and related compounds could be explained by actions at the unmodified GHSR1a, and some were independent of GHSR1a (Muccioli et al., 2007). Several previous reviews provide evidence for the existence of novel ghrelin and desacyl-ghrelin receptors (Muccioli et al., 2007; Soares and Leite-Moreira, 2008; Seim et al., 2011).

ABBREVIATIONS: BIBP3226, N²-(diphenylacetyl)-N-(4-hydroxy-phenyl)methyl]-L-arginine amide; DRD2, dopamine D2 receptor; ECL2, 2nd extracellular loop; eNOS, endothelial nitric oxide synthase; EP80317, (2S)-5-amino-1,2,3,4,6,7-hexahydro-azepino(3, 2, 1-hi)indol-4-one-2-carboxylic acid-d-2Me-Trp-d-Lys-Trp-d-Phe-Lys-NH2; ERK, extracellular signal-regulated kinase; GH, growth hormone; GHSR, growth hormone secretagogue receptor; GPCR, G protein–coupled receptor; GRLR, ghrelin receptor–like receptor; MSP, modified substance P; NF449, 4,4′,4′′-[carbonyl-bis[imino-5,1,3-benzenetriyl bis-(carbonylimino)]]tetraakis-benzene-1,3-disulfonate; P3K,phosphatidylinositol 3-kinase; PKA, protein kinase A; TM, transmembrane domain; UAG, unacylated ghrelin, also referred to as des-acyl-ghrelin.
### Ghrelin receptor ligands

Agonists: this is a selection of many hundreds of agonists in the patent literature. Except for AZP531, they were developed as GHSR1a agonists. We concentrate on those that have been trialed in human and/or are judged to be useful for biologic testing and receptor characterization. Antagonists: many antagonists of GHSR1a have been synthesized, but few have gone forward to clinical trial, and most of the compounds are not readily available. Compounds from Abbott, Bayer, Elixir, Merck, Tranzyme, and Zentaris have been reviewed (Zhao et al., 2008).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Properties, Characterization</th>
<th>Relevant Pharmacological Studies, Studies in Human</th>
<th>References</th>
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<tr>
<td><strong>Agonists</strong></td>
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<tr>
<td>AZP531</td>
<td>Cyclic ghrelin (and desacyl-ghrelin) 6-13. Not an agonist at GHSR1a.</td>
<td>Selective agonist for the UAG receptor. Phase 1 clinical trial commenced July 2013 to provide data on AZP531 actions on metabolic dysfunction, particularly on hyperglycemia (Alizé Pharma, Ecully, France). Anticipated use in Type 2 diabetes.</td>
<td>Julien et al., 2012</td>
</tr>
<tr>
<td>Capromorelin (CP424391)</td>
<td>Pyrazolinone-piperidine dipeptide. Centrally penetrant. Orally active.</td>
<td>Being tested for treatment of frailty (phase 2) and GERD (phase 1) (RaQualia Pharma Inc., Nagoya, Japan).</td>
<td>Pan et al., 2001; White et al., 2009</td>
</tr>
<tr>
<td>GSK894490 (Compound 24)</td>
<td>Nonpeptide. Orally active.</td>
<td>Shown to increase lean body mass. Originally produced by Pfizer (Groton, CT), now licensed to RaQualia.</td>
<td>Witherington et al., 2008; Atcha et al., 2009</td>
</tr>
<tr>
<td>CP464709</td>
<td>Nonpeptide.</td>
<td>Centrally penetrant agonist with long half-life</td>
<td>Carpino et al., 2002</td>
</tr>
<tr>
<td>GHRP-6</td>
<td>His-b-Trp-Ala-Trp-b-Phe-Lys-NH2</td>
<td>The first effective and commonly used growth hormone secretagogue. Short in vivo half-life. Stimulates GH secretion in vivo. EC50 = 1.5 nM.</td>
<td>Bowers et al., 1984; Raun et al., 1998</td>
</tr>
<tr>
<td>Hexarelin</td>
<td>His-2-methyl-b-Trp-Ala-Trp-b-Phe-Lys-NH2, Methylated analog of GHRP-6.</td>
<td>One of the most commonly experimentally used GHSR1a agonists. Increases GH release and muscle mass. Popular with body builders. EC50 2 nM in pituitary cells.</td>
<td>Ghigo et al., 1994</td>
</tr>
<tr>
<td>Ibutamoren (MK677, L163191)</td>
<td>Nonpeptide, substituted spiropiperidine sulfonamide. Orally active as mesylate.</td>
<td>Effective agonists of GHSR1a. MK677, GHRP-6, and L692585 have about 50% greater efficacy than ghrelin and have been termed super agonists. Ibutamoren increases GH, IGF-1, and IGFBP-3 levels.</td>
<td>Codner et al., 2001; Murphy et al., 2001; Nass et al., 2008</td>
</tr>
<tr>
<td>Ipamorelin</td>
<td>Synthetic pentapeptide (Alb-His-b-2-Nal-b-Phe-Lys-NH2), originally from Novo Nordisk (Copenhagen, Denmark).</td>
<td>Being tested for postoperative ileus—phase 2b (Helsinn Pharma, Lugano, Switzerland). Demonstrated to significantly increase the cumulative fecal pellet output, food intake, and body weight gain in rodent model of postoperative ileus.</td>
<td>Raun et al., 1998; Venkova et al., 2009</td>
</tr>
<tr>
<td>L163255</td>
<td>Spiropiperidine. Orally active.</td>
<td>Oral dose of 1 µg/kg increased GH release in pig. IGF-I also elevated. Shown to increase retroperitoneal and perirenal white adipose tissue mass without having any significant influence on inguinal or epididymal fat.</td>
<td>Chang et al., 1996; Davies et al., 2009</td>
</tr>
<tr>
<td>Macimorelin acetate (AEZ2130) (ARD07) (EP01572)</td>
<td>Synthetic small molecule. Orally active as acetate.</td>
<td>Phase 1 studies completed and phase 2 studies underway to use macimorelin as a treatment of cachexia. Phase 3 studies of a diagnostic test for growth hormone deficiencies in adults have been completed (Aeterna Zentaris, Quebec City, QC, Canada).</td>
<td>Guerlavais et al., 2003; MacLean et al., 2009</td>
</tr>
<tr>
<td>RM131 (BIM28131)</td>
<td>Pentapeptide, formerly called BIM-28131</td>
<td>Tested for diabetic gastroparesis in women. Currently in phase 2 clinical trials for diabetic gastroparesis and GI functional disorders (Rhythm Pharmaceuticals, Boston, MA).</td>
<td>DeBoer et al., 2008; Shin et al., 2013</td>
</tr>
<tr>
<td>SM130686</td>
<td>Oxindole derivative. Orally active.</td>
<td>Partial agonist. EC50 = 3.0 nM. From Sumitomo Chemical Co. (Osaka, Japan).</td>
<td>Nagamine et al., 2001</td>
</tr>
<tr>
<td>Tabimorelin (NN703)</td>
<td>Modified peptide. Orally active.</td>
<td>Tested in adults with GH deficiency. Therapeutic effects on growth-hormone deficient adults were limited.</td>
<td>Hansen et al., 1999; Svensson et al., 2003</td>
</tr>
<tr>
<td>Ulimorelin (TZP101)</td>
<td>Macrocyclic peptidomimetic.</td>
<td>K1 = 16 nM, EC50 = 29 nM. Long half-life in human, 13 hours (Lasseter et al., 2008). Tests in patients with gastroparesis showed minor improvement. Phase 3 trial for postoperative ileus demonstrated compound safety, but ulimorelin showed no advantage compared with placebo. It is reported to have low potency to release growth hormone (Fraser et al., 2008).</td>
<td>Höveyda et al., 2011; Shaw et al., 2013</td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
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<tr>
<td>(n-Lys3)-GHRP-6</td>
<td>Hexapeptide, His-b-Trp-b-Lys-Trp-b-Phe-Lys-NH2</td>
<td>Originally described by Cheng et al. (1989), this is one of the most commonly used antagonists in cell-based</td>
<td>Cheng et al., 1989</td>
</tr>
</tbody>
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TABLE 1—Continued

<table>
<thead>
<tr>
<th>Compound</th>
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<th>Relevant Pharmacological Studies, Studies in Human</th>
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<tbody>
<tr>
<td>YIL781</td>
<td>Piperidine-substituted quinazolinone derivative. Orally active.</td>
<td>Competitive antagonist with ( K_b ) of 7.54 in IP accumulation assay. Centrally penetrant and centrally effective antagonist in rat in vivo. Available commercially for experimental use.</td>
<td>Esler et al., 2007; Perdona et al., 2011; Callaghan et al., 2014</td>
</tr>
<tr>
<td>AEZS123 (JMV2959)</td>
<td>Nonpeptide with trisubstituted 1,2,4-triazole structure.</td>
<td>( K_b ) of 32 nM and ( K_a ) of 19 nM. No agonist action. Related antagonists are described by Moulin et al. (2008).</td>
<td>Salomé et al., 2009</td>
</tr>
<tr>
<td>GSK1614343 (Compound 18s2d in Sabbatini et al., 2010).</td>
<td>Carbohydryde dipeptide trifluoromethyl derivative.</td>
<td>Competitive antagonist with ( K_b ) of 8.03 in IP accumulation assay. Not able to antagonize ghrelin-induced food consumption in rats, but unexpectedly stimulated food intake and body weight gain in both rats and dogs, a profile associated with decreased ghrelin plasma levels.</td>
<td>Sabbatini et al., 2010; Costantini et al., 2011; Perdona et al., 2011</td>
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Inverse agonists acting at GHSR1a

<table>
<thead>
<tr>
<th>Modified Substance P (MSP)</th>
<th>Peptide ( EC_{50} = 5.2 ) nM</th>
<th>Modified substance P, [( \text{D-Arg}^1,\text{D-Phe}^5,\text{D-Trp}^7,9,\text{Leu}^{11} )] substance P, is a low-potency antagonist but a high-potency full inverse agonist. A range of related substance P derivatives also had inverse agonist properties.</th>
<th>Holst et al., 2006</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance P derivative, KwFwLL</td>
<td>Hexapeptide ( EC_{50} = 36 \pm 8 ) nM</td>
<td>A series of derivatives of MSP have been synthesized, leading to Lys-( \text{D-Trp-Phe-D-Trp-Leu-Leu-NH}_2 ), which has little remaining resemblance to substance P and is an inverse agonist with no residual agonist (or agonist) activity.</td>
<td>Holst et al., 2007</td>
</tr>
<tr>
<td>10n (Pfizer)</td>
<td>Spiro-azetidino-piperidine</td>
<td>One of the first in a series of Spiro-azetidino-piperidine derivatives developed at Pfizer. ( pK_i ) versus ghrelin ( 8.13 \pm 0.21 ).</td>
<td>Kung et al., 2012</td>
</tr>
<tr>
<td>12a (Pfizer)</td>
<td>Spiro-azetidino-piperidine-triazole</td>
<td>Orally active. ( pK_i ) versus ghrelin ( 7 ) nM.</td>
<td>McClure et al., 2013</td>
</tr>
<tr>
<td>PF5190457</td>
<td>Spiro-azetidino-piperidine</td>
<td></td>
<td>Bhattacharya et al., 2014</td>
</tr>
</tbody>
</table>

GERD, gastroesophageal reflux disease; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IP, inositol phosphate.

II. Properties of Growth Hormone Secretagogue Receptor 1a

A. The Receptor and Agonists

The molecularly identified ghrelin receptor GHSR1a is a typical GPCR. It is a family A receptor and a member of the ghrelin receptor group (Fig. 1), which includes receptors for motilin (\(-52\%\) homology with GHSR1a), neurotensin 1 and 2 (\(-35\%\) homology), neuremedin receptors 1 and 2 (\(-30\%\) homology), and GPR39 (\(-30\%\) homology) (Holst et al., 2004).

The original discovery paper showed that ghrelin at nanomolar concentrations activated GHSR1a in pituitary and in transfected CHO cells, but that GHSR1a did not respond to UAG (Kojima et al., 1999). Later studies revealed a weak but full agonism and low potency displacement of binding by UAG at GHSR1a (Bednarek et al., 2000; Matsumoto et al., 2001; Gauna et al., 2007b; Callaghan et al., 2014). A difference in potency of over 1000-fold was reported in two studies: UAG had an \( EC_{50} \) of between 1.6 and 2.4 \( \mu M \), compared with an \( EC_{50} \) of 2–2.6 \( nM \) for ghrelin at GHSR1a (Gauna et al., 2007b; Callaghan et al., 2014). In another study, the difference in potency was greater: 1–2 nM for ghrelin and >100 \( \mu M \) for UAG (Matsumoto et al., 2001). Radioligand binding experiments confirm a greater than 1000-fold difference in binding efficiency: UAG displaced labeled ghrelin from GHSR1a with an \( IC_{50} \) of 10–13 \( \mu M \), whereas the \( IC_{50} \) for ghrelin was 7–10 nM (Bednarek et al., 2000; Gauna et al., 2007b). Ghrelin administered to humans stimulates appetite and increases circulating GH, adrenocorticotropic hormone, cortisol, prolactin, and glucose (Garin et al., 2013). In contrast, growth hormone release in human is not affected by UAG (Broglio et al., 2004).

Thus, the potency of ghrelin at GHSR1a depends on the integrity of the octynol side chain at serine 3, although other hydrophobic side chains can be substituted.
with retention of activity (Bednarek et al., 2000). The minimum octanoylated sequence for effective stimulation of the receptor is the N-terminal 4 amino acids of ghrelin, Gly-Ser-Ser(\text{octanoyl})-Phe (Bednarek et al., 2000). The EC_{50} of this acylated tetrapeptide was about half that of full-length ghrelin and it produced 90% of the maximum response to ghrelin.

It is notable that circulating levels of ghrelin and UAG are in the range 0.1 to 0.5 nmol/l (Tschöp et al., 2001; Patterson et al., 2005; St-Pierre et al., 2007; Crujeiras et al., 2010), although higher levels (3–4 nmol/l) are measured by assays that detect bound peptide (Patterson et al., 2005; Lambert et al., 2011). Thus, UAG in the circulation is at a considerably lower concentration than that necessary to activate GHSR1a.

The hexapeptide growth hormone secretagogues, such as hexarelin, which were the first synthetic GHSR1a agonists (Bowers et al., 1984), have poor bioavailability and short in vivo half-lives. However, chemical screens soon identified potent nonpeptide, small molecule agonists (Table 1), including the Merck compounds, L163255, L163191 (MK677), and L692429 (Barakat et al., 1998; Smith, 2005). Development of agonists accelerated once the receptor was cloned, particularly after identification of its natural ligand, and soon numerous potent small-molecule agonists and some peptides with improved in vivo stability became available (Chollet et al., 2009, 2012). New compounds included NN703 from NovoNordsk (Hansen et al., 1999); capromorelin and related pyrazolinone-piperidine dipeptides from Pfizer (Carpino et al., 2002); GSK892491 (Witherington et al., 2001; Patterson et al., 2005; St-Pierre et al., 2007; Crujeiras et al., 2010), although higher levels (3–4 nmol/l) are measured by assays that detect bound peptide (Patterson et al., 2005; Lambert et al., 2011). Thus, UAG in the circulation is at a considerably lower concentration than that necessary to activate GHSR1a.

Cortistatin is also a natural ligand of GHSR1a. This peptide has close structural homology to somatostatin, with both peptides sharing the ability to bind and activate all five somatostatin receptor subtypes (Tringali et al., 2012). Because cortistatin and somatostatin display a number of different biologic actions, specific cortistatin receptors, able to bind selectively cortistatin peptide has close structural homology to somatostatin, with both peptides sharing the ability to bind and activate all five somatostatin receptor subtypes (Tringali et al., 2012). Because cortistatin and somatostatin display a number of different biologic actions, specific cortistatin receptors, able to bind selectively cortistatin.
that loss of constitutive activity throughout life does not cause weight loss, consistent with ghrelin and GHSR knockout models (Wortley et al., 2004; Zigman et al., 2005). Nevertheless, there is evidence that constitutive activity contributes to appetite when normal GHSR1a is present throughout life. Intracerebroventricular application (by minipump) of an inverse agonist (a modified substance P [MSP]; see Table 1) decreased food intake, reduced weight, and lowered hypothalamic neuropeptide Y expression in rats (Petersen et al., 2009). After 6 days, weight was about 5% less than in the controls.

C. Interaction Sites for Agonists and Inverse Agonists at Growth Hormone Secretagogue Receptor 1a

Site-directed mutagenesis and modeling have determined that the key sites for agonist binding differ from the sites that determine constitutive (ligand-independent) activity, although the sites are close to each other (Mokrosinski et al., 2012; Mear et al., 2013; Sivertsen et al., 2013). A number of modulatory sites have also been identified (Fig. 2). The discovery that a single mutation (Ala204Glu) in the 2nd extracellular loop (ECL2) of the human GHSR1a causes idiopathic short stature syndrome (Pantel et al., 2006) provided clear evidence that constitutive and ligand-dependent activation could be separated. When the properties of the native and mutated receptor were compared it was found that both responded equally to agonists but that constitutive activity was substantially diminished for the mutant (Pantel et al., 2006; Liu et al., 2007).

A critical agonist interaction site is the glutamic acid residue in the extracellular part of the 3rd transmembrane domain (TM3). Mutation of this residue, GluIII:09, using the numbering system of Schwartz (1994), to Gln rendered the receptor unresponsive to ghrelin and a range of GHSR1a agonists but did not change ligand-independent activity (Feighner et al., 1998; Holst et al., 2009). Detailed comparison of mutational effects on the agonism of ghrelin, GHRP-6, MK667, SM130686 (Table 1), and L692429 have identified residues in TM3 (including GluIII:09) and TM6 as essential to ghrelin potency and indicate that the most important sites for ghrelin agonist action occur in a restricted region within the center of the binding pocket, spanning TM3 and TM6 (Fig. 2). For the synthetic, nonpeptide agonists, sites in the ghrelin binding pocket (GluIII:09, PheVI:16, ArgVI:20) are also important for their agonist potency, but influential interaction sites also occur in the external parts of other helices, including TM2, TM 4, TM5, and TM7 (Holst et al., 2006, 2009). Some of these sites are important for nonpeptide agonists, such as AspII:20 whose mutation reduces MK677 potency over 1000-fold (Feighner et al., 1998; Holst et al., 2009), while having less influence on the potency of ghrelin. There are also interactions with the extracellular loops that may influence efficacy (Holst et al., 2006, 2009).

Substitutions revealed a critical role for constitutive activity of a cluster of aromatic amino acids in TM6 and 7 (Fig. 2). These were PheVI:16, plus the residues PheVII:06 and PheVII:09 on the inner faces of the external pocket of GHSR1a, distinct from the essential interaction sites for agonists (Holst et al., 2004). The close spatial proximity of these amino acids is hypothesized to place GHSR1a in its active conformation in the absence of agonist (Holst et al., 2004; Mokrosinski et al., 2012). The level of constitutive activity can be modified up or down by manipulating the size and hydrophobicity of the residue in position VI:16. It has also been suggested that the aromatic residue VI:16 may work as a tethered agonist located strategically at the interfaces between TM3, TM6, and TM7, locking the extracellular ends of these TM domains in a conformation that drives high constitutive activity of GHSR1a (Schwartz et al., 2006). A conserved aromatic link crucial for high basal signaling of GHSR1a is formed by the TrpVI:13 and PheV:16 residues (Holst et al., 2004). Specific residues in the vicinity of this cluster have been proposed to orchestrate finely tuned microswitches critical for setting the activation level in absence of ligand (Holst et al., 2004; Valentin-Hansen et al., 2012). Three deeply located residues, Trp276 (TrpVI:13), ValIII:16, and IleIII:19 also significantly impact on GHSR1a constitutive activity (Gozé et al., 2010). Gozé et al. (2010) introduced the mutation TrpVI:13Ala and mutated the two surrounding amino acid residues in adjacent TM3, ValIII:16, and IleIII:19. The TrpVI:13Ala mutation reduced the ligand-independent activity, but, surprisingly, the double mutation, ValIII:16Leu plus IleIII:19Met, increased GHSR1a ligand independent activity twofold, presumably by realigning TM3, 5, 6, and 7.

The molecular basis for reliance of ligand-independent activity on the ECL2 site (Ala204) identified by Pantel...
et al. (2006) has been recently investigated (Mokrosinski et al., 2012). The study revealed that restricting flexibility of ECL2, either by mutation or by ligand binding, reduces ligand-independent signaling (Mokrosinski et al., 2012). ECL2 links TM4 and TM5 and has a disulfide link to TM3 (Fig. 2). It is proposed that flexing of ECL2, in particular the propensity of the part of ECL2 that links TM3 and TM5 (ECL2b) to form an alpha-helix, is strongly influenced by Ala204, the mutation favoring the alpha-helix and restriction of the free movement of TM3 relative to TM5 (Mokrosinski et al., 2012).

Other studies showed that ligand properties can be switched from agonist to inverse agonist or from inverse agonist to agonist by a single amino acid or space generating substitution in the ligand peptide sequence depending on the residue mutated (Holst et al., 2007; Els et al., 2012). These studies identified the C-terminal heptapeptide (fQwFwLL) of MSP as the sequence necessary for inverse agonism, the D-Phe residue being essential (Holst et al., 2006).

III. Identification of Growth Hormone Secretagogue Receptor 1a and 1b Expression by Reverse-Transcription Polymerase Chain Reaction

GHSR1a in the human, pig, and rat is encoded by two exons, and GHSR1b is encoded by a single exon (Howard et al., 1996; McKee et al., 1997). A major issue with reverse-transcription polymerase chain reaction identification of the GHSR1a receptor transcript is that some studies have used primers that cannot distinguish between the 1a and 1b isoforms or genomic DNA because the forward and reverse primer are both targeted to the first exon. For example, Murata et al. (2002) reported expression of GHR1a mRNA in liver samples and liver cell lines using primers designed against exon 1 and not spanning the intron, whereas several other groups have not been able to detect GHSR1a mRNA in the same liver cell line or human liver samples using primers appropriate for distinguishing 1a and 1b (Gnanapavan et al., 2002; Thielemans et al., 2007; Ueberberg et al., 2009). Others have used primers that should be able to detect the 1a isoform over the 1b isoform because the primers select against regions in exon 2 (Iglesias et al., 2004; Beiras-Fernandez et al., 2010). However, as the primers do not span an intron, there is potential that the primers were detecting genomic DNA.

Two studies quantitatively profiled expression of GHSR1a and 1b in human tissues using intron-spanning primers (Gnanapavan et al., 2002; Ueberberg et al., 2009). The Gnanapavan studies used tissue obtained from surgery, whereas Ueberberg used Human Total RNA Master Panel II from BD Biosciences (San Jose, CA). Both studies found the highest levels of GHSR1a mRNA expression in pituitary, consistent with its role in regulating GH function. The only other organs in which GHSR1a expression has been consistently and reliably detected by these and other studies are brain, spinal cord, pancreas, adrenal glands, and heart. GHSR1b expression occurs in many more tissues and organs. Notable among organs where GHSR1b but not GHSR1a was expressed are adipose tissue, breast, liver, skeletal muscle, and prostate (Gnanapavan et al., 2002; Ueberberg et al., 2009). As detailed below, in each of these tissues that lack GHSR1a, ghrelin has effects on cell functions.

IV. Heteromeric Receptors

Several functional receptors, consisting of GHSR1a or GHSR1b in combination with another GPCR, have been identified (Jiang et al., 2006; Takahashi et al., 2006; Rediger et al., 2011; Kern et al., 2012; Park et al., 2012; Schellekens et al., 2013). Heterodimerization with GHSR can change G protein coupling (Park et al., 2012), can change agonist potency (Schellekens et al., 2013), and can create a receptor with pharmacology different to its component GPCRs (Takahashi et al., 2006). Experiments with pancreatic islet cells demonstrate change in G protein coupling. In normal pancreas, GHSR1a and the somatostatin receptor, SST5, are expressed together, and ghrelin inhibits insulin secretion. In contrast, ghrelin stimulated insulin secretion from pancreas-derived cells that did not express SST5, but when these cells where transfected with Sst5, insulin secretion was inhibited by ghrelin (Park et al., 2012). The authors present evidence that the excitation is through Go alpha q and that inhibition is via the GHSR1a/SST5 heterodimer, coupled through Go 2alpha, (Park et al., 2012). Taking clues from lung cancers, in which expression of the peptide neuromedin U and of the GHSR1b and neurotensin 1 receptors are increased, the GHSR1b/neuromedin 1 receptor combination was investigated. Quite surprisingly, this heterodimer functions as a neuromedin U receptor (Takahashi et al., 2006).

The dopamine D2 receptor (DRD2)/GHSR1a heterodimer occurs in hypothalamic neurons (Kern et al., 2012). In wild-type mice, but not in Ghstr null mice, the dopamine agonist cabergoline inhibited feeding, indicating that the combination with GHSR1a was necessary for DRD2 activation. The selective GHSR1a antagonist JMV2959 (Salome et al., 2009) (Table 1) blocked the anorectic effect of cabergoline, suggesting that by binding to GHSR1a, JMV2959 changes the conformation and responsiveness of the coupled DRD2 receptor. Dopamine D1 receptors also form heterodimers with GHSR1a (Jiang et al., 2006; Schellekens et al., 2013).

A heterodimer between GHSR1a and the melanocortin 3 receptor MC3R has been identified. Melanocortin signaling is enhanced twofold at this heterodimer, and
the enhancement is reduced if a constitutively inactive GHSR1a mutant (A204E; see section II.D) forms the heterodimer or if substance P, which is hypothesized to act as a GHSR1a inverse agonist, is added (Rediger et al., 2011).

The serotonin 2C receptor (5-HT2C) receptor occurs in several isoforms that are produced by RNA editing, which modifies the second intracellular loop and alters signaling properties. The unedited form, serotonin 2C receptor with amino acids isoleucine, asparagine, isoleucine at positions 156, 158, and 160 (5-HT2C-RED), has the highest constitutive activity. This isoform dimerizes with GHSR1a to form a receptor that responds to both 5-HT and ghrelin (Schellekens et al., 2013). The heterodimer was less responsive to ghrelin and the ghrelin receptor agonist MK677 (Table 1) than homomeric GHSR1a. Responsiveness was restored by a 5-HT2C antagonist. GHSR1a/D1 and GHSR1a/MC3 heteromeric receptors also exhibited lowered responses to GHSR1a agonists (Schellekens et al., 2013). An inverse agonist of the GHSR1a receptor increased responsiveness of the GHSR1a/5-HT2C-RED heterodimer to 5-HT2C agonism.

When GHSR1b was coexpressed with GHSR1a in HEK293 cells, the signal transduction capacity of GHSR1a was attenuated, suggesting that GHSR1b may interact with GHSR1a (Chan and Cheng, 2004). Heterodimerization was demonstrated in lipid rafts incorporating GHSR1a and GHSR1b (Mary et al., 2013). In these rafts, GHSR1b exerted a dominant negative inhibition of GHSR1a signaling.

### V. Evidence for Novel Receptors: Ghrelin Receptor–Like Receptors and Unacylated Ghrelin Receptors

We have grouped the evidence for the existence of ghrelin receptor–like receptors (GRLRs) by the cell types and organs where the evidence has been found. The most convincing evidence is from cells or tissues in which there is no detectable expression of GHSR1a or from experiments in which responses were observed after knockout of Ghsr. Some of the evidence comes from experiments in which ghrelin and UAG have parallel effects at similar concentrations, remembering that ghrelin is over 1000 times more potent than UAG at GHSR1a. The most convincing of these experiments are those in which competition for binding has been demonstrated, suggesting that the same receptor, a GRLR, is responsible for effects of ghrelin and UAG in these tissues.

Some ligands that stimulate GHSR1a also act at sites where ghrelin is not an agonist. The sites include the UAG receptors. A UAG receptor is a receptor for UAG and related compounds at which ghrelin is a much poorer agonist or not an agonist at all. In some cases, mimicry between ghrelin and other ligands in a particular tissue could be because both GHSR1a and UAG receptors are present.

#### A. Adipose Tissue

1. **Overview.** Published data indicate that proliferation, differentiation, lipid accumulation, and glycerol handling by preadipocytes and adipocytes are mediated by receptors that do not discriminate between ghrelin and UAG and by UAG-selective receptors. On the other hand, inflammation of adipose tissue involves GHSR1a expressed by macrophages and possibly also adipocytes, whereas UAG agonists are anti-inflammatory. GHSR1a may also have a role in thermogenesis in brown fat (Lin et al., 2011). It is feasible that agonists of UAG receptors could assist in maintaining fat stores in lean subjects and in reducing peaks in circulating free fatty acids, for example after meals (Kos et al., 2009; Benso et al., 2012), as well as reducing inflammation in fat stores, such as what occurs in metabolic disease (Delhanty et al., 2013).

Gnanapavan et al. (2002) reported that GHSR1a mRNA is not expressed in human adipose tissue although low levels of GHSR1b mRNA were present. On the other hand, in a study using intron-spanning primers, GHSR1a expression was found in rat adipose tissue (Choi et al., 2003). Although GHSR1a expression in white and brown adipose tissues is below the detectable level in young mice, GHSR1a expression was readily detectable in visceral white fat and interscapular brown fat of old mice (Lin et al., 2011). This may be contributed to by GHSR1a expression in inflammatory cells in fat from old mice (Ma et al., 2013). It would be desirable to use in situ hybridization histochemistry to determine the precise cellular sites of expression of Ghsr1a in adipose tissues of old animals.

2. **Lipid Accumulation.** Both ghrelin and UAG stimulate lipoprotein lipase levels and cause lipid accumulation in rat and human visceral adipocytes and rat bone marrow adipocytes (Thompson et al., 2004; Kos et al., 2009; Rodríguez et al., 2009). Ghrelin and UAG had similar potencies, with thresholds for increasing lipoprotein lipase levels of about 1–10 nM in human adipocytes (Kos et al., 2009). Labeled ghrelin binding in rat epididymal adipose tissue was displaced by ghrelin, UAG, MK0677, and hexarelin. All four agents inhibited β-adrenergic agonist–mediated lipolysis (Muccioli et al., 2004). Investigation of the second messenger pathway showed that both ghrelin and UAG blocked isoproterenol-induced cAMP accumulation via activation of phosphatidylinositol 3 (PI3)-kinase γ/AKT and phosphodiesterase 3B (Baragil et al., 2011). PI3-kinase γ, also known as a class Ib PI3-kinase, is reported to be strictly GPCR activated (Andrews et al., 2007). Ghrelin and UAG were also similarly potent in bone marrow, but their actions were not reduced by the GHSR1a antagonist L163255 (Table 1) (Thompson et al., 2004). In human adipocytes, this effect was blocked by the Y1 receptor blocker BIBP3226.
[N²-(diphenylacetyl)-N-[(4-hydroxy-phenyl)methyl]-d-arginine amide], suggesting that ghrelin and UAG may act indirectly by releasing a peptide of the pancreatic peptide, peptide YY, neuropeptide Y family (Kos et al., 2009). In human volunteers, a 16-hour infusion of UAG (1 μg/kg per hour) reduced plasma free fatty acid levels (Benso et al., 2012).

On the other hand, UAG, but not ghrelin, at concentrations of 10–100 nM, suppressed glycerol secretion and reduced the expression of glycerol-releasing hormone sensitive lipase, an effect not prevented by the Y1 antagonist BIBP3226 (Kos et al., 2009). Similarly, release of glycerol and nonesterified fatty acids was reduced by UAG (10–100 pM), but not ghrelin, in differentiated adipose 3T3-L1 cells (Miegueu et al., 2011). The stimulation of fatty acid uptake in an adipose cell line (3T3-L1) was inhibited by the antagonist (d-Lys3)-GHRP-6, a lipid raft disruptor, PI3-kinase, and phospholipase C blockade (Miegueu et al., 2011). As indicated below, in section V.C, (d-Lys3)-GHRP-6 appears to be an antagonist of ghrelin receptor-like receptors that respond to both ghrelin and UAG, as well as being a GHSR1a antagonist.

3. Adipose Tissue Inflammation. Adipose tissues tend to become inflamed with age, an effect that is exacerbated by a high-fat diet. Macrophages that infiltrate the fat express GHSR1a; the adipose inflammation caused by high fat diets in mice is reduced by UAG (100 μg/kg, twice daily) reduced fasting-induced skeletal muscle wasting in Ghsr knockout mice (Porporato et al., 2013). Both the acylated and unacylated forms of ghrelin reduced dexamethasone-induced skeletal muscle atrophy. In mice, muscle cell damage, including cell apoptosis, caused by doxorubicin, was abrogated by both ghrelin and UAG given 12 hours later as a single injection, then by repeated doses, twice daily, over 4 days (Yu et al., 2014). In another study, UAG reduced muscle protein loss in rats with burn injury to the skin (Sheriff et al., 2012).

In addition to its positive effects on muscle cell differentiation and its inhibition of muscle atrophy, ghrelin stimulates glucose uptake by myoblasts. In the mouse myoblast cell line C2C12, which does not express Ghsr (Filigheddu et al., 2007; Gershon and Vale, 2014), ghrelin stimulated glucose uptake with an EC50 of about 10 nM (Gershon and Vale, 2014). Surprisingly, the effect of ghrelin was inhibited by the corticotrophin releasing factor receptor 2 antagonist antisauvagine (Gershon and Vale, 2014). Both ghrelin and UAG, effective at 1 nM, increased corticotrophin releasing factor receptor 2 expression in the muscle cells.

Skeletal muscle is repaired after ischemic damage by recruitment of resident stem cells (satellite cells) that form myoblasts that are necessary for muscle regeneration. It has been found that UAG, but not ghrelin, facilitated the division of satellite cells and their involvement in the repair process (Togliatto et al., 2013). The enhanced repair was associated with the increased expression of superoxide dismutase-2 in satellite cells.

C. Bone

1. Overview. Ghrelin and UAG stimulate osteoblast activity, and compounds that act on the GRLR, common to ghrelin and UAG, in bone may be effective in combating age-associated bone loss.

GHSR1b mRNA, but not GHSR1a, occurs in human bone and osteoblasts, and both ghrelin and UAG stimulate osteoblast proliferation (Delhanty et al., 2006). Peak responses occurred at 1 and 10 nM for ghrelin and UAG, respectively. Proliferation was reduced by inhibition of ERK or PI3-kinase (Delhanty et al., 2006). In another study, which showed that Ghsr expression was undetectable in bone cells and cell lines from rat and mouse, ghrelin was also shown to increase mitogenic activity in osteoblasts (Costa et al., 2011). Ghrelin also increased the bone-resorbing activity of rat osteoclasts (Costa et al., 2011). The proliferative effect of ghrelin on rat osteoblast cell lines was prevented by the antagonist (d-Lys3)-GHRP-6 (Kim et al., 2005), but this antagonist does not appear to be specific for GHSR1a, myoblasts, using intron spanning primers (Filigheddu et al., 2007). The IC50 values for the displacement of labeled ghrelin from myoblasts were 1.20 ± 0.09 nM for ghrelin and 1.32 ± 0.08 nM for UAG. In another study it was found that both ghrelin and UAG (100 μg/kg, twice daily) reduced fasting-induced skeletal muscle wasting in Ghsr knockout mice (Porporato et al., 2013). Both the acylated and unacylated forms of ghrelin reduced dexamethasone-induced skeletal muscle atrophy.
because these cell lines do not express Ghsr. Ghrelin and UAG (1 nM) both reduced reactive oxygen species production and protected cells of the murine osteoblast cell line MC3T3-E1 from peroxidase-induced oxidative injury (Dieci et al., 2014). There was no effect of the potent GHSR1a agonist macimorelin (EP1572; van der Lely et al., 2004) and the effect was also not antagonized by (n-Lys3)-GHRP-6.

D. Chondrocytes

Human and mouse chondrocytes respond to ghrelin but do not express GHSR1a (Caminos et al., 2005). Binding studies revealed high affinity \((K_d = 3.8 \text{ nM})\) and lower affinity binding \((K_d = 130 \mu \text{M})\) sites (Caminos et al., 2005). Ghrelin increased cAMP with a threshold effect at about 1 nM. Ghrelin decreased chondrocyte metabolic activity and decreased basal and insulin stimulated fatty acid uptake. Whether chondrocytes respond to UAG was not tested.

E. Pancreatic Islets

1. Overview. Published evidence points to expression of GHSR1a, GRLR, and UAG receptors in the pancreas. GHSR1a is expressed by pancreatic \(\beta\) cells, where ghrelin inhibits insulin secretion (see section IV), and ghrelin, also through its GHSR1a-dependent action, leading to increased release of insulin-like growth factor, is diabetogenic (Vestergaard et al., 2008). However, through GRLR and UAG receptors in the islets, ghrelin and UAG increase glucose tolerance and insulin release. They also increase \(\beta\)-cell survival and proliferation. Compounds selective for non-GHSR1a receptors in the islets may have the potential to be antidiabetogenic.

It is well established that ghrelin, acting through GHSR1a, inhibits insulin secretion (Broglio et al., 2001; Dezaki et al., 2007; Tong et al., 2010). Ghsr null mice demonstrate a lower fasting blood glucose and increased insulin sensitivity relative to wild-type littermates when fed a high-fat diet (Lin et al., 2011; Qi et al., 2011). However, ghrelin has other effects that cannot be explained by actions at GHSR1a.

Both ghrelin and UAG increase cell survival and inhibit apoptosis of pancreatic \(\beta\) cells induced by serum starvation or inflammatory mediators, with similar potencies (Granata et al., 2007). Investigation of the pancreatic \(\beta\)-cell line HIT-T15 and human pancreatic \(\beta\) cells points to the presence of receptors that bind ghrelin, UAG, and UAG fragments and modified UAG fragments, notably cyclized ghrelin6–13 (AZP531; Table 1). The sequence of AZP531 is distinct from the sequence necessary for activation of GHSR1a, which is ghrelin1–4 (Bednarek et al., 2000). Moreover, neither GHSR1a mRNA nor protein was detected in HIT-T15 cells (Granata et al., 2007). Labeled ghrelin bound to the cells and was displaced by ghrelin (IC\(_{50} = 3.8 \pm 0.6\) nM), UAG (IC\(_{50} = 2.3 \pm 0.4\) nM), and hexarelin (IC\(_{50} = 20.8 \pm 2.3\) nM). Scatchard analysis indicated the presence of a single binding site with similar affinity for ghrelin and UAG. Ghrelin and UAG both caused proliferation of HIT-T15 and human pancreatic \(\beta\) cells (Granata et al., 2007, 2012). NF449 (4,4′,4′′,4′′′-[carbonyl-bis[imino-5,1,3-benzenetriyl bis-(carbonylimino)]tetrakis-benzene-1,3-disulfonate], a 

F. Heart, Cardioprotection, and Ionotropic Effects

1. Cardioprotection

a. Overview. Ghrelin protects the heart against isoproterenol-induced injury (Chang et al., 2004b) and ischemia-reperfusion injury (Chang et al., 2004a; Zhang et al., 2009) and reduces damage due to myocardial infarct
(Locatelli et al., 1999; Soeki et al., 2008). Evidence that is reviewed below indicates that there are several receptor types in the heart, including GLR for ghrelin and UAG, UAG receptors, and GHSR1a. Cardioprotection appears to be mediated primarily through nonselective receptors for ghrelin and UAG (GLRIs) on cardiomyocytes. Receptors on endothelial cells (see section V.H below) may also contribute to cardiac protection and repair.

When compared directly, ghrelin and UAG have similar potencies in protecting the myocardium against drug-induced injury, inhibition of cardiomyocyte apoptosis, and inhibition of cardiac endothelial cell death (Baldanzi et al., 2002; Pei et al., 2014). The use of cardiomyocyte cell lines has partially clarified the mode of action of ghrelin and UAG in cardioprotection. The cancer therapeutic doxorubicin exhibits cardiotoxicity and causes death of rat-derived H9c2 cardiomyocytes, an effect that is inhibited by both ghrelin and UAG (Baldanzi et al., 2002). Although H9c2 cardiomyocytes do not express Ghsr1a, ghrelin and UAG recognize a common high-affinity binding site (Kd = 4 nM) on these cells. Two other agonists, MK677 and hexarelin, recognized the common ghrelin and UAG binding sites. The affinity of ghrelin binding sites on H9c2 cardiomyocytes was about 10-fold lower than the affinity at GHSR1a in pituitary and hypothalamic membranes (Baldanzi et al., 2002). Ghrelin also protects H9c2 cells from H2O2-induced apoptosis (Zhang et al., 2011). Binding experiments on ventricular membranes from guinea-pigs found that ghrelin, UAG, and hexarelin recognized a common high-affinity (Kd = 0.51 ± 0.06 nM) binding site (Bedendi et al., 2003). Ghrelin binding to rat cardiomyocytes revealed a single binding site, with a Kd of 0.25 nM (Chang et al., 2004a).

In vivo studies confirm that UAG is cardioprotective (Pei et al., 2014). Mice in which cardiac function was compromised by doxorubicin were given UAG (100 µg/kg i.p. twice daily for 4 days). Decreases in ventricular fractional shortening, increased fibrosis, and apoptosis caused by doxorubicin were reversed by UAG. Cardiac protection by hexarelin, in vivo, after ischemia and reperfusion was observed in hypophysectomized rats and is thus not an indirect effect of GH release by ghrelin receptor agonists (Locatelli et al., 1999).

In vivo effects (D-Lys3)-GHRP-6 (Pei et al., 2014) differ from those observed in vitro (Baldanzi et al., 2002); the prevention of doxorubicin-induced myocardial fibrosis and apoptosis by UAG was not antagonized by (D-Lys3)-GHRP-6 applied in vivo (Pei et al., 2014), whereas this compound reversed UAG effects on cardiomyocytes in vitro (Baldanzi et al., 2002). Another study that provides contrasting data on cardioprotection is that of Frasacarelli et al. (2003). It was reported that neither the potent GHSR1a agonist MK667 (Table 1) nor UAG was cardioprotective, whereas ghrelin and hexarelin were cardioprotective in perfused rat hearts subjected to ischemia/reperfusion injury.

Other experiments have identified cardiac binding sites that do not recognize ghrelin. One site in rat cardiac membranes was identified as CD36, a glycoprotein that is expressed in adipose tissue, platelets, monocytes/macrophages, dendritic cells, and microvascular endothelium. Hexarelin binding to CD36 was displaced by hexarelin-related peptides, including the antagonist (D-Lys3)-GHRP-6, but not by ghrelin (Demers et al., 2004). The functional effects of hexarelin binding to CD36 of cardiomyocytes have not been reported.

A recent binding study (Lear et al., 2010) using a murine cardiomyocyte line, HL-1, and isolated mouse and rat cardiomyocytes, has identified two binding sites for UAG, with Kd values of 0.65 and 47 nM, that were not effectively displaced by ghrelin, ghrelin9–28, or motilin. Displacement of labeled UAG with cold UAG yielded IC50 values of 4 and 400 nM (Lear et al., 2010). In the HL-1 cells, UAG increased medium-chain fatty acid uptake, whereas ghrelin did not modify fatty acid uptake.

2. Inotropic Effects. Studies on the inotropic effects of ghrelin and UAG have showed that they both reduced guinea pig papillary muscle mechanical tension in a dose-dependent manner and their action depended on nitric oxide synthesis as well as on endocardial endothelium integrity (Bedendi et al., 2003). The negative inotropic effect of ghrelin on papillary muscle has been studied in rat, where it was also found to be an action independent of GHSR1a (Soares et al., 2006). In contrast to the endothelium-mediated, GHSR1a-independent, negative inotropic effects of ghrelin and UAG, ghrelin also has positive inotropic effects on isolated rat ventricular myocytes (Sun et al., 2010). This effect of ghrelin is GHSR1a dependent, involves augmented L-type Ca2+ channel currents, and was blocked by (D-Lys3)-GHRP-6.

G. Heart, Coronary Artery Constriction

Ghrelin, 0.1 and 1 nM, increased arterial resistance in the isolated perfused rat heart and 1 nM constricted isolated coronary artery segments (Pemberton et al., 2004). These effects were blocked by the L-type Ca2+ channel antagonist diltiazem and by the protein kinase C inhibitor bisindolylmaleimide. Both GHRP6 and hexarelin constricted rat coronary arteries (Xu et al., 2003). In anesthetized pigs, direct infusion of ghrelin also caused coronary artery constriction (Grossini et al., 2007). The effect was prevented by the antagonist of β-adrenoceptors, butoxamine. It was suggested that ghrelin blocks a tonic β2-adrenergic receptor–mediated vasodilatation. Radiolabeled ghrelin binds to human coronary arteries with a Kd of 0.2 nM and binding is competed by hexarelin (Katugampola et al., 2001). Binding was reported to be on the smooth muscle (Katugampola et al., 2001).

In mice, hexarelin caused coronary vasoconstriction that was lacking in CD36 knockout animals (Bodart
et al., 2002). Moreover, coronary arteries of spontaneous hypertensive rats, which are CD36 deficient, were substantially less responsive to hexarelin compared with normotensive rats. In a GHSR1a reporter mouse, no evidence of GHSR1a expression was found in coronary arteries (Callaghan et al., 2012).

H. Endothelial Cells: Nitric Oxide Production, Proliferation, and Angiogenesis

1. Overview. Protective effects of ghrelin and UAG against endothelial cell damage caused by serum starvation, oxidative stress, or hyperglycemia through as yet unidentified receptors have been reported. There is some evidence that the actions of UAG are through activation of endothelial nitric oxide synthase (eNOS). This effect could possibly be targeted to assist tissue revascularization or to attenuate endothelial cell damage in diabetes.

Protected effects were first reported in porcine aortic endothelial cells stressed by serum starvation (Baldanzi et al., 2002). Apoptosis was reduced by about 80% with either ghrelin or UAG (1 μM). It was later reported that UAG, but not ghrelin, attenuated circulating endothelial progenitor cell damage in diabetic patients (Togliatto et al., 2010). In these experiments, patients were given UAG (3 μg/kg per hour) or ghrelin (1 μg/kg per hour) as an intravenous infusion for 12 hours, and blood samples were collected to isolate endothelial progenitor cells and circulating angiogenic cells. UAG infusion doubled the number of viable cells, whereas ghrelin (at 1/3 the dose) had no effect. The cells were capable of de novo vessel formation, which was enhanced by UAG. Similar observations were made on cells from obese mice. Fluorescent-tagged UAG bound to endothelial progenitor cells and circulating angiogenic cells. It was displaced by unlabeled UAG but not by ghrelin. In experiments on mouse cells from wild type and eNOS knockouts, it was shown that the actions of UAG were dependent on rescue of eNOS activity (Togliatto et al., 2010). Several other studies have also shown that ghrelin activates eNOS, including in endothelial cells from human umbilical vein (Xu et al., 2008) and bovine, human (Iantorno et al., 2007), rat (Shimizu et al., 2003), and mouse (Xu et al., 2008) aortic endothelial cells. However, effects of UAG were not tested in these studies. In contrast, Shimada et al. (2014) confirmed that UAG protects human vascular endothelial cells, but comparison with ghrelin was not made. The activation of eNOS is caused through Akt and AMP kinase–mediated phosphorylation (Xu et al., 2008). The effects in bovine aortic endothelial cells were inhibited by knockdown of Ghsr1a by treatment with siRNA (Iantorno et al., 2007), suggesting that GHSR1a is involved in this species.

There is evidence that actions on the capillary endothelium within human pancreatic islets contribute to islet protective effects of both ghrelin and UAG (Favaro et al., 2012). Treatment with 3 ghrelin gene products, ghrelin, UAG, and obestatin, reduced NO and inflammatory cytokine release from the endothelium. Competitive binding data suggested that obestatin, but not ghrelin or UAG, exerted its effects through binding to the glucagon-like peptide 1 receptor. Ghrelin and UAG increased survival and significantly inhibited glucose-induced apoptosis of the endothelial cells through increased cAMP and activation of PI3-kinase/Akt, ERK1/2 phosphorylation.

I. Liver

The literature is consistent in failing to detect GHSR1a in liver. GHSR1b, but not 1a, expression was detected in human liver (Gnanapavan et al., 2002; Ueberberg et al., 2009), and GHSR1a could not be detected in the liver of mouse (Lim et al., 2013) or rat (Moreno et al., 2010). GHSR1a expression was low to undetectable in pig hepatocytes, whereas the authors found high levels of expression in the pancreas (Gauna et al., 2005). Western blot analysis failed to reveal GHSR1a protein in the mouse liver (McGirr et al., 2011), although the sensitivity is such that small amounts of protein would probably remain undetected.

Despite the absence of GHSR1a, ghrelin (100 nM) stimulates glucose production in isolated porcine liver cells, whereas they do not respond to the potent GHSR1a agonist hexarelin (Gauna et al., 2005). Similarly, hexarelin did not alter glucose levels in human (Brogio et al., 2004). Effects on liver are also observed in Ghsr null mice, in which UAG changes the hepatic expression of genes involved in cell death, growth, and proliferation (Delhanty et al., 2010).

Hepatic insulin sensitivity is decreased in obesity, an effect that is associated with decreased expression of insulin receptor substrate 1. In mice, a high-fat diet decreased Irs1 gene expression, which was prevented by infusing the mice with UAG or the UAG receptor agonist AZP531 (Delhanty et al., 2013). Consistent with effects on insulin/glucose pathways in liver, in Ghsr knockout mice UAG increased the hepatic expression of Lcn2 and Nox4, genes that are linked to insulin sensitivity (Delhanty et al., 2010).

J. Tumor Cells

1. Overview. Receptors for ghrelin, UAG, and related ligands are present in several tumor types and in cancer cell lines derived from tumors (Chopin et al., 2012). This includes cancers of breast (Cassoni et al., 2004; Jeffery et al., 2005), the gastroenteropancreatic system (Leontiou et al., 2007; Nikolopoulos et al., 2010), liver (Murata et al., 2002), pituitary (Korbonits et al., 2001), and prostate (Jeffery et al., 2002; Lanfranco et al., 2008), in all of which ghrelin influences cancer cell proliferation. However, both proliferative and antiproliferative effects are observed, and no useful therapeutic agents based on this pharmacology have emerged.
Cassoni et al. (2001) used displacement of $[^{125}\text{I}]\text{Tyr-Ala-hexarelin}$ to characterize GRLRs in breast tumor tissue and breast cancer cell lines. Ghrelin and UAG displaced hexarelin binding with IC$_{50}$ values of 190 and 800 nM. By contrast, UAG is over 1000 times less potent than ghrelin at GHSR1a (see above). Unrelated peptides did not displace hexarelin from the tumor cells. Binding to these GRLRs was found in well differentiated carcinomas of the breast and in several breast cancer–derived cell lines, including the commonly used MCF7 and MDA-MB-231 lines. Two laboratories showed that neither line exhibits detectable GHSR1a gene expression (Cassoni et al., 2001; Jeffery et al., 2005), whereas GHSR1b gene expression is readily detected (Jeffery et al., 2005; Ghahete et al., 2011). It is possible that the GRLRs of breast cancers are heterodimers of the nonfunctional GHSR1b with another GPCR (see section IV).

Proliferation of the estrogen-dependent breast cancer cell line MCF7 is inhibited by ghrelin (Cassoni et al., 2001). It is notable that ghrelin does not have an effect in the absence of estrogen (Jeffery et al., 2005). Consistent with an inhibitory role of ghrelin in breast cancers, ghrelin expression is significantly correlated to low histologic grade, estrogen receptor positivity, small tumor size, and low proliferation of human breast tumors (Grönberg et al., 2008). In contrast, ghrelin at concentrations of 10 and 100 nM significantly increased proliferation of the estrogen-independent breast cancer cell line MDA-MB-231 (Jeffery et al., 2005). Another product of the ghrelin gene, in1-ghrelin (in which intron 1 is translated), also increases proliferation (Ghahete et al., 2011). Because most breast cancers express the ghrelin precursor gene and the acylating enzyme ghrelin O-acyl transferase (Grönberg et al., 2008, 2011; Ghahete et al., 2011), it is a reasonable hypothesis that ghrelin or other ghrelin gene products modulate breast cancer growth, the literature indicating that growth is reduced in estrogen-dependent breast cancers and increased in aggressive estrogen-independent breast cancers.

In prostate cancers, there is absence of both GHSR1a and GHSR1b expression, although GHSR1b expression occurs in 50% of benign prostate hyperplasias (Cassoni et al., 2004). Ghrelin had a dose-dependent proliferative effect on the prostate cell line LNCaP, with a threshold of about 0.1 nM and a peak response with 10 nM ghrelin (Yeh et al., 2005). Both ghrelin and UAG, at 10–100 pM, increased proliferation of the PC-3 cell line (Cassoni et al., 2004). However, neither GHSR1a nor GHSR1b expression was found in the LNCaP or PC-3 cell lines (Cassoni et al., 2004). In the human prostate cell lines DU-145 and PC-3 and in cells from prostate carcinomas, both ghrelin and UAG displaced labeled (Tyr4) ghrelin (Cassoni et al., 2004). Unlike the other prostate cancer cell lines, DU-145 does express GHSR1a (Cassoni et al., 2004) and agonists of GHSR1a inhibit proliferation (Pellegrino et al., 2013). The displacement of label with UAG suggests that this cell line may express a GRLR.

K. Appetite

1. Overview. Consistence evidence indicates that the orexigenic effects of ghrelin released from gastric ghrelin stores are mediated through GHSR1a. However, a number of studies indicate that actions at both GRLR and UAG receptors, most likely in the hypothalamus, can influence appetite. Whether non-GHSR1a have physiologic roles in control of food intake has not been determined.

Comparison of the effects of the GHSR1a ligand BIM-28163 on GH release and appetite also revealed the presence of a novel GRLRs (Halem et al., 2005). BIM-28163 bound to GHSR1a and blocked ghrelin’s action at the GHSR1a receptor in transfected cells and it also blocked ghrelin’s stimulatory effect on GH secretion (Halem et al., 2005). In contrast, BIM-28163 and ghrelin both stimulated food intake and in the dorsomedial hypothalamus, and c-Fos, a marker of activated neurons, was induced by both ghrelin and BIM-28163 (Halem et al., 2005). This suggests that some of the receptors through which ghrelin stimulates appetite are different from GHSR1a. Consistent with the effect of ghrelin on appetite being mediated through a receptor other than GHSR1a, the GHSR1a antagonist GSK1614343 does not block ghrelin-induced feeding (Costantini et al., 2011).

Intracerebroventricular UAG, but not ghrelin, increases food intake in rats, wild type, and Ghsr knockout mice, suggesting that there are UAG receptors in central appetite-controlling circuits (Toshinai et al., 2005). In fasted wild-type male mice of the ddY strain, intracerebroventricular or intraperitoneal UAG (~3 nmol) was reported to decrease food intake (Asakawa et al., 2005); however, other studies found no effect of intracerebroventricular UAG (7.5 nmol) in fasted male C57Bl6 mice (Neary et al., 2006) or fasted ddY mice (Toshinai et al., 2006). Peripherally applied ghrelin, but not UAG, increases feeding, and Ghsr knockout prevents the orexigenic effect of peripherally administered ghrelin (Sun et al., 2004; Toshinai et al., 2006). In humans, food intake was decreased after UAG infusion compared with ghrelin or ghrelin plus UAG infusions, but was not different from saline control (Tong et al., 2014).

L. Other Evidence Suggesting Novel Receptors

Some other reports that suggest novel receptors are briefly summarized below. In each case these are isolated reports, and in some cases sites and/or mechanisms of effect are unknown.

UAG mimics ghrelin’s action in the nucleus tractus solitarius, where injection of either compound lowers blood pressure with almost equal potency (Tsubota et al., 2005). Intracerebroventricular UAG (0.1 and
0.5 nM solutions) reduced the temperature of the dorsal body surface and increased tail temperature, suggesting that a blood flow redistribution was elicited by UAG (Inoue et al., 2013). Intracerebroventricular ghrelin mimicked UAG. However, the effect of ghrelin, but not that of UAG, was blocked by the antagonist (D-Lys3)-GHRP-6 (Inoue et al., 2013). Intraperitoneal injection of UAG (0.03 and 0.1 mg/kg body weight) caused similar, but less potent effects. Both intracerebroventricular and intraperitoneal UAG induced c-Fos expression in neurons of the median preoptic area, a region known to regulate body temperature.

There are several reports of UAG attenuating effects of ghrelin, for example reducing the appetite stimulatory effects of ghrelin (Inhoff et al., 2008) and reducing its effect on colorectal propulsion (Hirayama et al., 2010) and having opposite effects on insulin secretion (see above). A transgenic mouse overexpressing UAG at levels 10- to 44-fold greater than in control mice exhibits a small phenotype with a shorter nose to anus length and lower body weights (Ariyasu et al., 2005).

It is reported that nanomolar concentrations of UAG, hexarelin, and EP08317 [(2S,5S)-5-amino-1,2,3,4,6,7-hexahydro-azepino(3, 2-1-h)indol-4-one-2-carboxylic acid-d-2Me-Trp-d-Lys-Trp-d-Phe-Lys-NH₂] counteract the stimulation of interleukin-1β and interleukin-6 synthesis in microglial cells by the Alzheimer’s disease–associated β-amyloid protein (Bulgarelli et al., 2009). Ghrelin was ineffective. Expression GHSR1a was undetectable in the microglial cells.

VI. Conclusions

The only molecularly defined receptor for ghrelin, GHSR1a, is important for many of its actions, but this receptor has very low affinity for UAG, too low for circulating UAG to be a hormone that acts at GHSR1a. However, there is clear evidence of cell and organ responses to low, generally nanomolar, concentrations of UAG, both at sites where ghrelin is also an agonist and at sites where UAG alone has agonist effects. Thus, we postulate that there are two families of ghrelin receptors that are yet to be identified at a molecular level: receptors at which ghrelin and UAG are both potent agonists (GRLRs) and receptors at which UAG, but not ghrelin, is a potent agonist (UAG receptors). GRLRs are expressed by adipocytes, myoblasts, osteoblasts, cardiac muscle cells, pancreatic islet cells (which also express GHSR1a), coronary arteries, vascular endothelial cells, and cancer cells (breast and prostate). UAG receptors are expressed by hepatocytes and pancreatic beta cells.

It is necessary to determine the molecular nature of the GRLRs that have been reported in many cells types that are essential to human health. Until this is done, there will remain uncertainty about whether the effects at what appear to be nonspecific ghrelin receptors or UAG receptors are truly at unique receptors. In the case of the UAG receptors, clinical trials are already underway, using a ghrelin analog whose molecular site of action is still unknown. No agonist other than UAG has yet been convincingly demonstrated to act at GRLRs but not at ghrelin receptors. Further development of selective ligands will be problematic until the receptors are isolated. It is notable that receptors for ghrelin on pancreatic islet cells are heterodimers of GHSR1a and the somatostatin receptor SST5. Further exploration of heteromericGPCRs may uncover other physiologically important GRLRs.

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Receptors for Ghrelin


