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Chapter 1

GHRELIN: A PEPTIDE INVOLVED IN THE CONTROL OF APPETITE

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ABSTRACT

Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor. Ghrelin is a peptide of 28 amino acids possessing an uncommon octanoyl moiety on the serine in position 3, which is crucial for its biological activity. Ghrelin is predominantly produced and secreted into the blood stream by the endocrine X/A like cells of the stomach mucosa. Besides, it is also expressed in other tissues like duodenum, jejunum, ileum, colon, lung, heart, pancreas, kidney, testis, pituitary and hypothalamus. Some of the major biological actions of ghrelin are the secretion of growth hormone, the stimulation of appetite and food intake, the regulation of gastric motility and acid secretion and the modulation of the endocrine and exocrine pancreatic functions. Ghrelin is an orexigenic peptide involved in the short-term regulation of appetite and food intake. The plasma ghrelin levels increase before meal and decrease strongly during the postprandial phase. Long-term body weight is also regulated by ghrelin, since it induces adiposity. The purpose of this chapter is to provide updated information on ghrelin, the role of ghrelin in the control of appetite, as well as the potential clinical applications of ghrelin agonists and antagonists in certain physiopathological conditions.

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1. DISCOVERY OF GHRELIN

Growth hormone releasing hormone (GHRH) is known to activate the pituitary secretion of growth hormone, while somatostatin inhibits it. Endorphin-derived peptides were shown to stimulate GH secretion and this led to the development of peptidic and non-peptidic synthetic molecules named growth hormone secretagogues (GHS). Both GHRH and GHS stimulate GH secretion via the cAMP and the inositol triphosphates/calcium pathways, respectively, suggesting they are acting on distinct receptors. In 1996, GHS receptor (GHS-R 1a) was cloned from human pituitary and remained an orphan G-protein coupled receptor (GPCR) until the discovery, in 1999, of its natural ligand, ghrelin [Kojima et al., 1999; Kojima & Kangawa, 2008].

Ghrelin was purified from rat stomach and its structure is unprecedented as this 28 amino acid peptidic hormone contains a unique modification: a *n*-octanoylation of serine in position 3 [Kojima et al., 1999; Kojima et al., 2001] (figure 1). This modification turned out to be essential for its known biological activity encountered by its binding to the GHS-R 1a receptor.

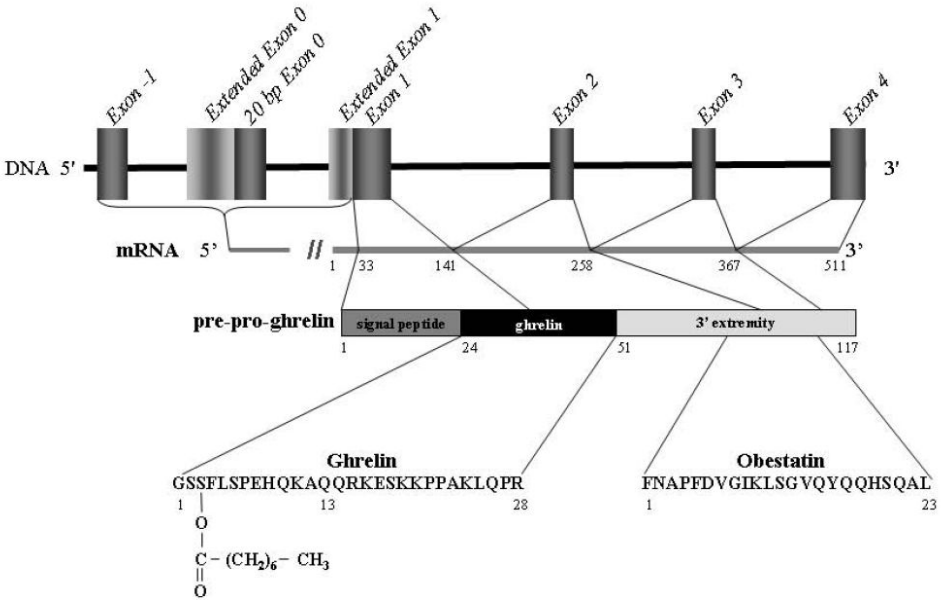

Figure 1. Human ghrelin gene structure contains 4 exons (boxes) and 3 introns (lines). After transcription, the mRNA is translated into prepro-ghrelin of 117 amino acids. Prepro-ghrelin is finally processed into ghrelin and/or obestatin.

2. HUMAN GHRELIN GENE STRUCTURE

The human ghrelin gene is located on chromosome 3 (3p25-26). Originally, human ghrelin gene was shown to contain a 20 bp non-coding exon, 4 coding exons and 3 introns and encoding a 511 bp mRNA [Kanamoto et al., 2004; Nakai et al., 2004]. Very recently, the

human ghrelin gene was shown to contain an additional novel exon (termed exon -1) and a 5' extension to exons 0 and 1 [Seim et al., 2007]. Therefore, a revised exon-intron structure proposes that the human ghrelin gene spans 7.2 kb and consists of six rather than five exons [Seim et al., 2007] (figure 1). Mature ghrelin is encoded by exons 1 and 2. Prepro-ghrelin contains 117 amino acids (AA), the first 23 AA represent the signal peptide, followed by 94 AA coding pro-ghrelin [Korbonits et al., 2004]. In 2005, another peptide, called obestatin, was shown to be derived from prepro-ghrelin [Zhang et al., 2005]. The sequence of obestatin starts at the 25th AA downstream of ghrelin and consists of 23 AA (figure 1). Another ghrelin gene-derived mRNA transcript, not coding for ghrelin but that may encode for the C-terminal region of the full-length prepro-ghrelin, was also identified [Seim et al., 2007]. Moreover, several spliced variants of human ghrelin gene were identified. A first group of transcripts include exons 1 to 4, coding for prepro-ghrelin, and varying only in the length of the sequence upstream of exon 1 (the prepro-ghrelin 5' untranslated region). Splice variant resulting from an alternative exon 2 splice site include des-Gln^{14}-ghrelin [Hosoda et al., 2000b]. A second group of transcripts contain splice variants that include exon -1 in various combinations with exons downstream of exon 1 (exons 2 to 4) [Seim et al., 2007]. Putative peptides encoded by these transcripts would include the sequence for C-ghrelin (exons -1, 2, 3, 4, including the coding sequence for obestatin as well) [Pemberton et al., 2003; Seim et al., 2007], the obestatin sequence (exons -1, 3, 4) [Seim et al., 2007; Zhang et al., 2005], and a novel C-terminal pro-ghrelin peptide lacking exon 3 (exons -1, 4) [Jeffery et al., 2005; Seim et al., 2007]. Interestingly, this latter transcript has been shown to be upregulated in two cancers, i.e. breast cancer [Jeffery et al., 2005] and prostate cancer [Yeh et al., 2005]. These various variants have been identified in different human tissues and cell lines, strongly suggesting a higher order of complexity of ghrelin gene expression. Indeed, an additional level of complexity may play an increasingly important role delivered by a third group of natural antisense transcripts (transcribed from the anti-sense strand), that were also recently identified [Seim et al., 2007], and subject as well to multiple transcription start sites and splice variants. However, these transcripts, to date do not seem to code for proteins, and may represent a source of non-coding RNAs implicated in gene regulation at the post-transcriptional and/or post-translational level.

Taken together, the data published to date on the human ghrelin gene structure strongly suggests that the ghrelin locus is far more complex than previously recognized. Further investigation will be necessary to understand the fine-tuning regulatory mechanisms that may be tissue specific, as well as dependent on physiological requirements. Likewise, it would be important to re-examine the ghrelin locus in order to identify and characterize novel peptides that may derive from it, and maybe other novel transcripts. This will raise challenging questions pertaining to their physiological functions in the light of ghrelin's pleiotropic actions. This in turn will open the way to their implication in various physiopathological conditions.

3. PREPRO-GHRELIN PROCESSING

Processing of the 117 AA prepro-ghrelin containing, containing pro-ghrelin (1-94), can lead to the formation of ghrelin 1-28 (in position 24-51 of prepro-ghrelin [Kojima et al.,

1999], obestatin 1-23 (in position 76-98 of prepro-ghrelin) [Zhang et al., 2005], a C-terminal peptide of 66 AA (in position 52-117 of prepro-ghrelin) [Bang et al., 2007], and peptides derived from the 66 carboxy-terminal AA of pro-ghrelin (termed C-ghrelin) [Pemberton et al., 2003] (figure 2). Several enzymes responsible for processing the ghrelin precursor have been identified. Knockout mice for prohormone convertase 1/3 (PC 1/3), 2 (PC 2) and 5/6A (PC 5/6A) revealed that PC 1/3, but not PC 2 or PC 5/6A, is involved in the conversion of pro-ghrelin to ghrelin by cleaving at a single Arg residue in the stomach [Zhu et al., 2006] (figure 2). It is worth noting that the ghrelin sequence ends at Pro27 and Arg28, two amino acids corresponding to a processing signal. A cleavage most frequently occurs following Pro27-Arg28, and rarely after Pro27, generating respectively ghrelin 1-28 (Kojima et al., 1999) and ghrelin 1-27 [Hosoda et al., 2003]. Cleavage following Pro27 is mostly the result of a carboxypeptidase-B like enzyme [Hosoda et al., 2003]. Either ghrelin 1-28 or ghrelin 1-27 are then subjected to a particular post-translational modification: the acylation of the hydroxyl group of Ser3. The acylation most frequently occurs with an octanoyl group (C8:0), and more rarely with a decanoyl (C10:0) or a decenoyl (C10:1) group [Hosoda et al., 2003]. Acylation of ghrelin can be increased by ingestion of either medium-chain fatty acids or medium chain triacylglycerols [Nishi et al., 2005].

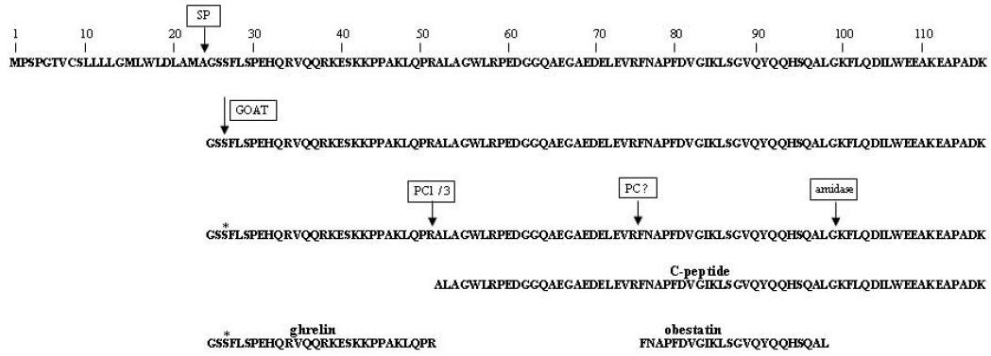

Figure 2. Processing of the prepro-ghrelin precursor. Prepro-ghrelin, composed of 117 amino acids, is processed into pro-ghrelin by the cleavage of the signal peptide by a signal peptidase (SP). In the endoplasmic reticulum, pro-ghrelin is octanoylated by the ghrelin *O*-acyl transferase (GOAT). Octanoylated pro-ghrelin is then processed into ghrelin and obestatin by prohormone convertase 1/3 (PC 1/3), followed by another prohormone convertase, and an amidase.

Very recently, the enzyme responsible for ghrelin octanoylation was simultaneously identified by two separate research groups as being an orphan membrane-bound *O*-acyl transferase (MBOAT) and renamed ghrelin *O*-acyl transferase (GOAT) [Gutierrez et al., 2008; Yang et al., 2008] (figure 2). Both teams based their reasoning on the same observations. Firstly, porcupine, an enzyme sharing structural similarities with MBOAT, is required for the acylation of serine in position 209 with palmitoleic acid and for transport of Wnt3a (member 3 of the wingless-type mouse mammary tumor virus integration site family) from the endoplasmic reticulum for secretion [Takada et al., 2006]. Secondly, porcupine was localized in the same cellular compartment through which ghrelin is expected to pass during its processing. These observations led to the hypothesis that an acyl transferase of the MBOAT family could be involved in the acyl modification of ghrelin [Gutierrez et al., 2008;

Yang et al., 2008]. Like porcupine, all other MBOATs are believed to transfer only long-chain fatty acids of 16-18 carbons. Both teams discovered GOAT using slightly distinct approaches.

Gutierrez et al. [Gutierrez et al., 2008] beneficited from the capacity of the human medullary thyroid carcinoma cells to produce ghrelin, allowing them to identify the ghrelin acyl transferase. Indeed, that cell line was used to perform gene-silencing of twelve candidate sequences. The selection was based on their similarity to known acyl transferases sequences, the presence of a human homologue, and the unknown function of the gene. Gene-silencing experiments revealed the knock-down of only one candidate gene encoding an uncharacterized protein containing structural motifs reminiscent of the MBOAT acyl transferase family. The authenticity of the human gene identified was verified using RT-PCR and 5' RACE reactions. The predicted protein encoded by the gene was named GOAT. Transient transfections of the GOAT cDNA were performed to assess the octanoylation of ghrelin. Mutation of the MBOAT-conserved histidine residue in position 338 of GOAT abolished the octanoylation of ghrelin. GOAT was also shown to acylate ghrelin with fatty acid ranging from C7 to C12.

Yang et al. [Yang et al., 2008] also sought for a cell line capable of octanoylating ghrelin in order to transfect cells with ghrelin cDNA and look at subsequent acylated ghrelin production by Western blot analysis. To confirm the presence of ghrelin in rat insulinoma Ins-1 cells, further transfections were performed using mutant forms of ghrelin cDNA encoding mutant forms of prepro-ghrelin with amino acid substitution at or near the arginine in position 28. To determine if any of the sixteen MBOATs, identified as containing 11 catalytic domains bearing conserved sequences, was capable of producing acylated ghrelin, Ins-1 cells were transfected with the corresponding sixteen MBOATs cDNA. Only one MBOAT cDNA led to ghrelin production. Furthermore, mutation of either serine in position 3 of ghrelin or of the MBOAT-conserved histidine residue in position 338 of GOAT abolished ghrelin acylation by GOAT. GOAT appears to be specific for medium chain fatty acids like octanoate. Moreover, [^{3}H]octanoate labeling experiments indicated that ghrelin is octanoylated before it translocates to the Golgi where it is cleaved by prohormone convertase 1/3 to form mature ghrelin. This suggested that GOAT is located in the endoplasmic reticulum.

GOAT, sharing structural similarities with members of the MBOAT family of acyl transferases [Chamoun et al., 2001; Hoffmann et al., 2000; Liang et al., 2004], is a highly hydrophobic protein with eight postulated membrane-spanning helices. Sequence conservation of GOAT across vertebrates and its tissue coexpression with ghrelin are consistent with the identification of ghrelin octanoylated forms in vertebrates. The presumed donor for octanoylation is octanoyl-CoA. However, how octanoyl Co-A gets into the endoplasmic reticulum lumen remains unclear. GOAT could possibly bind octanoyl CoA, and due to its hydrophobic nature, and allow transmembrane octanoylation of ghrelin

An in vitro biochemical assay for GOAT activity was recently developed using membranes from insect cells expressing mouse GOAT and purified recombinant pro-ghrelin as the acyl acceptor [Yang et al., 2008]. Using that assay, it was shown that GOAT recognizes several amino acids in pro-ghrelin surrounding the octanoylation site on serine-3. Glycine-1, serine-3 and phenylalanine-4 represent the crucial residues for the GOAT activity, in consistency with their strict conservation among all vertebrate ghrelins [Kojima & Kangawa, 2008]. A pentapeptide, corresponding to the first five N-terminal amino acids of ghrelin with its C-terminal end amidated, competitively inhibited GOAT activity and was used as a

substrate by GOAT. A more efficient inhibition was obtained when the pentapeptide contained an octanoyl group linked to serine-3 by an amide linkage, instead of an ester linkage. A similar pentapeptide, but containing an alanine-3, instead of serine-3, also inhibited GOAT, was not used as a substrate by the enzyme. These data suggest that GOAT is subjected to end-product inhibition [Yang et al., 2008].

Very recently, it was shown that gastric GOAT mRNA levels were similar in fed and 48h-fasted rats, and that exogenous leptin administration to fasted rats markedly increased GOAT mRNA levels [Gonzalez et al., 2008]. These data indicated that during fasting, low leptin levels prevent an increase in GOAT mRNA levels, and that GOAT is a leptin-regulated gene. Long-term chronic malnutrition (21 days) led to an increase in GOAT mRNA levels [Gonzalez et al., 2008] and this could represent the underlying mechanism responsible for increased acylated ghrelin levels in chronic undernutrition, such as anorexia nervosa [Soriano-Guillen et al., 2004].

GOAT knockout mice are therefore a valuable tool to determine the physiological consequences of a specific deficiency in acylated ghrelin. Moreover, GOAT represents a useful target for the development of anti-obesity and/or anti-diabetes drugs [Gualillo et al., 2008]. Much work remains to be done to fully understand how GOAT fits into the control of energy homeostasis.

4. GHRELIN-RELATED PEPTIDES

Ghrelin peptides can be classified into two group based on the length, ghrelin 1-28 and ghrelin 1-27, or into four groups based on the nature of the acyl group on Ser3 (non-acylated, octanoylated, decanoylated, decenoylated). Although the major active form is octanoylated ghrelin 1-28, decanoyl ghrelin 1-28, decenoyl ghrelin 1-28, octanoyl ghrelin 1-27 and decanoyl ghrelin 1-27 were also found in the stomach and in plasma [Hosoda et al., 2003] (figure 3). In the stomach, decanoyl ghrelin 1-28, decanoyl ghrelin 1-27, octanoyl ghrelin 1-27 and decenoyl ghrelin 1-28 represent respectively only 17%, 8%, 17% and 8% of the biologically active ghrelin. In contrast to rat stomach, human stomach is devoid of des-Gln14 ghrelin [Hosoda et al., 2000b]. It must be noted that the major circulating form of ghrelin is the des-acyl ghrelin, the so called biologically inactive form of ghrelin, at least on the GHS-R 1a [Hosoda et al., 2000a]. Besides, in human plasma, C-ghrelin also circulates at higher concentrations than octanoyl ghrelin [Pemberton et al., 2003].

A novel peptide derived from the carboxy-terminal of pro-ghrelin was identified in rat stomach and named obestatin, due to its appetite-suppressing potential [Zhang et al., 2005]. Obestatin is a 23 AA peptide that is amidated, notably due to the presence of C-terminal Gly-Lys motif following the C-terminal AA of obestatin [Zhang et al., 2005]. During the purification of obestatin from rat stomach, an obestatin truncated peptide was identified as containing the last 13 C-terminal AA of obestatin and named obestatin 11-23 [Zhang et al., 2005].

Ghrelin was identified in various mammalian species such as human [Kojima et al., 1999], rat [Kojima et al., 1999], mouse [Tanaka et al., 2001] (figure 4). In mammals, the first 10 N-terminal AA are identical and the acylation of Ser3 result principally in an octanoylation. Human ghrelin is identical to rat ghrelin, except for two AA in position 11 and

12. In ovines and bovines, ghrelin is composed of 27 AA, rather than 28, and is devoid of Gln14 similarly to rat des-Gln14-ghrelin.

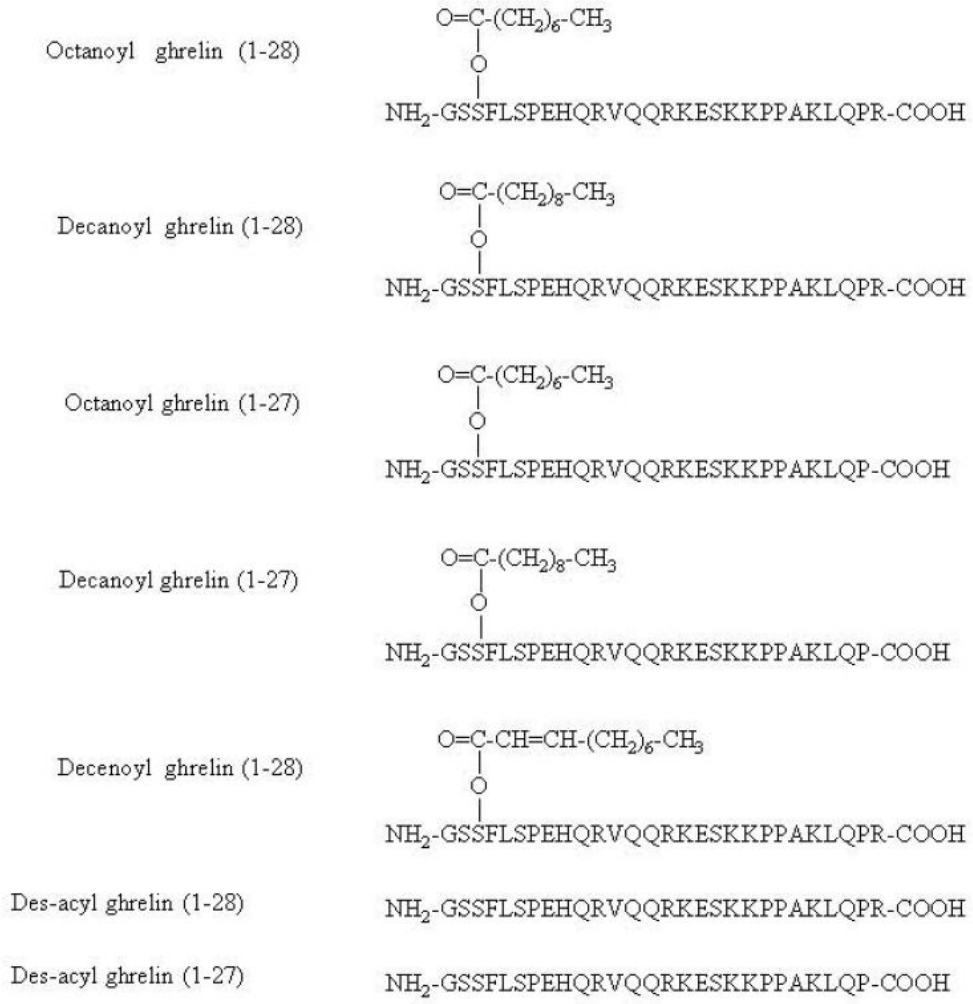

Figure 3. Ghrelin forms identified from human stomach.

Very recently, ghrelin and des-acyl ghrelin were shown to be phosphorylated by protein kinase C on the Ser in position 18. While ghrelin and des-acyl ghrelin bind to phosphatidylcholine:phosphoserine sucrose-loaded vesicles in a phosphatidylserine-dependent manner, this binding capacity was greatly lowered when the peptides were phosphorylated. A possible explanation is the disruption of the amphipathic helix formed by about two third of the C-terminal part of the peptide [Dehlin et al., 2008]. Further investigations will be required to determine if phosphorylation can occur in cells under specific conditions, and if so what would be the impact of such phosphorylation on the subcellular localization and function of the peptide.

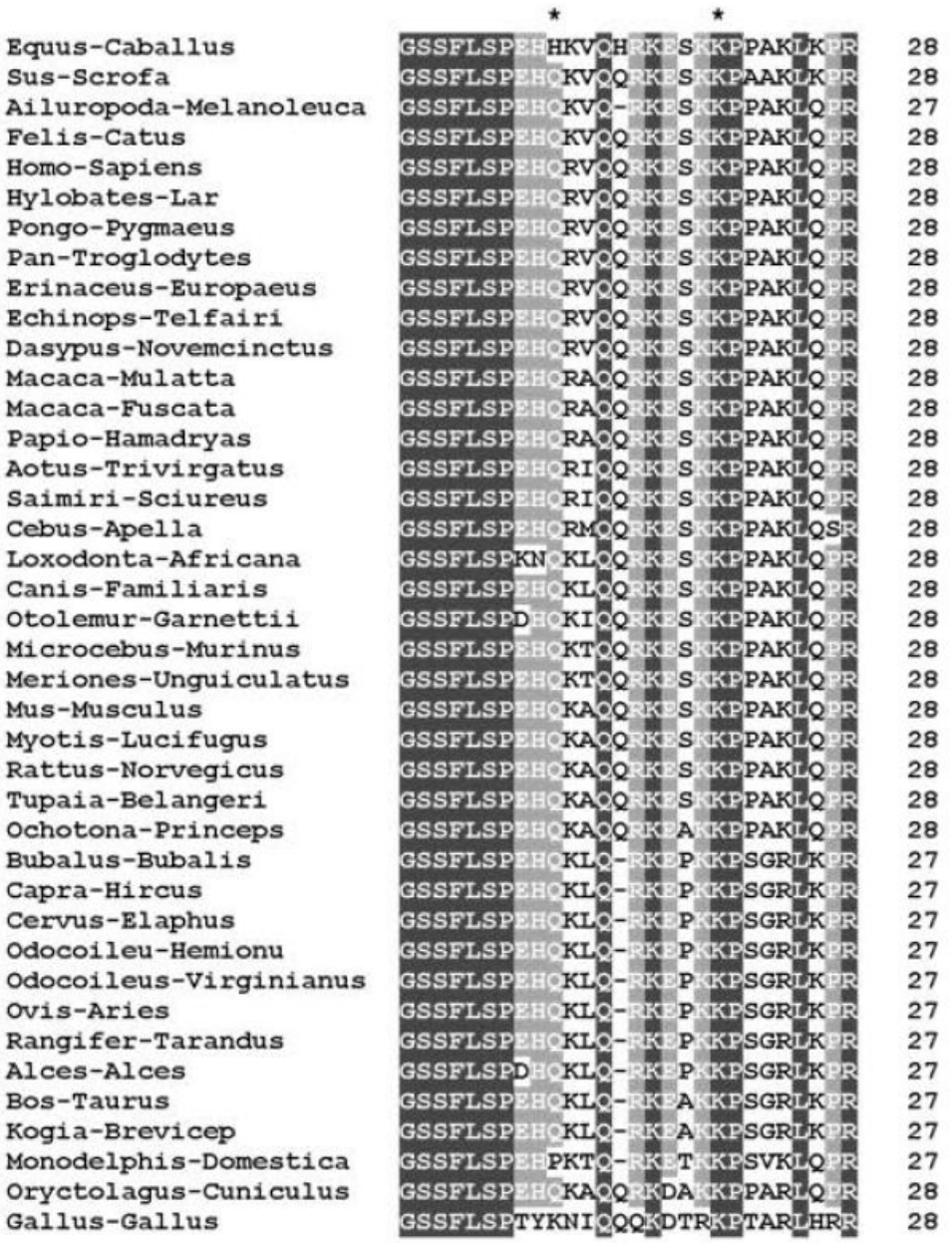

Figure 4. Amino acid sequence homologies among mammalian ghrelin sequences. Multiple sequence alignment of 40 mammalian ghrelin peptide sequences, available to date in the two public sequence databases sets (*Ensembl* release 50 and *NCBI* Nucleotide). Dark gray shading are residues sharing 100% conserved identity (40/40 sequences), light gray shaded residues share at least 80% identity (i.e. identity in at least 32/40 sequences). Note that the amino terminal hepta-peptides is conserved with 100% identity. Every 10 amino acids are indicated by *. Peptide lengths are indicated in the rightmost column.

5. HUMAN GHRELIN RECEPTOR GENE STRUCTURE

The ghrelin receptor, termed GHS-R, belongs to the family of G-protein coupled receptor, characterized by seven transmembrane helix domains [Howard et al., 1996]. GHS-R gene is located on chromosome 3 (3q26.2) and is composed of two exons separated by one intron. Exon 1 codes for the extracellular amino-terminal domain through to the fifth transmembrane helix of the GHSR. Exon 2 codes then for the sixth transmembrane helix through to the carboxy-terminal segment [McKee et al., 1997]. GHS-R has two variants resulting from alternative splicing of its mRNA: GHS-R 1a and GHS-R 1b. GHS-R 1a mRNA, encoded by the two exons and from which the intron is excised by splicing, codes for a protein of 366 AA comprising the seven transmembrane helix domains. GHS-R 1b mRNA is coded only by exon 1 and and followed by the non-excised intron. Thereby, GHS-R 1b codes for a protein of 289 AA comprising only five transmembrane helix domains. The nucleotide sequences are identical for AA 1 to 265 , thereafter GHS-R 1b mRNA nucleotide sequence is distinct from that of GHS-R 1a mRNA as of codon Leu265 and codes only for 24 additional AA using an alternative stop codon [McKee et al., 1997]. Since the coupling of G-protein coupled receptor with the G-protein involves the third intracellular loop, GHS-R 1b is unable to couple to G-proteins. Though there is no obvious role for such a truncated receptor, it has recently been proposed to act as a dominant negative mutant of GHS-R 1a by heterodimerization [Leung et al., 2007], attenuating the latter's constitutive activity. GHS-R 1b was also detected in PBMCs, in the absence of the functional GHS-R 1a [Mager et al., 2008].

GHS-R 1a is not obviously related to known families of G-protein coupled receptors, although it is often included in a small family of the class A receptors for small polypeptides comprising the motilin receptor (52% homology), neurotensin receptor-1 and neurotensin receptor-2 (33-35% homology), neuromedin receptor-1 and neuromedin receptor-2 (± 30% homology), and the orphan GPR39 (27-32% homology) [McKee et al., 1997; Tan et al., 1998]. Concerning GPR39, it must be cautioned that it was at first described as the receptor for obestatin [Zhang et al., 2005]. Nevertheless, recent controversy over obestatin binding to GPR39 has lead to the conclusion that GPR39 is ultimately not the obestatin receptor [Lauwers et al., 2006; Tremblay et al., 2007].

Likewise, it must be noted that the scavenger receptor CD36, involved in the endocytosis of the pro-atherogenic oxidized low-density lipoproteins, is a pharmacologically and structurally distinct receptor for peptidyl GHSs, but not for ghrelin [Bodart et al., 1999; Bodart et al., 2002].

6. ACTIVATION MECHANISM OF GHRELIN RECEPTOR

Binding of GHSs and ghrelin to GHS-R 1a leads to phospholipase C activation. Phospholipase C activation induces the hydrolysis of phosphatidylinositol 4,5-biphosphate and the subsequent formation of diacylglycerol and inositol 1,4,5-trisphosphate. Diacylglycerol activates protein kinase C and inositol 1,4,5-trisphosphate induces a calcium release from the endoplasmic reticulum [Smith et al., 1996]. Des-acyl ghrelin also seems to bind to GHS-R 1a but at very high concentration (micromolar range) [Gauna et al., 2007].

Neither ghrelin, nor GHSs, bind to GHS-R 1b, suggesting that GHS-R 1b is pharmacologically inactive (Howard et al., 1996). However, when GHS-R 1b is coexpressed with GHS-R 1a in HEK293 cells, the signal transduction of GHS-R 1a was decreased, suggesting that GHS-R 1b could form an heterodimer with GHS-R 1a [Chan & Cheng, 2004].

GHS-R 1a possesses constitutive activity [Holst et al., 2003] that might be mandatory for proper growth and development of the human body [Holst & Schwartz, 2006]. In HEK293 cells, GHS-R 1a possesses constitutive activity that is about 50% of the maximal agonist-induced activity [Holst et al., 2003; Holst et al., 2006]. The molecular basis for the constitutive activity appears to be related to three aromatic residues located in the sixth and seventh transmembrane helix domains that would promote the formation of a hydrophobic core between helices 6 and 7. This would ensure proper docking of the extracellular end of the seventh transmembrane helix domain into the sixth transmembrane domain, mimicking agonist activation and stabilizing the receptor in its active conformation [Holst et al., 2004]. However, a naturally occurring non-conservative mutation, A204E, occurring surprisingly in the second extracellular loope, that eliminates the constitutive activity without impairing stimulation by ghrelin [Holliday et al., 2007; Pantel et al., 2006].

7. GENETIC POLYMORPHISM OF GHRELIN AND GHRELIN RECEPTOR

Interestingly, both ghrelin and its receptor (GHSR) genes are located on chromosome 3, and the regions have been linked to obesity [Kissebah et al., 2000; Yeh et al., 2005]. A large number of polymorphisms have been identified in the ghrelin gene, not counting the transcript and splice variants described above. Several are found in the coding region of ghrelin, however a large number are in non-coding regions or in prepro-ghrelin but outside the ghrelin coding region.

Polymorphisms of both ghrelin and its receptor GHSR 1a have been studied in a wide series of disorders and pathologies, such as in obesity [Bing et al., 2005; Dardennes et al., 2007; Hinney et al., 2002; Korbonits et al., 2002; Larsen et al., 2005; Martin et al., 2008; Miraglia et al., 2004; Ukkola et al., 2001; Vivenza et al., 2004; Wang et al., 2004], in particular the Leu72Met polymorphism in prepro-ghrelin was associated with early onset of obesity [Korbonits et al., 2002; Miraglia et al., 2004; Ukkola et al., 2001; Ukkola et al., 2002]. Another polymorphism, 3056T>C single nucleotide polymorphism (SNP), has been reported to be correlated, in Japanese women, to higher body mass index, fat mass and eating disorders [Ando et al., 2007]. However, other studies yielded conflicting results [Bing et al., 2005; Hinney et al., 2002; Jo et al., 2005; Larsen et al., 2005]. Another example of conflicting results, with respect to weight, is the Arg51Gln allele, for which a positive association was found [Ukkola et al., 2001], but no correlation was found in another study [Hinney et al., 2002]. These authors also found that a frameshift mutation leading to ghrelin haplo-insufficiency was compatible with normal body weight. GH secretion was also studied in relation to short stature (height), as measured by IGF-1 and IGFBP-3 hepatic secretion in response to GH. A recent study showed lowered circulating levels of IGF-1 in ghrelin rs3755777 allele bearing subjects [Dossus et al., 2008], whereas decreased circulating levels of IGFBP-3 were correlated (though borderline) with the ghrelin rs2075356 SNP. Other

studies, however, found no correlation [Ukkola et al., 2002; Vartiainen et al., 2004; Vivenza et al., 2004]. On the other hand, 5 of the 15 alleles studied [Dossus et al., 2008] showed a correlation with height, whereas in a smaller study no significant association with genotype or haplotype were found with height, weight or body mass index (BMI) [Garcia et al., 2008]. Likewise, a study of the Leu72Met, Gln90Leu and Arg51Gln proposed that ghrelin levels rather than ghrelin/obestatin polymorphism would play a role in GH deficiency and thus height [Zou et al., 2008]. In contrast, the GHS-R 1a naturally occurring A204 mutation, leads to a loss of constitutive, activity, that decreases cell surface expression without impairing stimulation by ghrelin, and segregated with short stature in two unrelated families [Pantel et al., 2006]. A correlation between Leu72Met and bing eating disorder was found [Monteleone et al., 2007], whereas no association of Leu72Met and Arg-51-Gln could be found with anorexia nervosa or bulimia nervosa [Monteleone et al., 2006]. In contrast, the Leu72Met/Gln90Leu haplotype had an excess transmission in patients with anorexia nervosa [Dardennes et al., 2007]. These authors invoke in the former study a lack of comparison between the clinical subtypes studied. However, they also argue, that ethnic and international variations in ghrelin gene polymorphism must be taken into account [Cellini et al., 2006; Miyasaka et al., 2006; Ukkola et al., 2002; Zou et al., 2008]. Though, there is controvery as to the effects of ghrelin polymorphic variants on different disorders and pathologies, one must take into account the diversity of the subject panels used in the various studies, and the series of polymorphism investigated. The Leu72Met, as well other SNPs have been studied in relation to other disorders. The Leu72Met showed correlations with metabolic syndrome parameters, such as high-density-lipoproteins (HDL), triglycerides levels, blood pressure and increased risk for type 2 diabetes. However, in one report the levels of HDL/cholesterol were increased when compared to the Leu72Leu allele [Hubacek et al., 2007], whereas in another report it decreased in a study of older Amish [Steinle et al., 2005]. Likewise, the risk factor was positively associated to ghrelin gene polymorphism in a Finnish study [Mager et al., 2006], and was not in a Korean study [Choi et al., 2006]. Another study of 5 SNPs in exon 1, in introns and in the 3' untranslated region did not correlate with body fat percentage or serum lipid profiles [Martin et al., 2008]. A GHS-R 1a polymorphism, rs2232165, has also been shown to be associated with alcohol-abuse [Landgren et al., 2008]. Finally, ghrelin SNPs have been associated with various cancers, such as breast cancer [Dossus et al., 2008; Jeffery et al., 2005] and prostate cancer [Yeh et al., 2005].

In the light of an increasing body of data, displaying conflicting results, in the various areas investigated, a word of caution must be made regarding the polymorphisms in the prepro-ghrelin mRNA, outside of the ghrelin coding-region, e.g. Leu72Met. Indeed, these "mutated" sites may either affect proper processing of prepro-ghrelin, thereby potentially disrupting trafficking, posttranslational modification and secretion. However, most investigators did not see variations in circulating plasma levels. Though this may not be the best parameter, the acyl form is rapidly degraded in plasma and therefore plasma levels do not reflect local concentrations of the octanoylated form [De Vriese et al., 2004]. Moreover, the recent discovery of novel peptides stemming from the prepro-ghrelin RNA (as discussed above), containing polymorphic differences studied previously, might have an impact on the cellular processing and physiological effects of these peptides, independently of ghrelin itself. Consequently, the study of prepro-ghrelin will link observed effects/correlations to ghrelin in a misleading manner; as the studies are conducted at the mRNA level and not at the protein(s) level. Clearly, in view of ghrelin's pleiotropic effects and the high level of complexity, in the

ghrelin gene in terms of transcript variants, alternative splice variants, polymorphism, and multiple peptide products besides ghrelin, will require much more investigation and revisiting of previous studied areas. Furthermore, the polymorphic sites, thus far, described may affect the reverse strand transcript processing as well. Should the hypothesis that the anti-sense transcript harbor non-coding regulatory RNAs [Seim et al., 2007], such as microRNA, then such polymorphisms might alter considerably these regulatory molecules as well as potential target sequences on the sense strand. We speculate that these new findings will certainly open up future fascinating avenues of investigation in ghrelin gene expression and regulation.

8. TISSUE DISTRIBUTION OF GHRELIN

In the digestive system, ghrelin is predominantly synthesized in the stomach [Ariyasu et al., 2001; Kojima et al., 1999]. Five endocrine cell types have been identified in the gastric mucosa: enterochromaffin cells (EC), enterochromaffin-like cells (ECL), D cells, G cells and X/A like cells which respectively secrete serotonin, histamine, somatostatin, gastrin, GABA and ghrelin. Rat oxyntic cells display 60-70% of ECL cells, 20% of X/A-like cells, 2.5% of D-cells and 0-2% of EC and G cells. Human oxyntic endocrine cells display 30% of ECL cells, 20% of X/A-like cells, 22% of D-cells and 7% of EC and G cells [Simonsson et al., 1988; Solcia et al., 2000]. X/A like cells are gastric endocrine cells located in the fundus [Date et al., 2000b; Rindi et al., 2002]. They are round to ovoid cells, containing compact and dense secretory granules, located next to the capillary lumen, indicating ghrelin is secreted in an endocrine fashion into the plasma [Date et al., 2000b]. Immunoreactive ghrelin cells have also been located in the duodenum, jejunum, ileum and colon [Date et al., 2000b; Hosoda et al., 2000a; Sakata et al., 2002]. In the intestine, ghrelin concentration progressively decreases from the duodenum to the colon [Hosoda et al., 2000a]. Circulating ghrelin arises in majority from the gastric mucosa and the intestine [Ariyasu et al., 2001; Krsek et al., 2002]. In the endocrine pancreas, ghrelin-secreting cells have been colocalized with either glucagon in α-cells [Date et al., 2002b; Kageyama et al., 2005], or insulin in ß-cells [Volante et al., 2002], or in a new cell type called ε [Prado et al., 2004; Wierup et al., 2002; Wierup & Sundler, 2005]. In the exocrine pancreas, ghrelin has been located to acinar cells [Lai et al., 2007].

In the central nervous system, ghrelin is found in low amounts [Hosoda et al., 2000a]. However, neurons producing ghrelin have been identified in the arcuate nucleus of the hypothalamus, a region involved in the regulation of food intake [Kojima et al., 1999; Lu et al., 2002]. Moreover, ghrelin has also been identified in a particular area in the hypothalamus. By sending their efferent fibers, ghrelin-containing neurons could stimulate the release of peptides from neurons containing neuropeptide Y (NPY) and the agouti-related protein (AGRP), orexins, pro-opiomelanocortin (POMC), and cocaïn- and amphetamine-regulated transcript (CART) [Cowley et al., 2003]. Pituitary also contains ghrelin [Korbonits et al., 2001b; Korbonits et al., 2001a]. In vivo studies indicated that ghrelin stimulates GH secretion, suggesting that somatotropic cells are target cells for ghrelin [Date et al., 2000a; Takaya et al., 2000]. Furthermore, in the rat, ghrelin was found in cells secreting prolactin, GH, and thyroid stimulating hormone [Caminos et al., 2003].

Ghrelin is also expressed in other tissues such as in kidneys, adrenal glands, thyroid, breast, ovary, placenta, testis, prostate, liver, gallbladder, lung, skeletal muscle, myocardium, skin [Ghelardoni et al., 2006; Gnanapavan et al., 2002].

Studies related to tissue expression of obestatin remain limited. Obestatin expression was reported in the stomach, duodenum, jejunum, colon, myenteric plexus, pancreas, spleen, testis, cerebral cortex [Chanoine et al., 2006; Dun et al., 2006; Zhang et al., 2005; Zhao et al., 2008]. In the stomach, most of obestatin-producing cells are distributed in the basal part of the oxyntic mucosa, and are with prepro-ghrelin-producing cells more numerous than ghrelin-producing cells [Zhao et al., 2008]. In the pancreas, obestatin is present in the periphery of the islets, with a distribution distinct from that of α-cells, ß-cells, and δ-cells [Zhao et al., 2008].

9. GHRELIN IN CIRCULATION

Following surgical gastric mucosa removal, circulating ghrelin concentration is drastically reduced by about 80% in rat [Dornonville de la Cour et al., 2001] and human [Ariyasu et al., 2001; Jeon et al., 2004]. Human plasma ghrelin-immunoreactivity consists of more than 90% of des-acyl ghrelin [Patterson et al., 2005]. C-ghrelin also circulates at higher concentrations than octanoyl ghrelin in human plasma [Pemberton et al., 2003]. It is presently not yet well understood if both ghrelin and des-acyl ghrelin, which are present in the stomach, are both secreted into the bloodstream via the same or differently regulated pathway(s). In rat stomach, ghrelin is degraded by deacylation, as well as by N-terminal proteolysis [De Vriese et al., 2004; Shanado et al., 2004], and lysophospholipase I was identified as a ghrelin deacylation enzyme [Shanado et al., 2004]. Several mechanisms could account for the large presence of des-acyl ghrelin in the circulation: shorter half-life of ghrelin compared to des-acyl ghrelin [Akamizu et al., 2005] and plasma ghrelin deacylation [De Vriese et al., 2004; Hosoda et al., 2004]. In serum, ghrelin desoctanoylation is achieved in human by butyrylcholinesterase and other esterase(s), such as platelet-activating factor acetylhydrolase, and in rat by carboxylesterase [De Vriese et al., 2004; De Vriese et al., 2007]. Although paraoxonase was suggested to also participate to ghrelin deacylation [Beaumont et al., 2003], this hypothesis was not confirmed by us due to the lack of effect of EDTA on ghrelin deacylation in human serum and the negative correlation between des-acyl ghrelin and paraoxonase activity [De Vriese et al., 2004]. Due to ghrelin degradation by serum, it is difficult to accurately determine the ghrelin level and consequently its physiological and pathophysiological roles. Therefore, we suggested that addition of inhibitors of esterases and proteases to blood collected samples would be critical to ensure ghrelin stability.

It is interesting to note that butyrylcholinesterase knockout mice fed with a normal standard 5% fat diet had normal body weight, while mice fed with high-fat diet (11% fat) became obese. Since the obesity could not be explained by increased ghrelin, caloric intake, or decreased exercise, it is hypothesized that butyrylcholinesterase plays a role in fat utilization [Li et al., 2008].

While des-acyl ghrelin mostly circulates as a free peptide, the majority of circulating acyl ghrelin is bound to larger molecules. In particular, ghrelin was found to bind to lipoproteins [Beaumont et al., 2003; De Vriese et al., 2007]. The presence of the acyl group is necessary for ghrelin interaction with triglyceride-rich lipoproteins and low-density lipoprotein but not

high-density lipoproteins and very high-density lipoproteins. Ghrelin interacts via its N- and C-terminal parts with high-density lipoproteins and very high-density lipoproteins. These data suggest that, whereas triglyceride-rich lipoproteins mostly transport acylated ghrelin, high-density lipoproteins and very high-density lipoproteins transport both ghrelin and des-acyl ghrelin [De Vriese et al., 2007].

10. GHRELIN- AND GHRELIN RECEPTOR-NULL MICE PHENOTYPES

The phenotype of ghrelin knockout mice is indistinguishable from that of wild-type mice. Indeed, no differences were noticed concerning the size, growth rate, body composition, food intake, reproduction, bone density, activity, development, organs weight, tissular pathology [De Smet et al., 2006; Sun et al., 2003; Wortley et al., 2004]. Food intake of ghrelin knockout mice was similar to wild-type mice in response to starvation and to ghrelin injection. Though, in old ghrelin knockout mice interruption of the normal light/dark cycle triggers additional food intake [De Smet et al., 2006]. Pre- and post- prandial concentrations of glucose, insulin, leptin and GH were similar.

Ghrelin knockout mice fed for six weeks with a high fat diet displayed a decrease in the respiratory quotient and in the fat mass, independently of any body weight change [Wortley et al., 2004]. However, male ghrelin knockout mice submitted to a continuous high-fat diet three weeks after weaning (at approximately six weeks of age) are protected from the rapid weight gain occurring in wild-type mice [Wortley et al., 2005]. Very recently, it was shown that congenic adult ghrelin knockout mice submitted to either a positive (high-fat diet) or negative (caloric restriction) energy balance displayed similar body weight as wild-type littermates [Sun et al., 2008]. These contradictory data could be explained by differences in the mouse genetic backgrounds and/or the moment at which the high-fat diet was given to the animals. Further studies will be necessary to define the role of ghrelin in preventing or not preventing diet-induced obesity or weight gain after weight loss.

Ghrelin could modulate the type of metabolic substrate that is used preferentially to maintain energy balance, particularly under a high fat diet. These data are in agreement with the observed decreased use of fat in response to ghrelin administration in adult rats [Tschop et al., 2000]. Furthermore, the lack of modification body weight in ghrelin-null mice as in NPY- or AGRP-null mice, and in NPY- and AGRP-null mice, strongly suggest the existence of compensatory mechanisms [Erickson et al., 1996; Herzog, 2003].

GHS-R knockout mice displayed a slightly smaller size, without any modification in appetite, food intake and body composition. Their ghrelin, insulin and leptin levels in response to starvation were similar to wild-type mice. In response to ghrelin injection, GHS-R-null mice do not release GH and do not increase their food intake, indicating that these effects are mediated by the GHS-R [Sun et al., 2004]. A similar phenotype has been observed in transgenic rats expressing an antisense GHS-R mRNA, thereby attenuating GHS-R protein expression in the arcuate nucleus, also indicating that the GHS-R was involved in the regulation of GH secretion and food intake [Shuto et al., 2002]. More recently, it was shown that GHS-R-null mice eat less food, metabolize more fat, become less adipose, and are more insulin-sensitive [Zigman et al., 2005]. These latter data are consistent with the fact that

ghrelin-null mice resist weight gain induced by early exposure to high fat diets [Wortley et al., 2005], but not with the fact that congenic adult knockout mice for GHS-R are not resisting diet-induced obesity or weight gain after weight loss [Sun et al., 2008]. Interestingly, when fed with a standard chow diet, GHS-R and ghrelin double knockout mice display decreased body weight, increased energy expenditure, and increased motor activity [Pfluger et al., 2008].

Both ghrelin and GHS-R knockout mice submitted to caloric restriction displayed lower blood glucose levels, suggesting that the primary function of ghrelin in adult mice is to modulate glucose sensing and insulin sensitivity [Sun et al., 2008]. This hypothesis was supported by the fact that GHS-R knockout mice fed with high-fat diet had several fold greater insulin sensitivity, no hepatic steatosis, and lower total cholesterol [Longo et al., 2008].

11. PHYSIOLOGICAL FUNCTIONS OF GHRELIN IN FEEDING

Appetite and Food Intake

The control of food intake is under the control of complex physiological mechanisms. Food intake is regulated by the hypothalamus but also directly influenced by gastrointestinal peptides, which respond to nutritional status and body composition. To defend against starvation, the organism replies in an integrative fashion using central brain centers, gastrointestinal peptides and adipose-derived signals [Baynes et al., 2006]. In response to food intake, the concentrations of plasma glucose, free fatty acids and other nutrients increase, leading to hormones release. These hormones act on the tractus solitary nucleus, and on the arcuate nucleus of the hypothalamus to regulate the appetite and the energy metabolism at short and long terms. The arcuate nucleus of the hypothalamus contains two neuronal populations involved in the control of appetite: neurons synthesizing the neuropeptide Y (NPY) and agouti-related protein (AGRP) (their activation stimulates appetite) and neurons containing pro-opiomelanocortin (POMC), melanocortin (α-MSH) and cocaine- and amphetamine-regulated transcript (CART) (their activation inhibits appetite) [Ramos et al., 2005].

Intracerebroventricular, intravenous, or subcutaneous administration of ghrelin to rats leads to stimulation of food intake and decrease of energy expenditure, accounting for body weight increase [Kamegai et al., 2001; Nakazato et al., 2001; Shintani et al., 2001; Tschop et al., 2000; Wren et al., 2001]. Intravenous ghrelin administration of ghrelin in humans also increases appetite and stimulates food intake [Wren et al., 2001]. During fasting, before the onset of the meal, plasma ghrelin levels increase. After feeding, plasma ghrelin levels decrease strongly after 30 minutes [Cummings et al., 2001; Tschop et al., 2001]. In fasting subjects, ghrelin levels display a circadian pattern similar to that described in people eating three meals per day [Natalucci et al., 2005]. The preprandial increase and the postprandial decrease of plasma ghrelin levels strongly suggest that ghrelin might serve as a signal for meal initiation. Recently it has been demonstrated that the timing of ghrelin peaks is related to habitual meal patterns and may rise in anticipation of eating rather than eliciting feeding [Frecka & Mattes, 2008].

In contrast to ghrelin, most of the orexigenic peptides as NPY, AGRP, orexins, melanin-concentrating hormone (MCH) and galanin stimulate food intake when centrally administrated but not when peripherally administrated. Ghrelin is the only circulating hormone that stimulates appetite after systemic administration (table 1).

Among the anorexigenic peptides, several are synthesized in the hypothalamus, such as α-MSH, cocaine-and amphetamine-regulated transcript (CART), or corticotrophin-releasing hormone (CRH). Others are synthesized in endocrine cells of the gastrointestinal tractus, such as cholecystokinin (CCK), gastrin-related peptide (GRP), glucagon-like peptides (GLP-1 and GLP-2), pancreatic polypeptide (PP) and peptide YY (PYY). Leptin is synthesized in adipose tissue (table 1).

Table 1. Orexigenic and anorexigenic peptides

Orexigenic peptides	**Anorexigenic peptides**
Ghrelin	Melanocortin (α-MSH)
Neuropeptide Y (NPY)	Cocaïne- and amphetamine-regulated transcript (CART)
Agouti-related protein (AGRP)	Corticotropin-releasing hormone (CRH)
Melanin-concentrating hormone (MCH)	Cholecystokin (CCK)
Orexin A	Gastrin-related peptide (GRP)
Orexin B	Glucagon-like peptide 1 (GLP-1)
Galanin	Glucagon-like peptide 1 (GLP-2)
	Pancreatic polypeptide (PP)
	Peptide YY (PYY)
	Leptin

Mechanisms of Appetite Stimulation and Food Intake Control

Neurons expressing ghrelin have been identified in the arcuate nucleus of the hypothalamus, and in a previously uncharacterized group of neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei. These neurons send efferents onto neurons producing NPY, AGRP, POMC, and CRH [Cowley et al., 2003]. Ghrelin stimulates appetite and food intake through two different pathways:

1. Central Pathway

Ghrelin stimulates the activity of NPY/AGRP neurons [Cowley et al., 2003]. In rats, intracerebroventricular injection of ghrelin induces overexpression of NPY and AGRP mRNAs [Kamegai et al., 2001]. Inhibition of endogenous NPY and AGRP by anti-NPY and anti-AGRP antibodies, and by antagonists for Y1 and Y5 receptors abolished ghrelin-induced feeding [Nakazato et al., 2001]. Similarly, in NPY or AGRP null-mice, ghrelin-induced feeding is weakly attenuated, but completely abolished in mice lacking both NPY and AGRP [Chen et al., 2004]. Ablation of the NPY/AGRP neurons in mice completely suppress the feeding response to ghrelin [Luquet et al., 2007]. In rats, peripheral injection of ghrelin also activates the dorsomedial hypothalamic nucleus, which is innervated by projections from other brain areas like NPY/AGRP fibers arising from the arcuate nucleus [Kobelt et al.,

2008]. In NPY neurons, ghrelin interacts with the GHS-R and increases intracellular calcium via mechanisms depending on phospholipase C and adenylate cyclase-protein kinase A pathways [Kohno et al., 2007; Kohno et al., 2008]. In humans, ghrelin increases circulating NPY levels [Coiro et al., 2006]. All these data indicate that ghrelin activates hypothalamic NPY/AGRP neurons, stimulating the production of NPY and AGRP and therefore increasing food intake.

Intracerebroventricular administration of ghrelin activates not only the ARC but also the paraventricular nucleus and the lateral hypothalamus, including in orexin neurons [Scott et al., 2007]. Ghrelin activates in vitro neurons expressing orexins (hypothalamic orexigenic neuropeptides) [Yamanaka et al., 2003]. Appetite stimulation induced by ghrelin is inhibited in orexin-null mice and attenuated in mice pretreated with an anti-orexin antibody [Toshinai et al., 2003].

Peripheral ghrelin increases noradrenaline in the ARC and loss of neurons expressing dopamine β-hydroxylase abolishes ghrelin-induced feeding, suggesting that ghrelin stimulates food intake at least in part through the noradrenergic pathway [Date et al., 2006].

Ghrelin also inhibits POMC neurons, preventing the release of the anorexigenic peptide α-MSH [Riediger et al., 2003]. CART inhibits food intake and is expressed by both vagal afferent and hypothalamic neurons. Ghrelin administration decreases CART expression in rat vagal afferents neurons [de Lartigue et al., 2007] while peripheral ghrelin blockade using a specific anti-ghrelin antibody increases the expression of CART in the hypothalamic paraventricular nucleus [Solomon et al., 2005].

Although GHS-R mRNA is expressed in the tuberomammilary nucleus, ghrelin does not affect histamine release, and increases food intake in histamine H(1)-receptor knock-out mice. Thus, ghrelin expresses its action in a histamine-independent manner [Ishizuka et al., 2006].

2. Vagal Pathway

Ghrelin may also stimulate appetite via the vagus nerve. Detection of the ghrelin receptor in afferent neurons of the rat and human nodose ganglion suggests that the vagus nerve may transmit ghrelin signal from the stomach to the brain [Burdyga et al., 2006; Sakata et al., 2003]. In rats, blockade of the vagal afferent pathway, by vagotomy or perivagal application of an afferent neurotoxin, suppress ghrelin-induced feeding [Date et al., 2002a]. In the same way, patients with vagotomy and oesophageal or gastric surgery are insensitive to the appetite stimulatory effect of ghrelin [le Roux et al., 2005; Takeno et al., 2004]. Thus, through the activation of GHS-R on vagal afferent to the stomach, the signal induced by ghrelin may reach the nucleus of tractus solitarius, which communicates with the hypothalamus to increase food intake. However, intraperitoneal injection of ghrelin stimulates eating in rats with subdiaphragmatic vagal deafferentation, suggesting that the ghrelin signal does not involve vagal afferents [Arnold et al., 2006].

Regulation of Energy Homeostasis

In addition to its role in short-term regulation of food intake, ghrelin may also play a role in long-term body-weight regulation.

Plasma ghrelin levels are negatively correlated with BMI. Indeed, plasma ghrelin level is increased in anorexia nervosa and cachexia, and decreased in obesity. Moreover, ghrelin levels fluctuate in a compensatory manner to body weight variations [Soriano-Guillen et al., 2004]. Ghrelin levels decrease with weight gain resulting from overfeeding [Williams et al., 2006], pregnancy [Palik et al., 2007], olanzapine treatment [Hosojima et al., 2006], or high fat diet [Otukonyong et al., 2005]. Conversely, weight loss induces an increase of ghrelin levels. This effect is observed with weight loss resulting from food restriction [Purnell et al., 2007], long-term chronic exercise but not acute exercise [Kraemer & Castracane, 2007], cachectic states induced by anorexia nervosa [Soriano-Guillen et al., 2004], severe congestive heart failure [Nagaya et al., 2001], lung cancer [Shimizu et al., 2003], breast and colon cancers [Wolf et al., 2006]. However, data on ghrelin levels after weight loss induced by gastric bypass surgery are controversial. Some studies found a decrease [Chan et al., 2006; Cummings et al., 2002b; Fruhbeck et al., 2004; Korner et al., 2006], no change [Couce et al., 2006; Mancini et al., 2006; Stenstrom et al., 2006] or an increase of ghrelin secretion [Haider et al., 2007; Mingrone et al., 2006; Stratis et al., 2006].

In vivo, chronic ghrelin administration induces adiposity [Tschop et al., 2000; Tsubone et al., 2005]. Ghrelin increases body weight not only by stimulating food intake, but also by reducing energy expenditure, decreasing utilization of fat and increasing utilization of carbohydrates [Wortley et al., 2004]. Ghrelin may thus influence adipocyte metabolism. In vitro, ghrelin stimulates differentiation of preadipocytes [Thompson et al., 2004], inhibits adipocyte apoptosis [Kim et al., 2004] and antagonizes lipolysis [Muccioli et al., 2004]. Fat may be directed to either oxidation in skeletal muscle or brown adipose tissue, or to triglyceride storage in white adipose tissue. Chronic central infusion of ghrelin inhibits lipid oxidation and increases lipogenesis and triglyceride uptake in white adipocytes. An increase of the respiratory quotient indicates the decreased use of lipids for the generation of energy [Theander-Carrillo et al., 2006]. Ghrelin has also been shown to shift food preference towards diets high in fat [Shimbara et al., 2004]. Finally, ghrelin improves also lean body mass retention [Deboer et al., 2007]. In elderly subjects and after diet-induced weight loss, ghrelin levels increase with reduction of fat-free mass, specially skeletal muscle mass, but not with changes in fat mass [Purnell et al., 2007]. Recent data strongly support that ghrelin and des-acyl ghrelin modulate directly and positively adipogenesis and adipocyte function in rats, suggesting an important role for maintaining homeostasis [Giovambattista et al., 2008].

Ghrelin-null mice suggest a physiological role for ghrelin in energy homeostasis. The phenotype of ghrelin-null mice is similar to wild-type mice in terms of size, growth rate, food intake, body composition, reproduction, gross behavior, and tissue pathology [Sun et al., 2003]. However, young ghrelin-null mice are protected from the weight gain induced by chronic exposure to a high-fat diet. These mice have lower adiposity, higher energy expenditure and locomotor activity. This suggests that ghrelin plays a role in excess dietary fat storage [Wortley et al., 2005; Wortley et al., 2004].

In patients with Prader-Willi syndrome (PWS), a genetic disorder characterized by mental retardation and hyperphagia leading to severe obesity, plasma ghrelin levels are higher than in healthy subjects and do not decrease after a meal [Cummings et al., 2002a; DelParigi et al., 2002]. Other studies showed that ghrelin levels decreased postprandially in adult patients with PWS, but to a lesser extent than in obese and lean subjects [Gimenez-Palop et al., 2007; Paik et al., 2007]. This lesser postprandial ghrelin suppression may be due to a blunted postprandial response of PYY, an anorexigenic peptide that decreases postprandial

ghrelin levels. The low PYY levels could partially explain the high ghrelin levels observed in PWS [Gimenez-Palop et al., 2007]. Interestingly, children (5 years of age and younger) with PWS have normal ghrelin levels. Since these children have not yet developed hyperphagia or excessive obesity, it suggests that ghrelin levels increase with the onset of hyperphagia [Erdie-Lalena et al., 2006; Haqq et al., 2008]. In opposition with these data, Fiegerlova et al., showed that plasma ghrelin levels in children with PWS were elevated at any age, including the first years of life, thus preceding the development of obesity [Feigerlova et al., 2008]. Thus, ghrelin may be responsible, at least partially, for the insatiable appetite and the obesity of these patients.

Relationships between Ghrelin and Obestatin

As opposed to ghrelin, which increases food intake and body weight gain, Zhan et al. reported that intraperitoneal or intracerebroventricular injection of obestatin to mice decreased food intake and body weight gain [Zhang et al., 2005]. The initial observation that obestatin suppresses food intake in a time-dependent and dose-dependent manner ([Zhang et al., 2005] was reproduced in few studies ([Bresciani et al., 2006; Carlini et al., 2007; Green et al., 2007; Sibilia et al., 2006], but not by the majority of the studies carried out on rodents [Gourcerol et al., 2006; Gourcerol et al., 2007; Holst et al., 2007; Moechars et al., 2006; Nogueiras et al., 2007; Samson et al., 2007; Tremblay et al., 2007; Yamamoto et al., 2007; Zizzari et al., 2007]. This issue in reproducing the effects of obestatin has been partially clarified by the existence of a U-shaped dose-response relationship between intraperitoneal administration of obestatin and suppression of food intake and body weight gain in rodent [Lagaud et al., 2007]. The possible effect of obestatin on food intake might be secondary to an initial action in inhibiting thirst [Samson et al., 2007]. However, if obestatin only minimally affects food intake, obestatin does not seem to modify energy metabolism in long-term administration to rats [Sibilia et al., 2006]. In human, underweight anorectic and normal weight patients are characterized by higher plasma obestatin levels as well as an increased ghrelin to obestatin ratio, compared to obese patients. This suggests that obestatin might play a role in body weight regulation in these pathologies [Monteleone et al., 2008; Nakahara et al., 2008; Zamrazilova et al., 2008].

Relationships between Ghrelin and Leptin

Oppositely to ghrelin, leptin, a protein mainly produced by the adipose tissue, decreases food intake and increases energy expenditure to maintain the body fat stores. Fasting increases plasma ghrelin levels and decreases plasma leptin levels. Ghrelin expression in stomach cells, and plasma ghrelin levels are higher in leptin-null mice, the so called Ob/Ob mice which are hyperphagic and obese, than in healthy mice. However, ablation of ghrelin in Ob/Ob mice does not improve the obese phenotype, suggesting that the Ob/Ob phenotype is not a consequence of high ghrelin plasma levels [Sun et al., 2006].

After leptin administration, ghrelin concentration and food intake decrease, and energy expenditure increases [Nogueiras et al., 2008; Shintani et al., 2001]. Ghrelin and leptin have thus opposite effects on food intake. Leptin activates POMC neurons, stimulating release of

anorexigenic peptides α-MSH and CART, and inhibits NPY/AGRP neurons, preventing release of orexigenic peptides NPY and AGRP [Cowley et al., 2001]. Moreover, inhibition of NPY/AGRP neurons induced by leptin prevents γ-aminobutyric acid (GABA) release, leading to activation of POMC neurons. Oppositely, ghrelin activates NPY neurons and increases GABA release, leading to POMC neurons inhibition [Cowley et al., 2003; Nogueiras et al., 2008]. By activating NPY neurons, ghrelin increases intracellular calcium via mechanisms dependent on both the adenylate cyclase and phospholipase C (PLC) pathways. Leptin inhibits intracellular calcium increase induced by ghrelin via phosphatidylinositol 3-kinase- (PI3K) and phosphodiesterase 3- (PDE3) mediated pathways [Kohno et al., 2007]. Therefore, leptin acts on hypothalamic neurons by inhibiting the effects of ghrelin. Finally, inverse variations of ghrelin and leptin levels are clearly critical for energy homeostasis regulation.

12. Other Physiological Functions of Ghrelin

The pleiotropic physiological functions of ghrelin are summarized in figure 5.

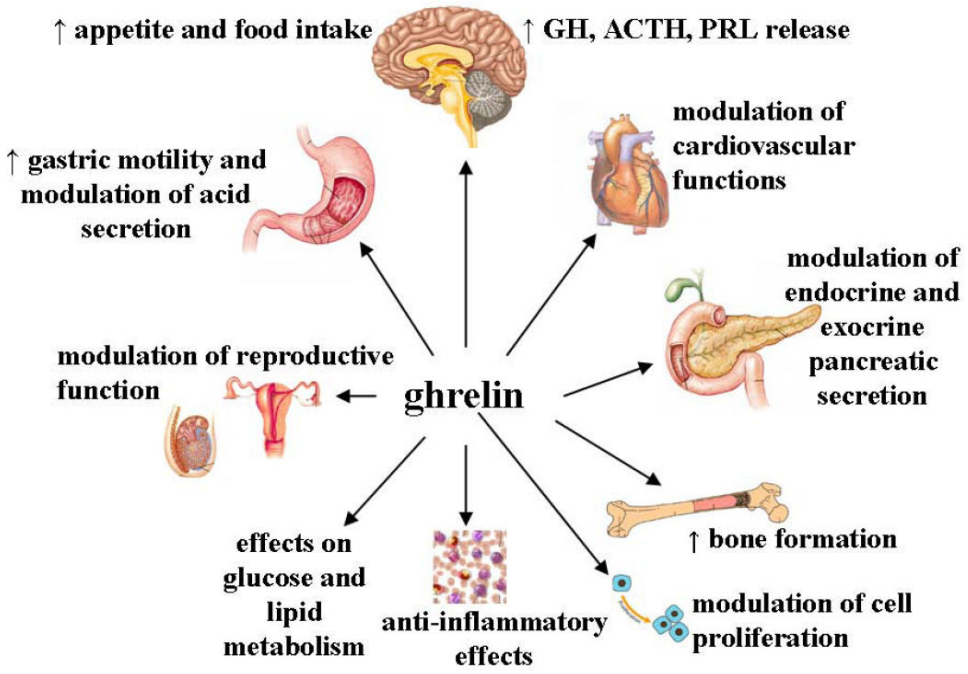

Figure 5. Pleiotropic biological functions of ghrelin. Major biological actions are summarized. GH: growth hormone; ACTH: adrenocorticotrophic hormone; PRL: prolactin.

Growth-Hormone Releasing Activity

Ghrelin strongly and dose-dependently stimulates growth hormone (GH) secretion, both in vivo and in vitro, in humans and animals by acting on GHS-R 1a present on pituitary somatotropic cells [Date et al., 2000a; Hataya et al., 2001; Malagon et al., 2003; Takaya et al., 2000]. To induce GH secretion from somatotropic cells, ghrelin activates the cGMP signal tranduction pathway but also requires activation of the nitric oxide synthase pathway [Rodriguez-Pacheco et al., 2008]. Combined administration of ghrelin and GH-releasing hormone (GHRH) displays synergistic effects, rather than additive effects, on GH release. Ghrelin action on GH release also seems to be mediated by the hypothalamus as patients presenting organic lesions in the hypothalamus region are still able to release GH in response to ghrelin [Popovic et al., 2003]. Apart from GH release, ghrelin stimulates adrenocorticotrophic hormone (ACTH), cortisol and prolactin (PRL) release [[Lengyel], 2006].

Des-acyl ghrelin is unable to stimulate GH secretion under physiological conditions, as it cannot bind to GHR-R. However, over-expression of des-acyl ghrelin in transgenic animals results in a small phenotype, maybe by modulation the GH-insulin growth factor 1 axis [Ariyasu et al., 2005].

Most studies have so far been unable to demonstrate that obestatin affects GH secretion in rats [Bresciani et al., 2006; Nogueiras et al., 2007; Samson et al., 2007; Yamamoto et al., 2007; Zhang et al., 2005]. However, obestatin might inhibit ghrelin action on GH secretion under certain conditions, but this remains to be confirmed [Zizzari et al., 2007].

Gastrointestinal Functions

Ghrelin modulates gastric acid secretion. In anesthetized rats, intravenous administration of ghrelin dose-dependently increases gastric acid secretion [Asakawa et al., 2001; Masuda et al., 2000]. This effect is abolished by vagotomy and by preliminary administration of atropine, suggesting that ghrelin might act through the vagus nerve [Masuda et al., 2000]. The intracerebroventricular administration of ghrelin in anesthesized rats also stimulates gastric acid secretion [Date et al., 2001]. However, other studies have shown that intracerebroventricular administration of ghrelin inhibited gastric acid secretion in conscious rats [Levin et al., 2005; Sibilia et al., 2002]. Recently, it has been shown that simultaneous administration of ghrelin and gastrin induced a synergistic increase of gastric acid secretion [Fukumoto et al., 2008]. Stimulatory or inhibitory effects of ghrelin on gastric acid secretion may depend on experimental conditions and models.

Ghrelin stimulates gastric motility by inducing the migrating motor complex and accelerating gastric emptying [Dass et al., 2003; Depoortere et al., 2005; Fujino et al., 2003; Peeters, 2003; Peeters, 2005]. By its prokinetic effect, ghrelin is able to reverse gastric postoperative ileus in rat [Trudel et al., 2002].

Moreover, ghrelin exerts a gastroprotective effect against stress-, ethanol- and cysteamine-induced ulcers [Konturek et al., 2004; Sibilia et al., 2003]. This effect depends on sensory nerve fiber integrity and is mediated by the nitric oxide system [Peeters, 2005].

Pancreatic Functions

Ghrelin seems to influence glucose metabolism. Acute ghrelin administration increases plasma glucose levels and amplifies the hyperglycemic effect of arginine. This effect could be due to glycogenolysis activation, indirectly by stimulation of catecholamine release, or directly by acting on hepatocytes where ghrelin might modulate neoglucogenesis. [Broglio et al., 2005; Gauna et al., 2005; Murata et al., 2002].

Ghrelin has been reported to influence the endocrine pancreatic function. Depending on the experimental conditions, ghrelin either stimulates [Adeghate & Ponery, 2002; Date et al., 2002b; Lee et al., 2002] or inhibits insulin secretion [Broglio et al., 2001; Colombo et al., 2003; Cui et al., 2008; Reimer et al., 2003]. Salehi et al. suggest that the effect of ghrelin on insulin secretion depends on concentration: ghrelin might have an inhibitory effect at low concentration and a stimulating effect at high concentration [Salehi et al., 2004]. Besides the direct effect of ghrelin on insulin secretion, a negative association between ghrelin secretion and insulin secretion has been observed [Ariyasu et al., 2001; Cummings et al., 2001; Saad et al., 2002; Toshinai et al., 2001]. This could be explained by the increase of plasma ghrelin levels and the decrease of plasma insulin levels during fasting, but this does not involve a direct inhibitory effect on insulin secretion.

The effects of ghrelin on exocrine pancreatic function are also controversial. In rat pancreas, ghrelin was shown to inhibit pancreatic protein secretion or to increase protein output. These effects are indirect and may involve cholecystokinin and reflex vagal pathways [Jaworek, 2006].

Cardiovascular Functions

Ghrelin has diverse cardiovascular effects. In vitro, ghrelin inhibits apoptosis of cardiomyocytes and endothelial cells. Moreover, by inhibiting NF-κB activation in human endothelial cells and mononuclear cell adhesion, ghrelin might oppose inflammation of the cardiovascular system. Ghrelin exerts vasodilatory effects by an endothelium-independent mechanism. Administration of ghrelin decreases mean arterial pressure without changing the heart rate. Ghrelin improves cardiac contractility and left ventricular function in chronic heart failure and reduces infarct size [Isgaard & Johansson, 2005]. In rats with myocardial infarction, ghrelin suppresses cardiac sympathetic activity and prevents early left ventricular remodeling, suggesting the potential usefulness of ghrelin as a new cardioprotective hormone early after myocardial infarction [Soeki et al., 2008].

Anti-Inflammatory Functions

Ghrelin has anti-inflammatory effects. Indeed, ghrelin inhibits production of proinflammatory cytokines in human endothelial cells, T cells, monocytes, and in a rat model of endotoxic shock [Chang et al., 2003; Dembinski et al., 2003; Dixit et al., 2004; Li et al., 2004; Nagaya et al., 2001; Xia et al., 2004]. Also, ghrelin attenuates the development of acute pancreatitis in rats [Dembinski et al., 2003]. Ghrelin dose-dependently inhibits proliferation of anti-CD3 activated splenic T lymphocytes in mice [Dixit & Taub, 2005]. Ghrelin possesses

a neutrophil-dependent anti-inflammatory effect that prevents burn-induced multiple organ injury and protects against oxidative organ damage [Sehirli et al., 2008]. Ghrelin attenuates lipopolysaccharide-induced acute lung inflammation and suppresses lipopolysaccharide--induced proinflammatory cytokine production in lung macrophages, which is partially mediated by increased NO production [Chen et al., 2008].

Other Functions

Ghrelin participates in the regulation of the reproductive function. In the testis, ghrelin inhibits testosterone secretion. Besides having direct gonadal effects, ghrelin may participate in the regulation of gonadotropin secretion and may influence the timing of puberty. In the pituitary, ghrelin inhibits luteinizing hormone secretion [Garcia et al., 2007].

Ghrelin stimulates bone formation by stimulating in vitro osteoblastic cell proliferation and differentiation, inhibiting apoptosis, and increasing in vivo bone mineral density in both normal and GH-deficient rats [Fukushima et al., 2005].

In addition to these functions, ghrelin also modulates cell proliferation in various cell types [De Vriese et al., 2005; De Vriese & Delporte, 2007; Korbonits et al., 2004].

13. Potential Clinical Applications of Ghrelin

In search of GHS-R 1a agonists and antagonists, numerous structure-function studies have been performed using peptidic and non-peptidic ghrelin analogues. Given that ghrelin stimulates food intake, ghrelin agonists or antagonists are mainly developed for cachexia treatment and for obesity treatment, respectively. Although patients with cachexia have increased plasma ghrelin levels, reflecting a compensatory response to weight loss, ghrelin administration still improves food intake and weight gain in a rat model of cancer cachexia [Deboer et al., 2007], and in patients with congestive heart failure or chronic obstructive pulmonary disease [Nagaya et al., 2006]. In patients with cachexia associated with anorexia nervosa, the effect of ghrelin administration is controversial [Miljic et al., 2006].

GHS-R 1a Agonists

As an alternative to growth hormone replacement therapy, an effort has been made to identify peptidomimetic and nonpeptidic small molecular growth hormone secretagogues (GHSs). Synthetic ghrelin agonists existed long before ghrelin was discovered. Indeed, in search for hypothalamic factor controlling the release of growth hormone, enkephalin analogues were synthesized as they induced weak GH release. This led to the discovery of GHSs, peptidic or non peptidic molecules inducing potent growth hormone release, such as growth hormone-releasing peptide-2 (GHRP-2), growth hormone-releasing peptide-6 (GHRP-6), hexarelin, MK-0677, CP-424,391 and NNC-26-073 [Smith et al., 1997; Smith et al., 2007; Wu et al., 1996]. Several GHSs have advanced to clinical studies including MK-677 (Merck), CP-424391 (Pfizer), LY-444711 (Lilly).

In the full ghrelin sequence, the presence of the voluminous hydrophobic groups on Ser3 is critical for the biological activity of the peptide [Bednarek et al., 2000; Matsumoto et al., 2001]. Maximal activity is reached with the acylation of Ser3 by an octanoyl group (C8:0). A substantial activity is maintained by the acylation of Ser3 by a decanoyl (C10:0), lauryl (C12:0) or palmitoyl (C16:0) group. However, the activity is largely decreased by the acylation of Ser3 by a butyryl (C4:0) or an acetyl (C2:0) group. Ser3 modification by a polyunsaturated fatty acid, such as 3-octenoyl (C8:1), or by a fatty acid containing a lateral chain, such as a 4-methylpentanoyl, maintain the activity [Matsumoto et al., 2001]. Whereas the replacement of Ser3 by a Trp maintains the activity of ghrelin, its replacement by aliphatic AA such as Val, Leu or Ile totally inhibits the activity of ghrelin [Bednarek et al., 2000]. Short peptides encompassing the first four or five residues of ghrelin remained functionally active, nearly as efficiently as the full-length ghrelin, on the on GHS-R 1a [Bednarek et al., 2000]. Ala-scan of ghrelin 1-14 revealed the importance of the amino-terminal positive charge and Phe in position 4 for the receptor interaction. Furthermore, analogs of ghrelin 1-14 modified in position 8 (Glu) by Ala or Tyr were more potent that ghrelin 1-14 [Van Craenenbroeck et al., 2004].

BIM-28125, a peptidic analogue of ghrelin, was reported as a potent stimulator of growth hormone secretion [Rubinfeld et al., 2004]. BIM-28131, a compound related to BIM-28125, induced body weight gain in rats [Strassburg et al., 2008]. Certain oxindole derivatives such as SM-130686((+)-6-carbamoyl-3-(2-chlorophenyl)-2-diethylaminoethyl)-4-tri fluoro methyloxindo-le) had potent growth hormone releasing activity, and induced body weight as well as fat-free mass gain [Nagamine et al., 2001; Tokunaga et al., 2001]. Structure-activity relationship of the C3-aromatic part of SM-130686 was examined and a series of 3-dichlorophenyl analogues were identified as potent ghrelin agonists [Tokunaga et al., 2005].

Among a series of GHS analogues synthesized based on the 1,2,4-triazole structure, some behaved as GHS-R 1a agonists such as JMV2873 [Demange et al., 2007]. Some 3,4,5-trisubstituted 1,2,4-triazoles were synthesized and two possessed potent GHS-R 1a agonist activity [Moulin et al., 2008].

From a series of pseudopeptidic analogues derived from EP-51389, based on a gem-diamino structure, JMV1843 was identified as a potent GHS-R 1a agonist [Guerlavais et al., 2003]. This molecule is currently under a phase III clinical trial in the US for the diagnosis of growth hormone deficiency in adults.

Diltiazem and some of its metabolites were reported to behave as GHS-R 1a agonists [Ma et al., 2007].

A series of small molecules GHS-R 1a agonists, SB-791016, were identified but suffered from poor oral absorption which may be attributed to the relatively high lipophilicity and poor solubility [Heightman et al., 2007]. Subsequent structure-activity relationship optimization of these compounds and in vivo properties were recently reported, and a potent GHS-R 1a agonist was identified: GSK899490A [Witherington et al., 2008].

The potential use of GHS-R 1a agonists as therapeutic agents for the treatment of gastrointestinal motility disorders has also been investigated [Peeters, 2006]. GHRP-6 is a GHS-R 1a agonist able to increase gastric emptying in normal rats [Depoortere et al., 2005], as well as in animal models of postoperative ileus [Trudel et al., 2002], septic ileus [De Winter et al., 2004], burn-induced slow gastrointestinal transit [Sallam et al., 2007], diabetes mellitus [Qiu et al., 2008a; Qiu et al., 2008b; Zheng et al., 2008]. RC-1139, a GHS-R 1a

agonist, was reported to behave as a potent gastrokinetic in rats, and it also reversed postoperative ileus, even in the presence of opiates [Poitras et al., 2005].

TZP-101, a small GHS-R 1a agonist displaying superior bioavailability than the ghrelin peptide, increases gastric emptying and intestinal transit in normal rats [Fraser et al., 2008], in a rat model of postoperative ileus [Venkova et al., 2007], and in patients with diabetic gastroparesis [Madsen et al., 2007]. In a clinical phase I study, the safety, pharmacokinetics and pharmacodynamic of TZP-101 were recently evaluated in healthy volunteers and suggested that this compound could be used for gastrointestinal motility disorders [Lasseter et al., 2008].

GSK894281 (N-[5-(cis-3,5-dimethyl-1-piperazinyl)-2-(methyloxy)phenyl]-3-fluoro-4-(5-methyl-2-furanyl)benzenesulphonamide) is a GHS-R 1a agonist triggering defecation in rats [Shafton et al., 2008].

GHS-R 1a Antagonists

A GHS-R 1a antagonist, D-Lys^3-GHRP-6 delayed gastric emptying induced by GHS-R 1a agonist [Depoortere et al., 2006; Qiu et al., 2008a], but this could be due at least in part by interacting with the serotonergic 5-HT2B receptors [Depoortere et al., 2006].

The potential benefits of using GHS-R 1a antagonists for the treatment of type 2 diabetes and obesity, particularly in Prader-Willi syndrome, have been investigated. Ghrelin-receptor antagonists, as [D-Lys-3]GHRP-6, decrease food intake in lean mice and obese mice, and reduce weight gain [Asakawa et al., 2003; Beck et al., 2004].

Piperidine-substituted quinazolinone derivatives were identified as a novel class of small-molecules GHS-R 1a antagonists [Rudolph et al., 2007]. Phenyl or phenoxy groups were identified as optimal substituents at position 6 of the quinazolinone core, and the replacement of phenyl groups in position 2 by small alkyl substituents were proven to be beneficial [Rudolph et al., 2007]. YIL-781, a piperidine-substituted quinazolinone derivative acting as potent GHS-R 1a antagonist, was shown to improve glucose tolerance due to increased insulin secretion, to reduce food intake and to promote weight loss in diet-induced obese mice [Esler et al., 2007].

Among a series of GHS analogues synthesized based on the 1,2,4-triazole structure, some behaved as GHS-R 1a antagonists such as JMV2866 and JMV2844 [Demange et al., 2007]. Some 3,4,5-trisubstituted 1,2,4-triazoles were synthesized and most of them possessed potent GHS-R 1a antagonist activity [Moulin et al., 2008].

Inverse Agonists

Consistent with the high constitutive activity of the ghrelin receptor, inverse agonists of the receptor, decreasing its constitutive activity, may be useful for the treatment of obesity [Holst et al., 2003]. During long fasting, GHS-R 1a expression increases in the hypothalamus, leading to an increase of GHS-R 1a signaling, a higher appetite, and a decrease of energy expenditure. The decrease of the GHS-R 1a constitutive activity by an inverse agonist could increase the sensitivity to anorexigenic hormones like leptin or PYY, and prevent food intake between meals [Holst & Schwartz, 2004]. [D-Arg^1, D-Phe^5, D-Trp7,9, Leu11]substance P

was identified as an inverse agonist on GHS-R 1a [Holst et al., 2006]. The use of inverse agonists of the GHS-R 1a in obesity treatment needs to be further investigated.

Others

Spiegelmers, antisense polyethylene glycol-modified L-oligonucleotides capable to specifically bind a target molecule, have been synthesized to neutralize ghrelin and inhibit its binding to the GHS-R 1a. The spiegelmer NOX-B11-2 decreased food intake and body weight in diet-induced obese mice [Asakawa et al., 2003; Kobelt et al., 2006; Shearman et al., 2006]. Another Spiegelmer, NOX-B11-3 was also shown to inhibit ghrelin-induced GH release in rats [Helmling et al., 2004]. NOX-B11-3 exerted long-lasting action after a single peripheral injection: it blocked ghrelin, but not fasting-induced neuronal activation in the hypothalamic arcuate nucleus [Becskei et al., 2008]. The neutralization of circulating ghrelin by these Spiegelmers may be useful to treat diseases associated with high ghrelin levels such as the PWS.

Ghrelin hapten immunoconjugates lead to the production of antibodies specifically directed against acylated ghrelin. In rats with strong anti-ghrelin immune responses, body weight gain is reduced with preferential reduction of fat mass compared to lean mass, by decreasing feed efficiency (weight gain per kilocalorie of food) [Zorrilla et al., 2006].

14. CONCLUSION

The importance of ghrelin in appetite stimulation and body weight regulation is intensively investigated. The effect of ghrelin on appetite is mediated in hypothalamus through stimulation of NPY, AGRP and orexin release, and inhibition α-MSH and CART release, and through activation of GHS-R on vagal afferents in the stomach. The effect of ghrelin on body weight is mediated by stimulating food intake, but also by reducing energy expenditure and promoting adiposity. Ghrelin agonists seem useful for treatment of gastrointestinal motility disorders, cancer-induced cachexia, congestive heart failure or chronic obstructive pulmonary disease. Ghrelin-receptor antagonists, Spiegelmers and anti-ghrelin vaccine reduce body weight gain and might be useful for type 2 diabetes and obesity treatment, particularly in PWS. Recently, the enzyme responsible for the octanoylation of ghrelin, GOAT, was identified and could represent an additional interesting therapeutic target for the treatment of type 2 diabetes and obesity. All these clinical applications of ghrelin will require further intensive investigations.

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